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The roles of CARMA1 and PKC θ in T cell function and immunity

Katy Korzekwa

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Katy Korzekwa

Candidate

Biomedical Sciences

Department

This thesis is approved, and it is acceptable in quality and form for publication:

Approved by the Thesis Committee:

Judy Cannon , Chairperson

Carolyn Mold

Bridget Wilson

Terry Wu

**THE ROLES OF CARMA1 AND PKC θ IN T CELL FUNCTION
AND IMMUNITY**

by

KATY KORZEKWA

**B.S., MOLECULAR BIOLOGY, ADAMS STATE COLLEGE, 2005
B.S., BIOCHEMISTRY, ADAMS STATE COLLEGE, 2005**

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THE ROLES OF CARMA1 AND PKC θ IN T CELL FUNCTION AND IMMUNITY

By
Katy Korzekwa

B.S. Molecular Biology, Adams State College, 2005
B.S. Biochemistry, Adams State College, 2005
M.S. Biomedical Sciences, University of New Mexico, 2012

Abstract

T cells are critical players in adaptive immunity. They migrate throughout the body scanning the lymph nodes for antigen, then migrate to an infection site to help clear pathogen. Migrating T cells use multiple receptors and adhesion proteins to migrate to and enter different tissues. While the cell surface molecules involved in T cell motility are well understood, less is known about intracellular signaling pathways involved in T cell migration. PKC θ and CARMA1 signal downstream of TCR activation to induce NF- κ B activation. However, the roles of PKC θ and CARMA1 in other T cell functions are unknown. In this study we investigate the roles of PKC θ and CARMA1 in regulating T cell migration. We found that CARMA1 deficiency led to defective regulation of cell surface adhesion molecules, increased migration speed of activated T cells in vitro, and mislocalization of the actin regulatory ERM proteins. In contrast, PKC θ deficiency did not affect the regulation of adhesion molecules or the migration of T cells. However PKC θ ^{-/-} T cells also showed mislocalization of ERM proteins. Although CARMA1 is directly phosphorylated downstream of PKC θ following TCR activation, our data show

that CARMA1 likely regulates T cell migration in a PKC θ independent manner.

We also investigated the role of PKC θ in immunity to infection with the intracellular bacteria *Francisella tularensis*. PKC θ is important in immunity to some pathogens, but in certain cases, PKC θ deficiency is not important or can even be protective. T cells are crucial in *F. tularensis* immunity, with multiple subsets playing an important role. We found that PKC θ deficiency was protective against LVS, an *F. tularensis* strain highly infectious in mice, with PKC $\theta^{-/-}$ mice surviving longer than the WT mice upon LVS infection. We found the organs of the infected PKC $\theta^{-/-}$ mice had a smaller percentage of CD4⁺ T cells and a larger percentage of CD4⁻CD8⁻ T cells compared to WT mice. These results suggest that PKC $\theta^{-/-}$ mice are protected against *F. tularensis* infection and that PKC θ may contribute to the development of different T cell populations which contribute to protective immunity against *F. tularensis* infection.

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Introduction

T cells

The immune system is important for protecting an organism against disease. There are two layers of protection: the innate immune system and the adaptive immune system.

The innate immune system provides a non-specific but immediate response. The adaptive immune system provides a response that is specific to a pathogen, but it takes time to generate this response. T cells are important players in the adaptive immune response. They are a diverse group of cells that perform many different roles in immunity. They can act as “helpers”, termed CD4 helper T cells, helping to activate B cells, other T cells, or other immune cells such as macrophages. They can also act as “killers”, CD8 or cytotoxic T cells, directly killing infected cells.

T cells originate from hematopoietic stem cells and develop in the thymus. Mature T cells then leave the thymus into circulation via the bloodstream. These unactivated, or naïve, T cells move throughout the body scanning the lymph nodes for antigen presented on major histocompatibility complex molecules (MHC) that will bind to their T cell receptors (TCRs). To ensure that there will be a T cell that can bind to a wide variety of pathogenic antigen, TCRs are very diverse; only a few naïve T cells circulating at any time can bind a given antigen. Dendritic cells (DCs) collect antigen from a site of infection and then migrate to the nearest lymph node where they are scanned by T cells moving through. As lymph nodes are the location where DCs and T cells interact to initiate a T cell response, it is very important that the T cells are able to migrate efficiently to and through each lymph node to increase the chance of a productive

encounter.

When a T cell encounters a dendritic cell presenting cognate antigen, the T cell is activated via the TCR and begins to proliferate and produce cytokines. The activated T cells can then migrate to affected sites and assist in fighting the infection. At the infection site the T cells fight the infection as either helper T cells or cytotoxic T cells. When the infection is cleared, most of the T cells die, but some of these T cells become memory T cells that can persist long after an infection is cleared and can then respond more quickly if there is a secondary infection with the same pathogen (U.H. von Andrian & Mackay, 2000).

T cell homing and migration

Circulating T cells must be able to move from the blood into lymph nodes to scan for antigen, or into other tissues once activated. What tissues the T cells migrate into is regulated by the cell surface molecules expressed on the T cells and is called “homing” (Butcher & Picker, 1996). The T cells must adhere to the wall of the blood vessel by binding to endothelial cells (ECs) using adhesion molecules so that they can migrate into the tissue. Binding to ECs and migrating into tissue involves two major adhesion events (Figure 1). The first adhesion event involves a group of molecules called selectins. Selectins bind to sialyl-Lewis^x-like sugars (U.H. von Andrian & Mackay, 2000). The adhesion of the selectin to its ligand tethers the cell to the endothelium, and the shear stress from the blood flow causes the cell to start rolling. The rolling T cells encounter chemokines on the endothelium, and when these chemokines bind to their receptors

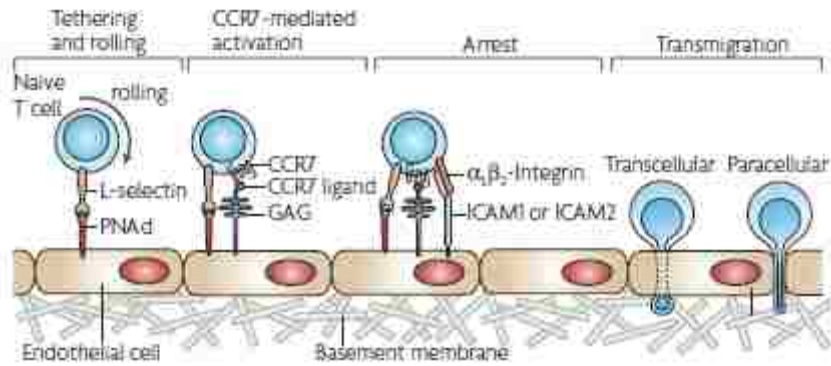


Figure 1. Naïve T cells adhere to the endothelium in the blood stream in a multi-step process before entering the lymph node. L-selectin binds to PNAd on the endothelial cell, slowing the T cell and causing it to roll. CCR7 ligand (CCL21) binds to CCR7, leading to signaling that activates the $\alpha_L\beta_2$ (LFA-1) integrin on the T cell so it can bind to ICAM on the endothelial cell. The T cell is arrested, and can now migrate into the lymph node (Förster, Davalos-Miszlitz, & Rot, 2008).

expressed on T cells, integrins on the T cells become activated. Integrin activation leads to the second adhesion event and the integrins on the T cell bind to adhesion molecules on the endothelium, arresting the cell. The arrested cell can then migrate into the tissue (U.H. von Andrian & Mackay, 2000).

The tissue that a T cell homes to is determined by the selectin and selectin ligands, chemokine receptors, and integrins expressed on the T cell and endothelium lining the tissue. There are three types of selectins involved in leukocyte migration: L-selectin, E-selectin, and P-selectin. L-selectin (CD62L) is expressed on most leukocytes, including naïve T cells, but is down-regulated on activated and memory T cells (Kansas, 1996). L-selectin binds to sulphated sialyl-Lewis^X-like sugars, called peripheral node addressin (PNAd). PNAd sugars are found on the high endothelial venules (HEV) of lymph nodes, and so T cells expressing L-selectin will home to lymph nodes (Marelli-Berg, Cannella, Dazzi, & Mirenda, 2008). E-selectin is expressed on endothelial cells in response to inflammatory signals, such as IL-1 or TNF- α (Kansas, 1996). P-selectin ligand 1 (PSGL-1), CD43, and CD44 are all molecules expressed on T cells, particularly Th1 cells, that have been shown to bind to E-selectin and help mediate migration into inflamed tissue, particularly in the skin (Hirata et al., 2000; M. Matsumoto et al., 2007; Náchter et al., 2011). P-selectin is expressed by platelets and endothelial cells, and may be expressed very early in an inflammatory response because it is stored within granules in the cell (Kansas, 1996). P-selectin binds to PSGL-1 on T cells to mediate activated T cell migration into inflammatory sites.

Chemokines are cytokines that can activate and induce movement in leukocytes. T cells can express a variety of chemokine receptors to help the cells home to different tissues. A chemokine that is highly expressed on HEVs is CCL21 (SLC), and when CCL21 binds to its receptor CCR7 on T cells, CCR7 signaling activates the integrins on the T cell (Ulrich H von Andrian & Mempel, 2003). CCL21 and CCL19 (ELC), another agonist for CCR7, are also expressed within the lymph nodes and contribute to T cell migration within the lymph nodes. Other chemokines are also found on HEVs, such as CXCL12, which some memory T cells use to enter lymph nodes, as memory T cells express the chemokine receptor CXCR4, which binds CXCL12, instead of CCR7 (Scimone et al., 2004). Some chemokines, such as CXCL9 and CCL2, are expressed on HEVs only when there is inflammation (Cyster, 2005). The expression of other chemokine receptors will direct T cells to other tissues. CCR4, CCR8, and CCR10 expression directs T cells to the skin, where their ligands are expressed, while CCR9 expression leads to migration to the lamina propria, and others such as CCR5 and CXCR3 direct T cells to sites of inflammation (Bromley, Mempel, & Luster, 2008).

Integrins are important not only for arrest in the bloodstream, but also for migration within tissues. Integrins are composed of two chains, an α and a β chain, and there are 24 known $\alpha\beta$ pairs in vertebrates (Luo, Carman, & Springer, 2007). On T cells integrins are in an inactive state and have little affinity for their ligands until they are activated through signaling from the TCR or chemokine receptors (Kinashi, 2005). Integrins bind to endothelial immunoglobulins, including intercellular adhesion molecule 1 (ICAM-1), mucosal addressin-cell adhesion molecule type 1 (MAdCAM-1), and vascular-cell

adhesion molecule 1 (VCAM-1). An important integrin for T cell migration to lymph nodes is LFA-1 ($\alpha_L\beta_2$), which binds ICAM-1 and is involved in arresting the cells in the bloodstream and in transmigration into the lymph nodes (Andrew et al., 1998; DeNucci & Mitchell, 2009; Warnock, Askari, Butcher, & von Andrian, 1998). LPAM ($\alpha_4\beta_7$) is important for migration to mesenteric lymph nodes (MLNs) and Peyer's patches (PPs) as well as inflamed gut tissue; LPAM binds to MAdCAM-1, which is largely expressed in the gut (DeNucci & Mitchell, 2009). MAdCAM-1 has also been shown to bind to L-selectin, indicating that it can function in the rolling stage of the adhesion cascade (Berg, McEvoy, Berlin, & Bargatze, 1993). The integrin $\alpha_4\beta_1$ binds to VCAM-1, which is involved in trafficking to many tissues, including bone marrow (Koni et al., 2001) and the CNS (Engelhardt & Ransohoff, 2005), and is upregulated in response to inflammation (DeNucci & Mitchell, 2009).

ICAM-1 and VCAM-1 are both expressed within the lymph node, but T cell motility within lymph nodes is not strictly integrin dependent (DeNucci & Mitchell, 2009); T cells lacking LFA-1 were shown to migrate in lymph nodes similarly to WT T cells in an in vivo study (Woolf et al., 2007). However it was shown that interaction between ICAM-1 on a dendritic cell and LFA-1 on a T cell is important for strong signaling with CD8⁺ T cells and the generation of memory T cells, so integrins do play an important role in the initiation of the T cell response within the lymph node (Scholer, Hugues, Boissonnas, Fetler, & Amigorena, 2008).

The combination of these different receptors and adhesion molecules expressed on the T

cells determines the destination of the cell. Naïve T cells rely on CD62L, CCR7, and LFA-1 to enter the lymph nodes. When a T cell is activated, the expression of adhesion molecules and chemokine receptors is changed. As T cells activate and become effector T cells (T_{EFF}), they downregulate CCR7 and CD62L, so they no longer circulate through lymph nodes (Mora & von Andrian, 2006). T_{EFF} also have increased expression of molecules that will direct them to other tissues, such as E-selectin ligands including CD44, and chemokine receptors such as CCR4 and CCR10 (Marelli-Berg et al., 2008). There are also differences in expression of receptors and adhesion molecules in memory T cells. Effector memory T cells (T_{EM}) have similar expression to T_{EFF} , but central memory T cells (T_{CM}) do express CCR7 and CD62L, and so are able to home to lymph nodes as well as peripheral tissues (Mora & von Andrian, 2006). This differential expression of chemokine receptors and adhesion molecules on different T cells ensures that the T cells will migrate to the tissue where they are needed, either to respond to an infection or to antigen presented in the lymph nodes.

T cell polarization and motility

T cells must migrate between the endothelial cells of the blood vessel wall and through the basement membrane, made up of a matrix of collagen and other fibers, to arrive at their target tissues. Therefore they must adopt a more flexible form to move through the small spaces created by the matrix mesh. Furthermore, the cells need to generate force in order to push through this matrix towards DCs within lymph nodes or towards infection sites. Chemokines play a major role in this process; aside from activating the integrins and inducing chemotaxis, they also induce changes in the cytoskeleton and polarization

of the cell (Sanchez-Madrid & del Pozo, 1999).

T cells are polarized into two distinct regions: the leading edge and the uropod (del Pozo, Sánchez-Mateos, Nieto, & Sánchez-Madrid, 1995) (Figure 2). The microtubule organizing center (MTOC) localizes to the uropod and the actin cytoskeleton is redistributed throughout the cell; the actin cycle providing the extension and contraction that moves the cell forward (Sánchez-Madrid & Serrador, 2009). Many cell surface receptors and other molecules are also polarized in motile T cells. Chemokine receptors localize to the leading edge and are responsible for the chemotaxis of the cell (Sanchez-Madrid & del Pozo, 1999). Adhesion molecules localize to the uropod, including ICAMs and L-selectin (Sánchez-Madrid & Serrador, 2009). Another group of molecules that localize to the uropod are the ezrin/radixin/moesin (ERM) proteins. These closely related proteins are actin regulatory proteins and can bind the actin to the plasma membrane, associating with transmembrane proteins such as CD43 and CD44 (Tsukita, Yonemura, & Tsukita, 1997). Moesin is the most abundant of these proteins in T cells (Schwartz-Albiez, Merlini, Spring, Möller, & Koretz, 1995). This linking of the actin filaments to the plasma membrane is important for ensuring continuous movement of the T cell (Sanchez-Madrid & del Pozo, 1999).

T cell activation and protein localization

Protein localization is not only important for T cell motility, but also for T cell activation. When a T cell interacts with an antigen-presenting cell, a junction called the immunological synapse (IS) is formed. Different components are localized to distinct

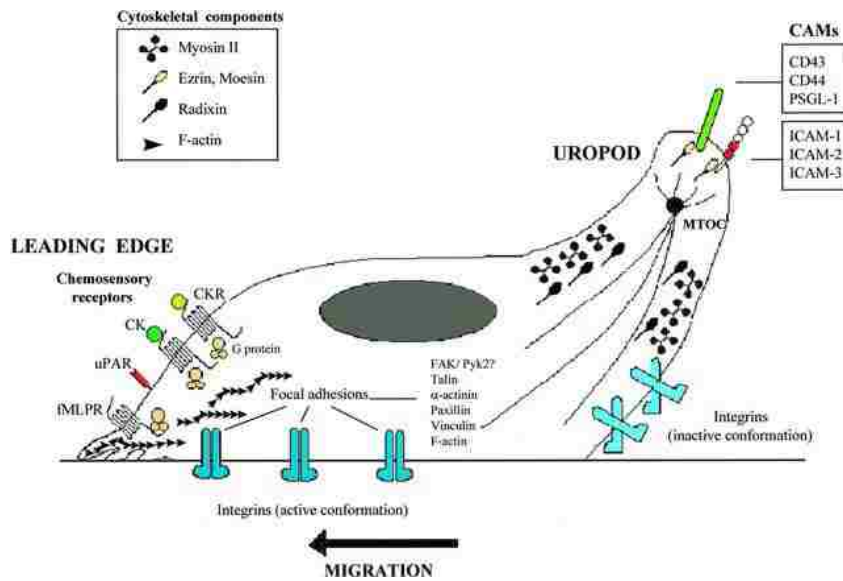


Figure 2. A polarized T cell migrating, with localization of adhesion molecules, chemokine receptors, and cytoskeletal components localized to the leading edge and uropod (Sanchez-Madrid & del Pozo, 1999).

regions of the IS called supramolecular activation clusters (Dustin, 2009). The TCR and costimulatory molecule CD28 localize to the IS as well as adhesion molecules like LFA-1 and cytoskeletal components including talin and F-actin. Signaling molecules downstream of the TCR also localize to the IS upon TCR activation. One such molecule is protein kinase C (PKC) θ (Monks, Kupfer, Tamir, & Barlow, 1997).

PKC θ is in the novel PKC (nPKC) family of PKCs, which do not require Ca²⁺ for activation (Hayashi & Altman, 2007). PKC θ is found mainly in T cells, as well as platelets and muscle cells. Following TCR activation, hydrolysis of PIP₂ generates DAG, which activates PKC θ (Isakov, 2002). PKC θ has been shown to be involved in several signaling pathways downstream of the TCR. PKC θ was shown to lead to the activation of AP-1 (Baier-Bitterlich et al., 1996), and PKC θ along with calcinurin leads to the activation of JNK and NF-AT (Werlen, Jacinto, Xia, & Karin, 1998). In 2000, PKC θ was found to be involved in NF- κ B activation (Coudronniere, Villalba, Englund, & Altman, 2000; X. Lin, O'Mahony, Mu, Geleziunas, & Greene, 2000). These are all pathways very important to T cell proliferation and survival. AP1, NF-AT, and NF- κ B are all involved in IL-2 production, which is important for T cell homeostasis (Altman, Isakov, & Baier, 2000; Murrack et al., 2000). Mice that are PKC θ -deficient appear normal and are fertile, and there is no apparent defect in T cell development in the thymus or in numbers of T cells in the thymus, lymph nodes, and spleen (Sun et al., 2000). However, it was shown that T cells from mice lacking PKC θ have impaired IL-2 production (Sun et al., 2000).

Downstream of PKC θ following TCR activation is CARMA1. CARMA1 (also called CARD11) is a membrane-associated guanylate kinase (MAGUK) that also contains a caspase-associated recruitment domain (CARD), so that it can both associate with the cytoplasmic membrane and bind to other molecules containing CARD domains (Blonska & Lin, 2009) (Figure 3). CARMA1 is largely only expressed in hematopoietic cells. CARMA1 plays a key role in NF- κ B activation in T cells; it is required for the recruitment of PKC θ to the immune synapse (Wang et al., 2002, 2004). Upon TCR activation, CARMA1 is phosphorylated in the linker domain by activated PKC θ , and once phosphorylated can recruit the other components necessary for signaling, including Bcl10, IKK β , and MALT1, to the IS (Che et al., 2004; R. Matsumoto et al., 2005; Wang et al., 2004). Protein phosphatase 2A (PP2A) dephosphorylates CARMA1 at the site of PKC θ -dependent phosphorylation, acting as a negative regulator for NF- κ B activation (Eitelhuber et al., 2011). CARMA1 is also shown to be involved in JNK2 activation (Blonska & Lin, 2009). CARMA1^{-/-} T cells develop normally in the thymus, but activation is severely impaired, with reduced proliferation and IL-2 production and defective upregulation of CD25, CD69, and CD44 (Hara et al., 2003).

PKC θ and CARMA1 roles in T cell immune responses

PKC θ has different roles in immunity depending on the type of T cell response. PKC θ ^{-/-} mice have been shown to have reduced Th2 responses; they were protected from an allergic hypersensitivity response in an inhaled allergen model, and the Th2 response to a helminth infection was greatly reduced (Marsland, Soos, Späth, Littman, & Kopf, 2004).

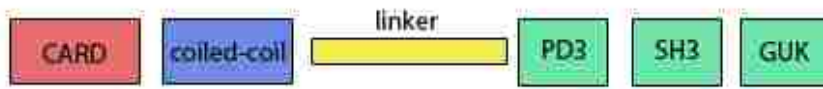


Figure 3. Domains of the CARMA1 protein.

The same mice, however, were able to produce an effective response to *Leishmania major*, which requires Th1 cells and IFN γ . In another study, using an airway inflammation model, it was shown that PKC θ is required for a good Th2 response, but that while the Th1 response was slowed initially, PKC θ was not required and IFN γ production was normal (Salek-Ardakani, So, Halteman, Altman, & Croft, 2004). These studies indicate that PKC θ is more critical for Th2 responses than Th1.

PKC θ was also shown to be important for CD8⁺ effector T cells; PKC θ ^{-/-} CD8⁺ T cells did not survive as well as wild-type T cells (Barouch-Bentov et al., 2005). However, other studies have shown that PKC θ is not required for an effective CD8⁺ response to viral infections (Giannoni, Lyon, Wareing, Dias, & Sarawar, 2005; Marsland et al., 2005). The role that PKC θ plays in any given immune response appears to vary greatly, even among those that appear to require a similar response. PKC θ is required for the development of experimental allergic encephalomyelitis (EAE), which depends on a Th1 and Th17 response (Salek-Ardakani, So, Halteman, Altman, & Croft, 2005). PKC θ is also required for graft-versus-host disease (GVHD), but not for graft-versus-leukemia (GVL) or an effective immune response to *Listeria monocytogenes* (Valenzuela et al., 2009). PKC θ is also shown to be important for the production of IFN γ ; in *Toxoplasma gondii* infection PKC θ ^{-/-} mice succumbed to infection with reduced IFN γ production, while in another study PKC θ ^{-/-} mice were partially protected from cerebral malaria, which was attributed to reduced Th1 type cytokine production, including IFN γ (Nishanth et al., 2010; Ohayon et al., 2010). These various studies show that the role of PKC θ in T cell responses is varied, and will require further study to be fully understood.

CARMA1, like PKC θ , is shown to have varying importance in different types of T cell responses. CARMA1 appears to play a more important role in the development of some T cell subsets than in others; CARMA1^{-/-} mice exhibited defective development of Tregs, and reduced memory CD4⁺ T cells in the periphery, but showed normal development of NKT cells (Medoff et al., 2009). In a murine model of allergic airway inflammation, CARMA1 was found to be required for an inflammatory response, and CARMA1^{-/-} mice had reduced Th2-type cytokines (Medoff et al., 2006). In a later study where CARMA1 was deleted from T cells after activation, it was shown that CARMA1 is also important for reactivation in memory T cells; these mice did not develop airway inflammation in response to a secondary challenge (Ramadas et al., 2011). The defect in Th2 responses is likely due to a decrease in JunB and GATA3; a recent study reveals that CARMA1 regulates Th2 cytokine production through expression of JunB and GATA3 (Blonska, Joo, Zweidler-McKay, Zhao, & Lin, 2012).

Francisella tularensis

Francisella tularensis is a facultative intracellular bacterium that causes the disease tularemia. There are four subspecies of *F. tularensis*: *tularensis*, *holarctica*, *mediasiatica*, and *novicida*, the first two of which are known to cause disease in humans (Sjöstedt, 2006). Subspecies *tularensis* is much more virulent in humans than *holarctica* and is more frequently fatal if untreated (Oyston, Sjöstedt, & Titball, 2004). The low dose of *F. tularensis* that is needed to cause tularemia, as well as the fact that it can be transmitted by an airborne route, has made it an agent of interest as a potential biological weapon

(Oyston et al., 2004). In the 1950's a live vaccine strain (LVS) was developed from a strain of subspecies holarctica, and it is frequently used in *F. tularensis* research because while it has low virulence in humans, it is very virulent in mice. The potential use of *F. tularensis* as a biological weapon has increased the interest in researching this pathogen, particularly in developing effective vaccines to protect against attacks.

Immune response to *Francisella tularensis*

F. tularensis is resistant to some of the usual mechanisms of innate immunity. It is resistant to the lethal components of complement, and in fact can use the opsonizing components to enter and infect cells (Cowley & Elkins, 2011). *Francisella* LPS has also been found to not be recognized by human or murine immune systems, and so it does not induce a pro-inflammatory response. However, there is still an innate response mounted against *Francisella*; TLR2 and MyD88 have both been shown to play a role in early cytokine production in response to *Francisella* infection.

F. tularensis can infect many different types of cells, but they mainly infect macrophages. Once infection occurs, the bacteria are able to quickly escape the phagosome and replicate freely within the cytoplasm (Oyston et al., 2004). The bacteria eventually induce apoptosis in the macrophages by inducing the release of cytochrome c from the mitochondria and the activation of caspases 9 and 3 (Lai & Sjöstedt, 2003). If the infected macrophages are activated by either IFN γ or TNF α , they are able to control the bacterial replication (Sjöstedt, 2006). Pro-inflammatory and Th1-type cytokines, including IFN γ , TNF α , IL-6, and IL-1 β , are very important for controlling *F. tularensis*

infection, but they are not present in detectable levels until 48 hours after infection at the earliest, and this can often be too late for protective immunity (Cowley & Elkins, 2011). The cytokines that seem to be the most important are IFN γ and TNF α ; the absence of either makes mice susceptible to any dose of LVS. IL-17A and IL-12 are also important to a lesser degree to clear infection (Cowley & Elkins, 2011).

The role of T cells in *F. tularensis* immunity

Several different immune cell types are important for controlling and clearing a *F. tularensis* infection. Neutrophils, mast cells and NK cells play a role in controlling *Francisella* infection, though none are essential (Cowley & Elkins, 2011). B and T cells, however, seem to be the important players for effective immune response and protection from repeat infections. The role of B cells is not completely understood, but it seems B cells are less important in primary *F. tularensis* infections, and play a more important role in secondary infections that is not entirely antibody-mediated (Karen L Elkins, Cowley, & Bosio, 2007).

T cells are required to clear an infection of *F. tularensis*; while mice lacking T cells are able to control a low dose LVS infection at first, they eventually succumb to the infection after about 1 month (K L Elkins, Rhinehart-Jones, Nacy, Winegar, & Fortier, 1993; K L Elkins, Rhinehart-Jones, Culkin, Yee, & Winegar, 1996). Either CD4⁺ or CD8⁺ T cells are individually able to clear an infection (Yee, Rhinehart-Jones, & Elkins, 1996). Another population of T cells, CD4⁻ CD8⁻ NK1.1⁻ TCR $\alpha\beta$ ⁺ Thy1.2⁺ T cells, or “DN” T cells, is also able to control *F. tularensis* infection but not clear it; the mice survive for

months with a chronic infection (Cowley et al., 2005). DN T cells do seem play an important role in anti-*Francisella* immunity; they are found in greater numbers in pulmonary infections than in intradermal, and they help produce IL-17A along with CD4⁺ and CD8⁺ T cells (Cowley, Meierovics, Frelinger, Iwakura, & Elkins, 2010).

IL-17A was shown to be very important in *F. tularensis* infection for helping to regulate the Th1 responses needed to control the infection (Y. Lin et al., 2009). CD4⁺, CD8⁺, and DN T cells all also produce IFN γ and TNF α to control the growth of *F. tularensis* in macrophages (Cowley & Elkins, 2003). A $\gamma\delta^+$ T cell population in humans can also help control *F. tularensis* infection; $\gamma9\delta2$ T cell numbers increase when exposed to *F. tularensis* antigen, and the $\gamma9\delta2$ T cells produce both IFN γ and TNF α (Poquet et al., 1998; Rowland et al., 2012). Together, these data show that T cells are very important for an immune response to *F. tularensis* and that a variety of populations can play a role in protection against *Francisella tularensis* infection.

Purpose

While a lot is known about the cell surface molecules and receptors involved in T cell migration, not much is known about the intracellular signaling pathways that may be involved in T cell migration. PKC θ and CARMA1 are both well established as signaling mediators downstream of TCR activation, but their potential role in other T cell functions, including migration, is unknown. In this thesis we investigate their roles in different aspects of T cell migration. We also investigate the role of PKC θ in the immune response to *Francisella tularensis*.

Methods

Mice

C57BL/6 mice, B6.Ly5.1 and B6.PKC θ -deficient mice were from Jackson Laboratories (Bar Harbor, ME). B6.CARMA1-deficient mice were a kind gift of Dr. Marisa Alegre from the University of Chicago. All mice were bred and/or maintained in a specific pathogen-free condition in barrier facilities (Albuquerque, NM) and conform to the principles outlined by the Animal Welfare Act and the National Institutes of Health guidelines.

Reagents and antibodies

Antibodies were purchased from the following: Fluorescent conjugated α -CD4, α -CD62L, and α -CD25 were from eBiosciences (San Diego, CA); fluorescent conjugated α -CD44, α -CD4, α -CD19, α -CD11b, α -CD4, α -CD8, α -CD62L were from Biolegend (San Diego, CA); α -tubulin was from Thermo Fisher (Lab Vision, Fremont CA); Santa Cruz Biotechnology (Santa Cruz, CA); α -moesin and α -pERM were from Cell Signaling Technology (Beverly, MA); and α -CD43 antibody S11 was produced in the laboratory of Dr. Anne Sperling at the University of Chicago. CCL21 was from Peprotech (Rocky Hill, NJ), ICAM-Fc from R&D Systems (Minneapolis, MN), CFSE and Collagenase 1 were from Invitrogen (Carlsbad, CA). IL-2 was from the NCI Preclinical Repository, α -CD3 antibody 2C11 and α -CD28 antibody PV1 from BioXCell (West Lebanon, NH), and pertussis toxin was from Sigma-Aldrich (St. Louis, MO). Secondary fluorescently tagged antibodies for immunofluorescence were purchased from Jackson ImmunoResearch (West Grove, PA).

T cell isolation and activation

Primary T cells from murine lymph nodes were purified by non-adherence to nylon wool, which results in >85% CD3⁺ T cells, as described (Sperling et al., 1995). Briefly, lymph nodes from mice were removed, cells dissociated, and then incubated on nylon wool. After 30 minutes, non-adherent cells were collected and used for assays. CD4⁺ T cells were isolated using CD4⁺ T cell Isolation Kit II according to manufacturer's protocols (Miltenyi Biotec, Auburn, CA). For activated cells, primary T cells were isolated by nylon wool activated on plates coated for 2 hours with 0.5ug/ml 2C11 and 1.0ug/ml PV1. 10U/ml IL2 was added to activating cells after 24 hours. Activated cells were used between days 2-9 and cells were split at days 3-4 as needed.

Immunofluorescence Staining and Microscopy

Purified primary murine T cells were placed on poly-L-lysine coated coverslips and then fixed for 20 min in 3% paraformaldehyde (PFA) in PBS, quenched with 50 mM NH₄Cl/PBS, permeabilized for 1 min with 0.3% Triton-X100, and blocked with a PSG solution (PBS, 0.01% saponin, 0.25% aqueous cold fish gelatin, and 0.02% NaN₃ [all from Sigma, St. Louis, MO]). Fixed cells were incubated with primary antibodies for 1 hour, washed 5 times with PSG, and incubated for 30 minutes with fluorochrome labeled secondary antibodies. Coverslips were washed 5 times with PSG, rinsed with ddH₂O, and then mounted on slides with Prolong Gold (Invitrogen, Carlsbad, CA). Cells were visualized using a 63xDIC Oil objective on a Zeiss Axioplan 2 MOT upright LSM510

Confocal microscope. Images were obtained using the Zeiss LSM 510 Image Acquisition software and analyzed with the Zeiss LSM Image Browser. Cells were analyzed for intensity of the fluorescent signal at multiple points on the cell and the intensity of the signal at the uropod was compared to that at the leading edge.

In vitro Live Cell Imaging

50 mm glass bottom culture dishes (MatTek, Ashland, MA) were coated with 6 μ g/ml Recombinant Intercellular Adhesion Molecule-1 (ICAM-1Fc) in PBS for 2 hours, blocked for 1 hour with 2.5% bovine serum albumin (BSA) in PBS, then stored at 4°C in PBS until use. Isolated primary T cells were incubated at 37°C for 1 hour with 300ng/ml CCL21 on ICAM-1 coated dishes, and then imaged. Activated T cells (7-9 days post activation) were isolated using a Ficoll gradient to remove dead cells, and then incubated at 37°C for 15 min with 300ng/ml CCL21 on ICAM-1 coated dishes and imaged. T cells treated with pertussis toxin were incubated for 2 hours in 0.5 μ g/ml pertussis toxin, then treated with CCL21 before being placed on the ICAM-1 coated dish for imaging. T cells were imaged every 2 seconds for 10 minute intervals on the Olympus DSU Spinning Disk Confocal/Stereology IX-81 inverted microscope with a 60xDIC Water objective and live cell incubation system at 37°C with 70% humidity and 5% CO₂. Cells were individually tracked on Slidebook 5.0 software (Intelligent Imaging Innovations Inc., Denver, CO, USA). Cells were tracked for 5-7 minutes each, and only cells that were continuously in frame and had no collisions with other cells were tracked.

Inoculation of mice with *Francisella tularensis*

1×10^5 cfu or 1×10^4 cfu LVS was delivered intranasally in 50 μ l to mice sedated with isoflurane. The animals were then observed for mortality, or sacrificed at indicated days for analysis.

Processing of infected mouse organs

Animals infected with *Francisella tularensis* were sacrificed at day 7 post infection, and lymph nodes, spleens, lungs, and livers removed. Lymph nodes were dissociated under Nitex mesh in media, then cells in media were recovered. Spleens were dissociated under Nitex mesh, cells in the media were recovered, then red blood cells (RBC) were lysed. Remaining cells were recovered. Livers were dissociated under Nitex mesh, cells in the media were recovered, RBC lysed, and cells were centrifuged through a 35% Percoll gradient. Lungs were minced into small pieces, then digested with 150 U/ml Collagenase 1 for one hour, and cells were centrifuged through a 44%/66% Percoll gradient.

Flow Cytometry

Primary and activated T cells (taken between 2 and 9 days post activation) and total leukocytes were incubated with fluorescent antibodies for 20 min at 4°C. Data were acquired using the BD LSRFortessa. Data were analyzed using FlowJo software (Tree Star Inc., Ashland, OR).

Statistical analysis

All graphs were generated and statistics were done using Graphpad Prism 5 (Graphpad

Software, Inc., La Jolla, CA, USA). Unpaired or paired Student two-tailed t tests were used as indicated in figure legends. Error bars represent SEM.

RESULTS

1. The role of CARMA1 in T cell migration

CARMA1 is very important for recruiting signaling components such as Bcl10 and MALT1 to the TCR upon activation, and mediating signal transduction pathways leading to NF- κ B activation, resulting in increased T cell survival, proliferation, and IL-2 production. However, nothing is known about whether CARMA1 might regulate other aspects of T cell function, including T cell migration. We hypothesized that CARMA1 would also be important for regulating T cell migration.

CARMA1 regulates the expression of migration markers upon T cell activation.

T cells lacking CARMA1 have impaired activation, and have been shown to have reduced proliferation, poor IL-2 production, and defective up-regulation of the cell surface markers CD25, CD69, and CD44, which are associated with activation (Hara et al., 2003). However, nothing is known about whether CARMA1 might regulate other aspects of T cell function such as T cell migration. T cell migration is critically dependent on cell surface expression of selectins, chemokine receptors, integrins, and other molecules that act to adhere T cells to endothelial cells to mediate T cell entry into tissues such as lymph nodes and inflammatory sites.

To determine whether CARMA1 might regulate T cell migration, we first investigated whether CARMA1 also regulates the expression of cell surface markers involved in T cell migration. We focused particularly CD62L and CD44, which are both differentially expressed in naïve and activated T cells and important for T cell homing. CD62L is

highly expressed on naïve T cells and helps them home to lymph nodes; upon T cell activation CD62L is down-regulated so that the T cells will no longer home to the lymph nodes and instead migrate to peripheral tissues. On the other hand, CD44 is not highly expressed in naïve T cells, but is upregulated in activated T cells to help them migrate into inflamed tissue where E-selectin, which binds CD44, is expressed.

To examine the expression of CD44 and CD62L on activated T cells, we isolated T cells from WT and CARMA1^{-/-} mice using nylon wool, which results in >85% CD3⁺ T cells, including both CD4⁺ and CD8⁺ T cells. We stained T cells isolated from WT and CARMA1^{-/-} mice with Carboxyfluorescein succinimidyl ester (CFSE), a fluorescent dye taken up into cells which can track cell division. CFSE stained cells evenly split the dye between daughter cells during cell division so proliferation can be tracked by flow cytometry as the signal is diluted (Lyons & Parish, 1994). We activated CFSE stained WT and CARMA1^{-/-} T cells via antibody to CD3, the component of the TCR that induces the signaling downstream of TCR activation, and to CD28, which is a co-stimulatory molecule that enhances the signaling from the TCR (Acuto & Michel, 2003). This combination of anti-CD3 and anti-CD28 leads to activation of multiple signaling pathways, including NF-κB and JNK, inducing T cell proliferation and IL-2 production.

It has previously been shown that CARMA1^{-/-} T cells do not proliferate as well as WT T cells (Hara et al., 2003). Thus, we added exogenous IL-2 to help compensate for the deficient IL-2 production of the CARMA1^{-/-} T cells. We found that without the supplemental IL-2 the CARMA1^{-/-} T cells only survive for 2-3 days in culture (data not

shown), however with additional IL-2 survival improved. To assess effects of CARMA1 on the expression of T cell migration markers, we isolated activated WT and CARMA1^{-/-} T cells at three days post activation. At day 3, we analyzed the T cells using flow cytometry for proliferation by assessing the level of CFSE dilution. In addition, we also labeled the cells for CD44 and CD62L expression. We had previously assessed proliferation at days 1 and 2 post activation and found little proliferation by CFSE dilution at these time points (data not shown). By the third day after activation the WT T cells showed significant proliferation and regulation of expression of cell surface molecules (See Figure 4). Thus we determined that day 3 was the optimal day to analyze differences between activated WT and CARMA1^{-/-} T cells.

Upon activation, T cells upregulate CD44, which acts as a ligand for E-selectin which directs the T cells to inflamed tissue (Nácher et al., 2011). Previously Hara et al. showed that there was a defect in upregulation of CD44 in CARMA1^{-/-} T cells 24 hours after activation. Similar to Hara et al., we found that 3 days after activation CARMA1^{-/-} T cells did not upregulate CD44 to the same extent as WT T cells (Figure 4A,B). The difference in upregulation was not due to impaired proliferation however; while the CARMA1^{-/-} T cells did not proliferate as well as the WT T cells, even CARMA1^{-/-} T cells that had proliferated still showed defective CD44 upregulation compared with WT T cells (Figure 4A).

When T cells are activated, they also downregulate CD62L, which normally enables homing of T cells to the lymph nodes (Jung, Gallatin, Weissman, & Dailey, 1988). We

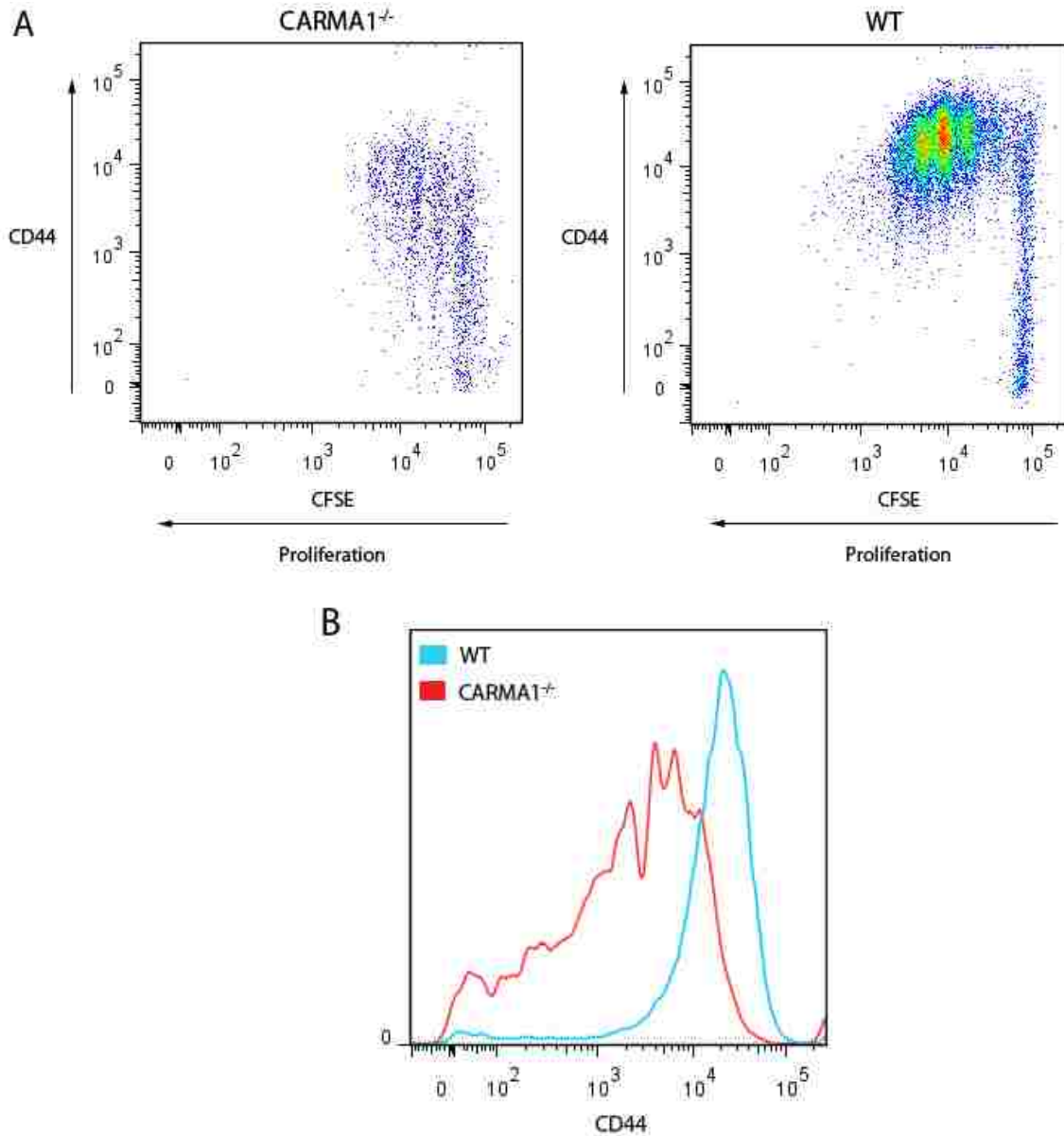


Figure 4. CARMA1^{-/-} T cells have defective upregulation of CD44 compared with WT T cells. Naive T cells isolated from lymph nodes were stained with 5 μ M CFSE and activated with α -CD3 and α -CD28 for 3 days before analysis. Activated T cells were stained with fluorescent α -CD44 prior to analysis with flow cytometry. (A) Decreased CFSE intensity indicates cells that have proliferated. Arrows indicate the approximate average CD44 intensity. (B) Histogram showing CD44 intensity. Proliferated CARMA1^{-/-} T cells have lower expression of CD44 than the proliferated WT T cells.

found that while the majority of the proliferated WT T cells had down-regulated CD62L, most of the CARMA1^{-/-} T cells were still CD62Lhi (Figure 5A,B). As we saw with CD44, the lack of down-regulation was not simply due to the impaired proliferation; even the CARMA1^{-/-} T cells that had proliferated, most still showed CD62Lhi expression profile. These data show that CARMA1 plays a role in regulating the change in expression of migration markers upon T cell activation that is independent of proliferation, regulating CD62L downregulation and CD44 upregulation, likely leading to changes in the migration capacity of T cells upon activation.

CARMA 1 controls the motility of activated T cells in vitro

CARMA1^{-/-} T cells show major defects in activation, including defective regulation of the expression of cell surface molecules involved in T cell migration. This led us to investigate whether CARMA1^{-/-} T cells have defective motility as well. To do this, we used an in vitro migration assay to assess the effect of CARMA1 on T cell motility. We activated WT T cells and CARMA1^{-/-} T cells as previously described and seven to nine days post activation we isolated the activated T cells with Ficoll gradient to remove dead cells. We then imaged the activated T cells in the presence of CCL21 on dishes coated with ICAM-1 to induce migration. T cells that adhered to the dish were captured by time-lapse in 2 second increments for up to 10 minutes and videos of cell motility were analyzed for multiple parameters (Figure 6). Individual cells were tracked for 5 to 7 minutes; cells that moved out of the field of view or collided with other cells were not counted.

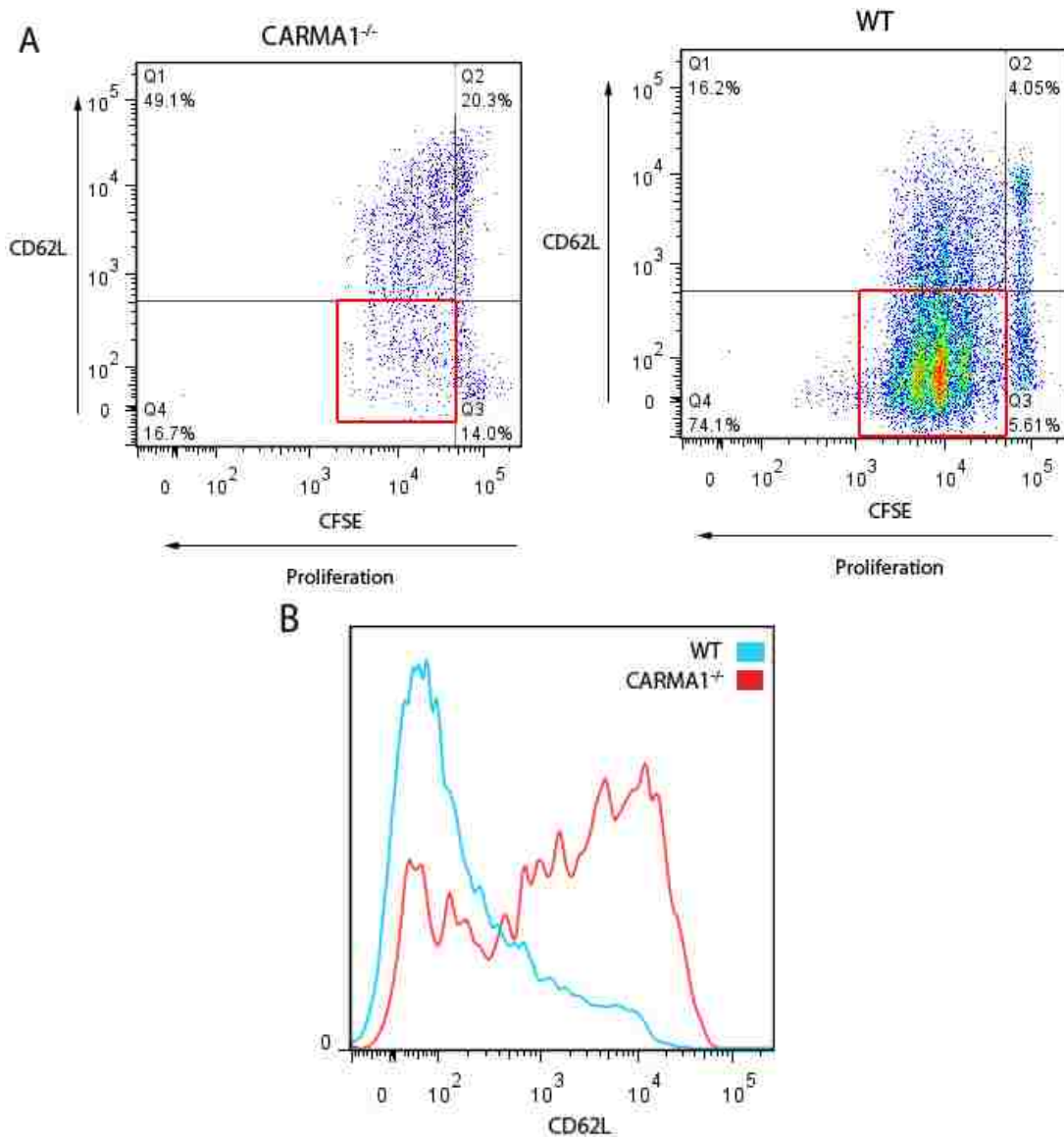
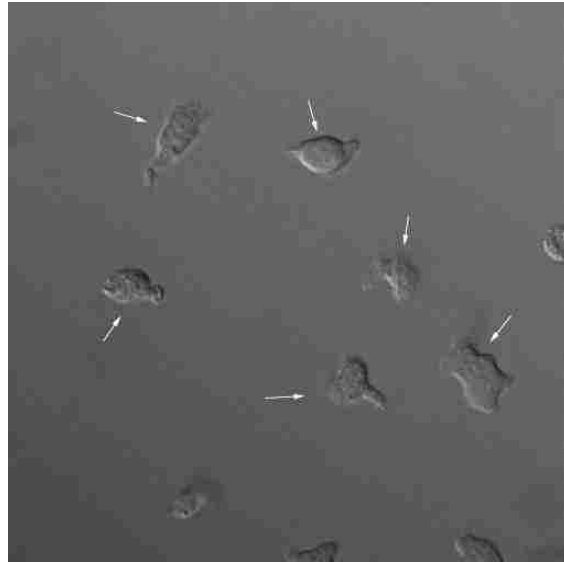
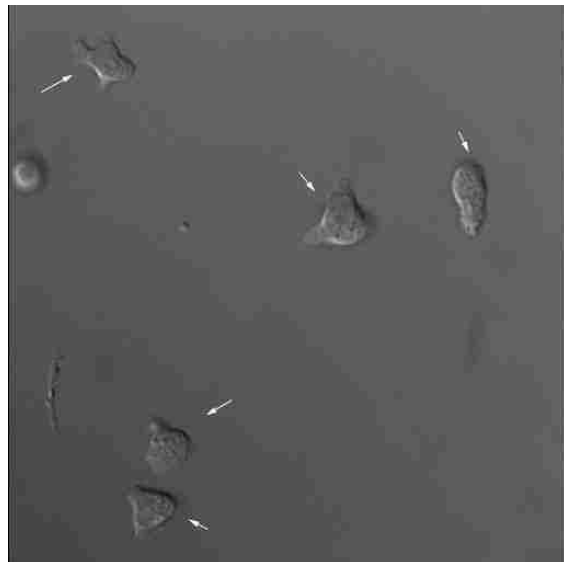


Figure 5. CARMA1^{-/-} T cells show defective downregulation of CD62L. Naive T cells isolated from lymph nodes were stained with 5 μ M CFSE and activated with α -CD3 and α -CD28 for 3 days before analysis. Activated T cells were stained with fluorescent α -CD62L prior to analysis with flow cytometry. (A) Decreased CFSE intensity indicates cells that have proliferated. The areas indicated with the red square show cells that have proliferated and have downregulated CD62L. Only 19% of CARMA1^{-/-} T cells proliferated and downregulated CD62L compared with 75% of WT T cells. (B) Histogram showing CD62L intensity.



WT



CARMA1^{-/-}

Figure 6. Activated WT and CARMA1^{-/-} T cells migrating on ICAM-1. WT and CARMA1^{-/-} T cells were activated with α -CD3 and α -CD28 7-9 days prior to the in vitro migration assay. T cells were suspended in media with 300ng/ml CCL21 and placed on dishes coated with ICAM-1. T cells were imaged in a 37°C 5% CO₂ humidified chamber starting 15 minutes after being placed in the chamber, taking time-lapse images in 2 second increments for up to 10 minutes. One frame of a video is shown; arrows indicate migrating cells.

We found that surprisingly, the activated CARMA1^{-/-} T cells had a much higher average speed at 22.1 μm/min than the WT T cells at 14.2 μm/min, as well as a higher maximum speed (57 μm/min vs. 46 μm/min) (Figure 7A,B). We then analyzed the chemotactic index, the distance a cell moved in a given direction divided by the total displacement of the cell (Moghe, Nelson, & Tranquillo, 1995). While CARMA1^{-/-} T cells moved at a higher velocity than WT T cells, we found no difference in chemotactic index between WT and CARMA1^{-/-} T cells (Figure 7C). As we found differences in migration of activated WT and CARMA1^{-/-} T cells, we also took naïve WT and CARMA1^{-/-} T cells purified from mouse lymph nodes on nylon wool and analyzed their migration on ICAM-1 in the presence of CCL21. Interestingly, there was no difference in average or maximum speed or chemotactic index between naïve WT T cells and CARMA1^{-/-} T cells (Figure 8). These data indicate that CARMA1 does play a role in regulating the motility of activated T cells, but not naïve T cells.

CARMA1 does not affect T cell dependence on CCR7 signaling for activated T cell motility in vitro

Naïve T cells have been shown to require CCL21 activation of CCR7 to induce adhesion and motility on ICAM-1 in vitro (Stachowiak, Wang, Huang, & Irvine, 2006). Activated T cells are not as dependent on CCR7, since signaling through the TCR complex upon activation can also enhance the binding of LFA-1 to ICAM-1 (Van Kooyk, Van De Wiele, van Kemenade, Weder, Kuijpers, & Figdor, 1989). However it is unknown whether the signaling through CCR7 might still play a role in migration of activated T cells. Our previous results in Figures 7 and 8 showing that CARMA1 plays a role in regulating the

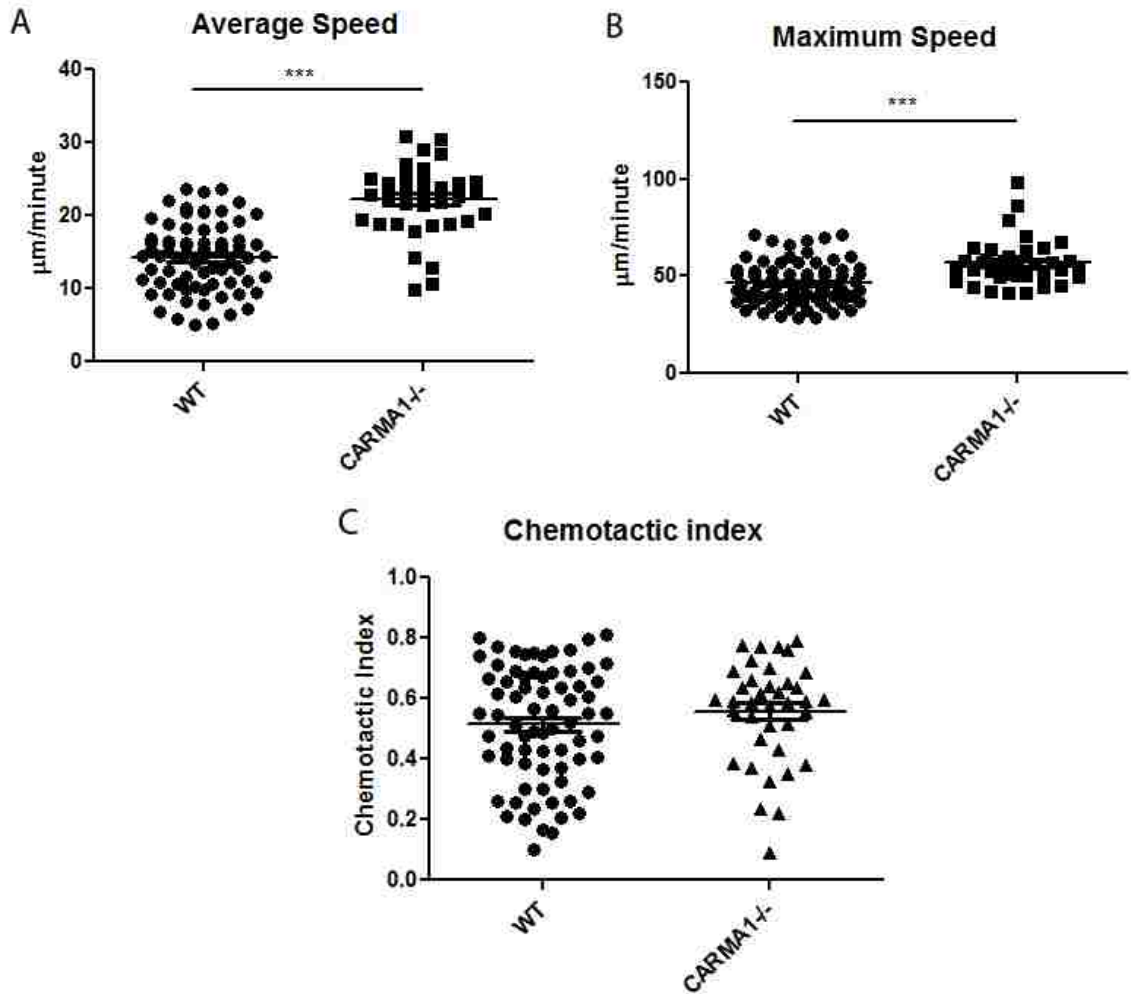


Figure 7. Activated CARMA1^{-/-} T cells have a higher average and maximum speed than activated WT T cells. Videos of activated WT and CARMA1^{-/-} T cells, as shown in Figure 6, were analyzed and individual cells tracked for 5-7 minute intervals. (A) CARMA1^{-/-} T cells had an average speed of 22 µm/min which was much higher than WT T cells at 14 µm/min. (B) CARMA1^{-/-} T cells had a higher maximum speed than WT T cells, at 57 µm/min compared to 46 µm/min. (C) Chemotactic index is defined as distance migrated from starting point / total displacement; there was no significant difference between CARMA1^{-/-} T cells and WT T cells. Each dot represents a single cell; plots show data from 3 experiments. *** indicates $p < 0.0001$. Differences were considered statistically significant with $p < 0.05$.

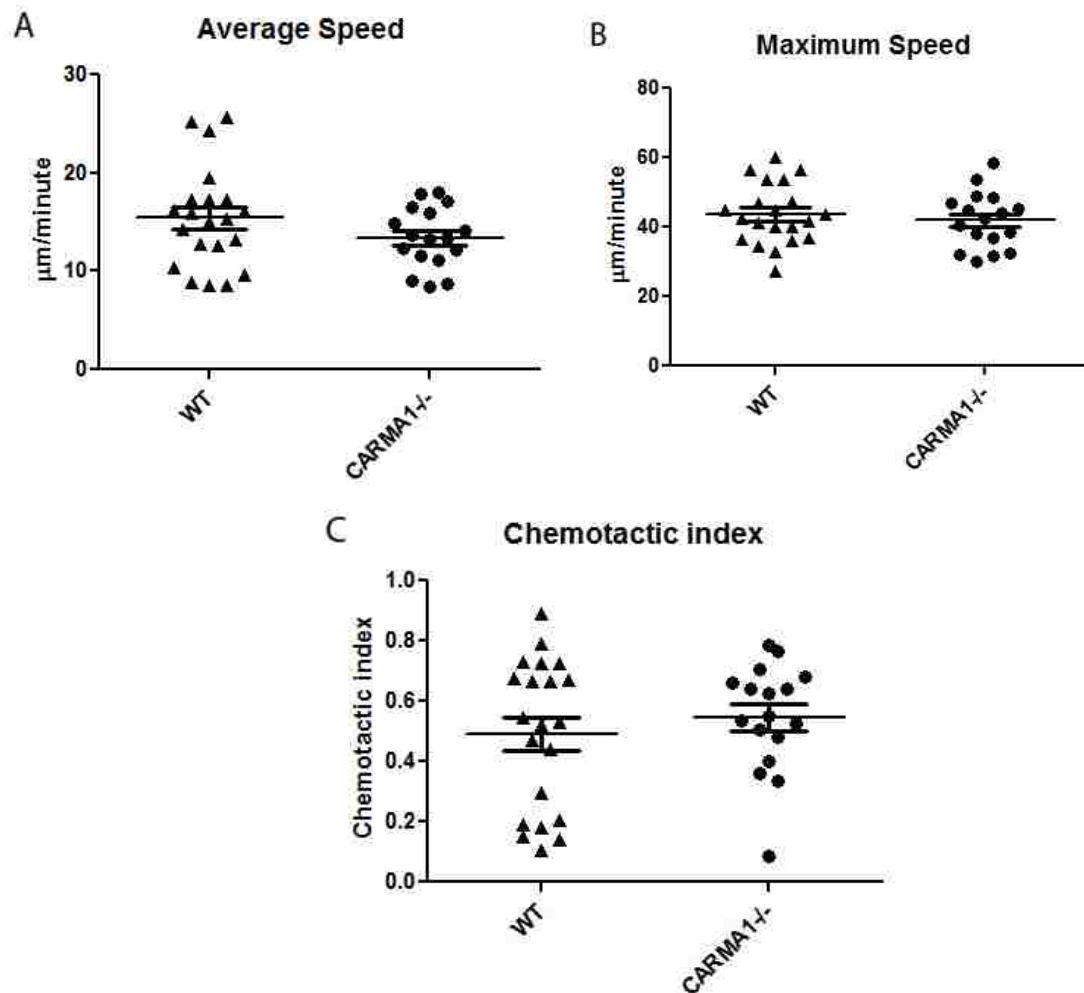


Figure 8. Naïve CARMA1^{-/-} and WT T cells migrate similarly on ICAM-1. Naïve WT and CARMA1^{-/-} T cells were isolated and suspended in media with 300ng/ml CCL21 and placed on dishes coated with ICAM-1. T cells were imaged in a 37°C 5% CO₂ humidified chamber starting 1 hour after being placed in the chamber, taking time-lapse images in 2 second increments for up to 10 minutes. Average speed (A), maximum speed (B), and chemotactic index (C) were determined. No significant difference was seen for any of these parameters.

motility of activated but not naïve T cells suggests that CARMA1 may play differential roles in regulating T cell migration, possibly due to differences in CCR7-dependent versus CCR7-independent migration.

To determine whether CARMA1 played a role directly in CCR7-driven T cell motility, we tested the role of CARMA1 in CCR7 mediated T cell migration by using the in vitro motility assay described in Figure 6. We activated WT and CARMA1^{-/-} T cells as previously described and at 7-9 days post activation purified the live T cells and assayed their motility on ICAM-1. To test the role of CCL21, we assayed the motility of activated WT and CARMA1^{-/-} T cells in the absence of CCL21. We found that WT and CARMA1^{-/-} T cells both still adhered and were motile in the absence of CCL21 (data not shown). However, the WT T cells had a lower average speed than in the presence of CCL21, migrating at 9.9 $\mu\text{m}/\text{min}$ down from 14.2 $\mu\text{m}/\text{min}$ (Figure 9A). Likewise, CARMA1^{-/-} T cells migrated with a lower average speed in the absence of CCL21 than when CCL21 was present, migrating at 15.2 $\mu\text{m}/\text{min}$ down from 22.1 $\mu\text{m}/\text{min}$ (Figure 9A). While both WT and CARMA1^{-/-} T cells had decreased migration speed in the absence of CCL21, CARMA1^{-/-} T cells still migrated faster than the WT T cells. These data indicate that activated WT T cells and CARMA1^{-/-} T cells are both partially dependent on CCL21 for migration on ICAM-1.

We also asked whether the in vitro motility in our assay were due to chemokine receptor signaling. Chemokine receptors belong to the 7 trans-membrane spanning G-protein coupled receptors (GPCRs). To test whether the effects of CCL21 were through

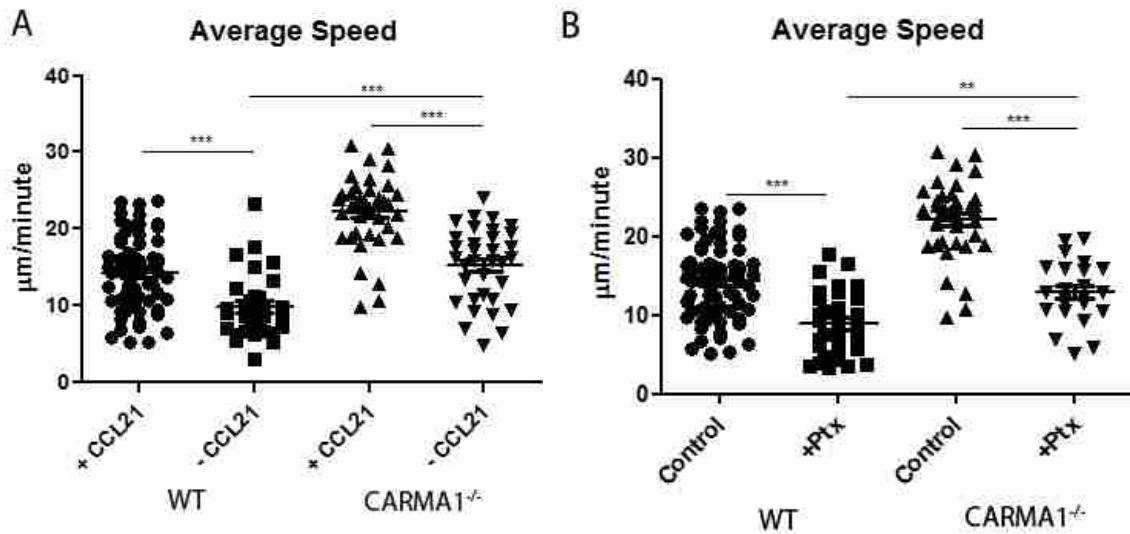


Figure 9. Activated WT and CARMA1^{-/-} T cells are partially dependent on CCL21 and CCR7 signaling for migration on ICAM-1. WT and CARMA1^{-/-} T cells were activated with α -CD3 and α -CD28 7-9 days prior to the in vitro migration assay. T cells were imaged on dishes coated with ICAM-1 in a 37°C 5% CO₂ humidified chamber starting 15 minutes after being placed in the chamber, taking time-lapse images in 2 second increments for up to 10 minutes. Videos of activated WT and CARMA1^{-/-} T cells were analyzed and individual cells tracked for 5-7 minute intervals. (A) WT and CARMA1^{-/-} T cells were imaged either in media with 300ng/ml CCL21 (+CCL21) or in media with no CCL21 (-CCL21). Both WT and CARMA1^{-/-} T cells had slower average speeds in the absence of CCL21. (B) WT and CARMA1^{-/-} T cells were either treated with 0.5 $\mu\text{g}/\text{ml}$ pertussis toxin (+Ptx) or imaged without treatment (Control). Both conditions were imaged in media with 300ng/ml CCL21. WT and CARMA1^{-/-} T cells both moved with a slower average speed following Ptx treatment than the untreated T cells. **: p=0.0006, ***: p<0.0001. Differences were considered statistically significant with p<0.05.

chemokine receptors, we also assayed the motility of activated WT and CARMA1^{-/-} T cells treated with pertussis toxin (Ptx). Ptx ADP-ribosylates the α subunit of Gi proteins, locking them in the inactive state, thus inhibiting signaling through all GPCRs coupled with Gi proteins, which includes CCR7, the receptor for CCL21 as well as all other chemokine receptors (Locht, Coutte, & Mielcarek, 2011). Again, we isolated activated WT and CARMA1^{-/-} T cells, then treated each cell type with 0.5 μ g/ml Ptx, then imaged the T cells on ICAM-1 in the presence of CCL21. We found that the WT T cells and CARMA1^{-/-} T cells both had a decrease in speed when treated with Ptx as they had in the absence of CCL21; the T cells adhered and were motile (data not shown), but had a lower average speed than with normal treatment. The WT T cells migrated at an average speed of 8.9 μ m/min vs. 14.2 μ m/min, and the CARMA1^{-/-} T cells migrated at an average speed of 12.9 μ m/min vs. 22.1 μ m/min (Figure 9B). Again, even with the decrease in speed with Ptx treatment, the CARMA1^{-/-} T cells still migrated faster than the WT T cells. These data indicate that both activated WT and CARMA1^{-/-} T cells are partially dependent on CCR7 signaling for motility on ICAM-1.

Together, these data indicate that activated WT T cells and CARMA1^{-/-} T cells respond similarly to CCL21 signaling through CCR7 and that they rely partly on CCR7 signaling for motility on ICAM-1, and that likely CCR7 is the major chemokine receptor that acts in both WT T cells and CARMA1^{-/-} T cells to induce motility, as lack of CCL21 and Ptx treatment showed similar effects. However, even in the absence of CCR7 signaling activated CARMA1^{-/-} T cell migration is still faster than WT T cell migration, indicating that the increased speed of the CARMA1^{-/-} T cells is likely not due to chemokine receptor

signaling, but is due to some other mechanism.

CARMA1 regulates the localization of ERM proteins to the uropod

The data above show that CARMA1 plays a role in expression of migration cell surface molecules and in T cell motility on ICAM in response to CCL21. T cell motility is intimately associated with T cell polarization, or the specific localization of proteins to discrete domains on the migrating T cell. As a T cell migrates, cell surface proteins and intracellular molecules such as chemokine receptors, actin regulatory proteins, and cytoskeletal components segregate to the leading edge or uropod to induce T cell migration. The actin regulatory molecules ERM proteins have been shown to be important in T cell uropod formation, and thus we asked whether CARMA1 might be involved in controlling ERM localization.

To do this we used immunofluorescence to analyze the localization of ERM proteins in naïve WT or CARMA1^{-/-} T cells. We isolated WT and CARMA1^{-/-} T cells from mouse lymph nodes using nylon wool and placed the T cells on coverslips coated in poly-L-lysine, which induces the attachment of T cells. We treated isolated WT and CARMA1^{-/-} T cells with CCL21 to induce the migratory phenotype which leads to polarization of ERM proteins to the T cell uropod. The T cells were fixed and then incubated with antibodies to either moesin or phospho-ERM (p-ERM), followed by a secondary fluorescent antibody to detect localization of ERM proteins. We assayed the localization to the uropod of both moesin and of p-ERM1 in the T cells, since ERMs are phosphorylated in their active form and phosphorylated ERM proteins have been shown to

1 Immunofluorescence data for pERM localization was obtained and analyzed by Ivy Brown.

localize to the T cell uropod as well (Allenspach et al., 2001).

We found that T cells treated with CCL21 show a typical migratory phenotype, with a leading edge and the smaller protruding uropod (Figure 10A, B). We counted the number of T cells which showed the fluorescent signal at the smaller end of the migrating T cell, which we presumed to be the T cell uropod. We found that a majority of WT T cells with the migratory phenotype showed moesin localization to the uropod (55%, Figure 10C). We found that in the *CARMA1*^{-/-} T cells the localization of moesin to the uropod was significantly decreased compared to WT (55% WT to 27% *CARMA1*^{-/-}) (Figure 10C). When we determined the localization of p-ERM proteins, we found that there was a slight decrease in p-ERM localization in *CARMA1*^{-/-} T cells compared to WT cells, however, the decrease was not statistically significant (Figure 10D). These results indicate that *CARMA1* is important for the localization of the total ERM proteins but affects the localization of p-ERMs to a lesser degree.

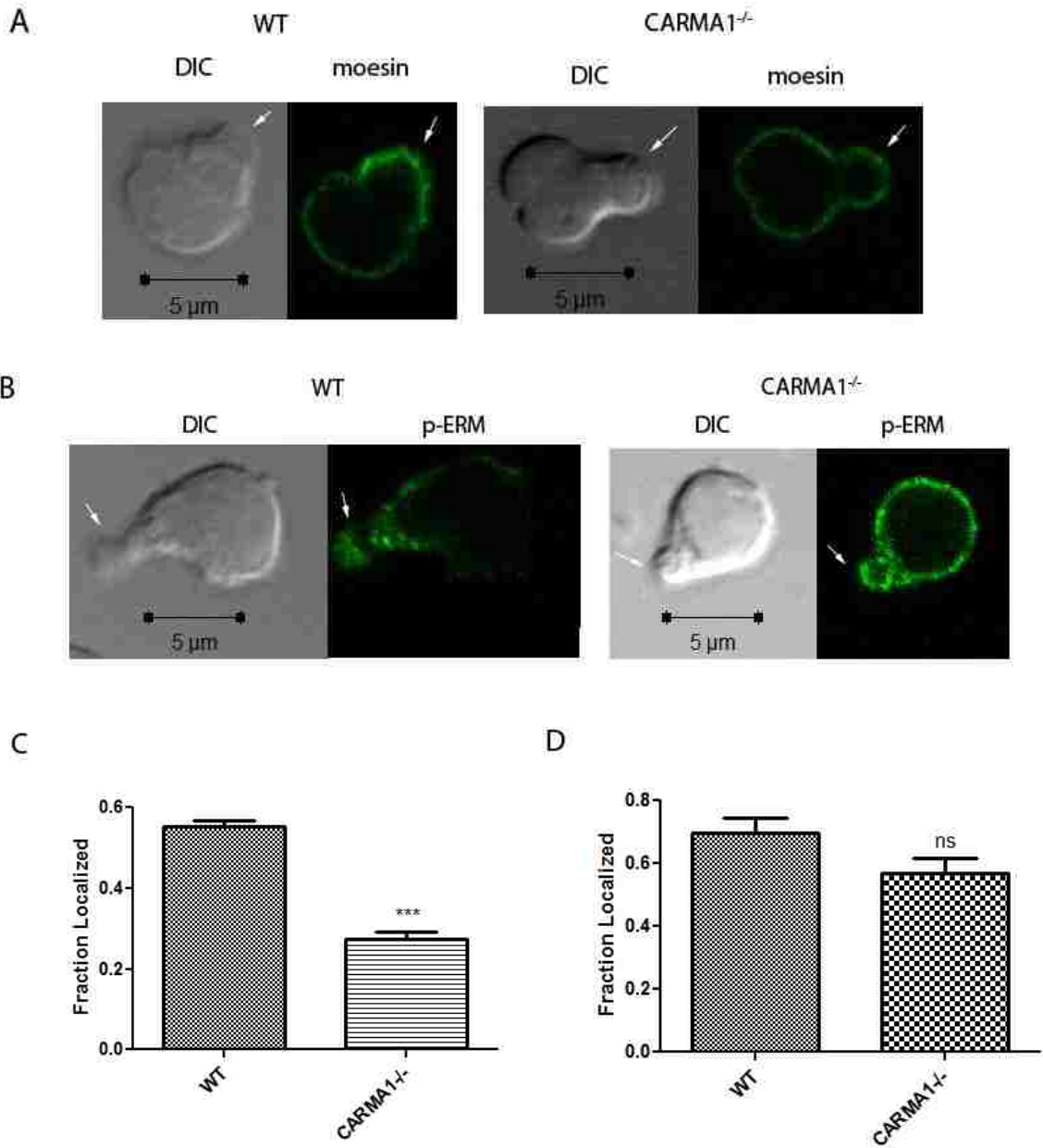


Figure 10. Localization of moesin and p-ERM in WT and CARMA1^{-/-} T cells. WT and CARMA1^{-/-} T cells were treated with 300 ng/ml CCL21 and placed on coverslips coated in poly-L-lysine. The T cells were fixed and labeled with antibodies to detect localization of moesin and p-ERM. (A&B) Localization of moesin (A) and p-ERM (B) in WT and CARMA1^{-/-} T cells; the uropod is indicated with an arrow. Moesin and p-ERM are localized to the uropod in the WT T cells while it is evenly distributed around the CARMA1^{-/-} T cells. (C&D) Fraction of localization of moesin (C) and p-ERM (D) to the uropod. Significantly fewer CARMA1^{-/-} T cells had moesin localized to the uropod than WT T cells. *** indicates p=0.0002, ns = not significant. Differences were considered statistically significant with p<0.05.

Summary

These data show for the first time a role for CARMA1 in T cell migration, regulating multiple aspects of migration. We show that CARMA1 is involved in regulating the expression of cell surface migration molecules upon activation, specifically regulating both CD44 and CD62L. In addition CARMA1 is important for localization of ERM proteins to the uropod. We also show that activated CARMA1^{-/-} T cells move with increased speed in vitro compared to WT T cells and that activated WT and CARMA1^{-/-} T cells both rely partly on signaling from CCL21 through CCR7 for motility. These data suggest that activated CARMA1^{-/-} T cells may have defective migration to non-lymphoid tissues, as they do not upregulate CD44 or downregulate CD62L properly. As the WT and CARMA1^{-/-} T cells respond similarly to lack of CCL21 and to inhibition of chemokine receptor signaling, this is not likely the cause of the increase in activated CARMA1^{-/-} T cells migration speed. A possible mechanistic basis for the increased speed that we see in the CARMA1^{-/-} T cells could be the mislocalization of ERM proteins, as this may lead to improper regulation of the actin cytoskeleton which could affect migration speed.

2. The role of PKC θ in T cell migration

PKC θ is activated following TCR activation and is involved in signaling pathways leading to activation of NF- κ B, AP-1 and JNK and increased survival, proliferation, and cytokine production. In these signaling pathways, PKC θ directly phosphorylates CARMA1, which we have just shown is involved in many aspects of T cell migration. Because we have shown a novel role for CARMA1 in regulating T cell migration, we hypothesized that PKC θ would also regulate various aspects of T cell migration.

PKC θ does not regulate expression of migration markers upon T cell activation

Like CARMA1^{-/-} T cells, PKC θ ^{-/-} T cells have defective activation and reduced IL-2 production upon activation (Sun et al., 2000). Also as with CARMA1, little is known about the role of PKC θ in other aspects of T cell function, such as T cell motility. In Part 1, we found that upon T cell activation CARMA1 regulates the expression of CD44 and CD62L, two cell surface molecules involved in T cell migration that are expressed differently in naïve and activated T cells. Because PKC θ is known to phosphorylate CARMA1, we hypothesized that PKC θ would also be involved in regulating the expression of these migration markers. To investigate the role of PKC θ in the expression of CD44 and CD62L, we isolated WT and PKC θ ^{-/-} T cells on nylon wool and stained them with CFSE in order to track their proliferation as done in Figure 4. We activated the T cells as previously described, adding IL-2 to compensate for the decreased IL-2 production of the PKC θ ^{-/-} T cells. PKC θ ^{-/-} T cells, like CARMA1^{-/-} T cells, were previously shown to have reduced IL-2 production (Altman et al., 2000). Three days post activation the cells were analyzed using flow cytometry for proliferation using CFSE

dilution. We also labeled the cells for CD44 and CD62L expression.

Interestingly, while CARMA1^{-/-} T cells showed defects in CD44 upregulation upon T cell activation, and we found no defect in the upregulation of CD44 in PKCθ^{-/-} T cells compared with WT T cells (Figure 11A,B). Similarly, while CARMA1^{-/-} T cells had defects in CD62L downregulation following T cell activation, we saw that PKCθ^{-/-} T cells showed similar downregulation of CD62L as WT T cells (Figure 12A,B). While the PKCθ^{-/-} T cells did proliferate, there were fewer cells recovered at day 3 than in WT T cells, but there did not appear to be a significant defect in proliferation, at least as assessed by CFSE dilution (see Figure 12A). These data show that unlike CARMA1, PKCθ is not involved in regulating the expression of CD44 or CD62L upon T cell activation.

PKCθ does not affect the motility of activated T cell in vitro

We had previously found that CARMA1^{-/-} T cells showed differences in in vitro motility compared to WT T cells (Chapter 1 Figure 7). Thus, we also asked whether PKCθ might also regulate the activity of CARMA1 to control T cell motility. We previously showed that CARMA1^{-/-} T cells have increased speed in the presence of CCL21 on ICAM-1 in vitro, so this led us to investigate whether PKCθ^{-/-} T cells show a similar change in motility. To do this we used the in vitro motility assay as described in Figure 6 and 7. We activated WT and PKCθ^{-/-} T cells as previously described and 7-9 days post activation isolated the live cells on a Ficoll gradient. Again we imaged the WT and PKCθ^{-/-} T cells on ICAM-1 coated dishes in the presence of CCL21. We analyzed individual cells in the

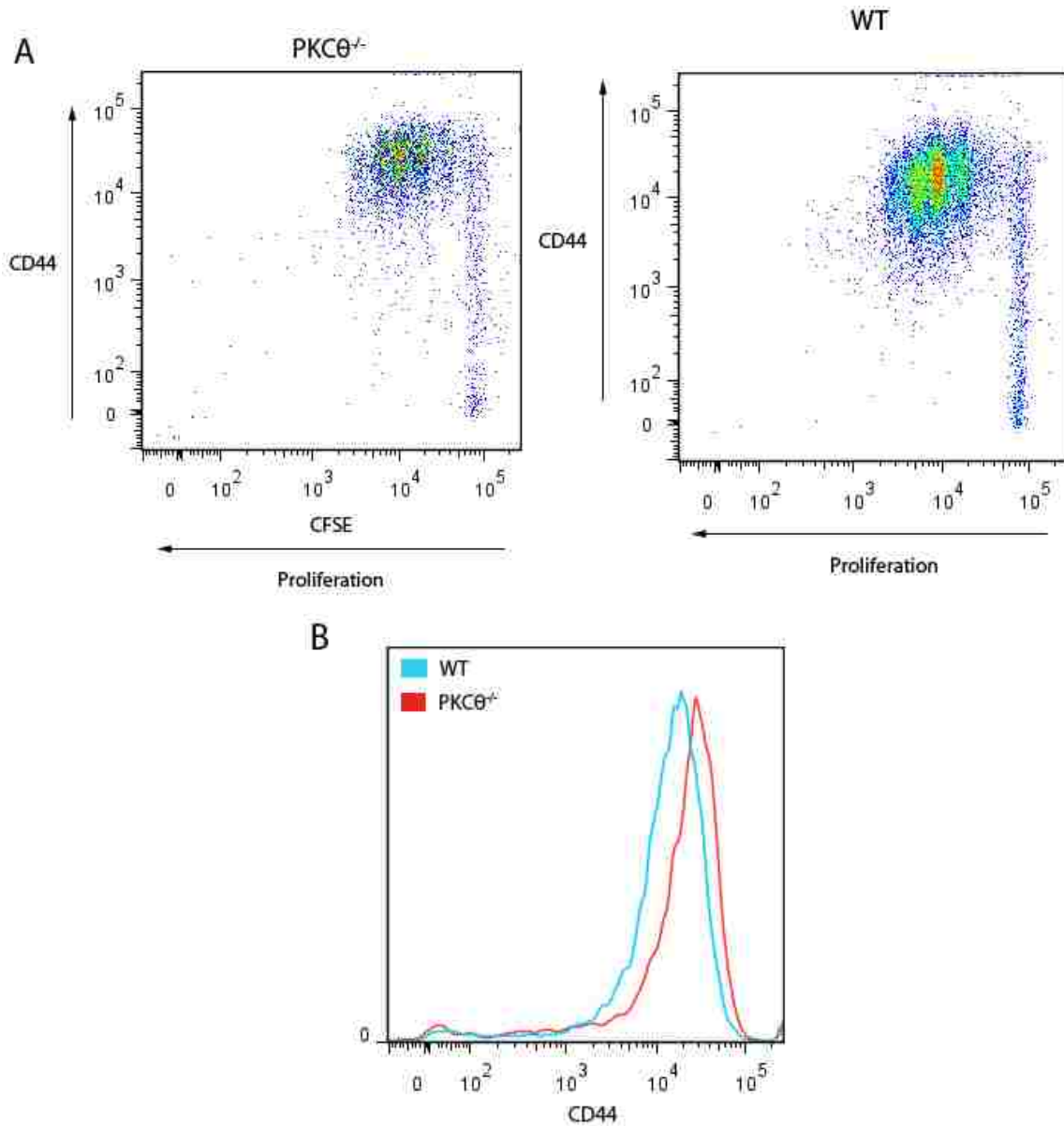


Figure 11. PKC $\theta^{-/-}$ T cells upregulate CD44 like WT T cells. PKC $\theta^{-/-}$ T cells upregulate CD44 like WT T cells. Naive T cells isolated from lymph nodes were stained with 5 μ M CFSE and activated with α -CD3 and α -CD28 for 3 days before analysis. Activated T cells were stained with fluorescent α -CD44 prior to analysis with flow cytometry. (A) Decreased CFSE intensity indicates cells that have proliferated. (B) Histogram comparing CD44 intensity. No difference was seen in CD44 intensity between WT and PKC $\theta^{-/-}$ T cells.

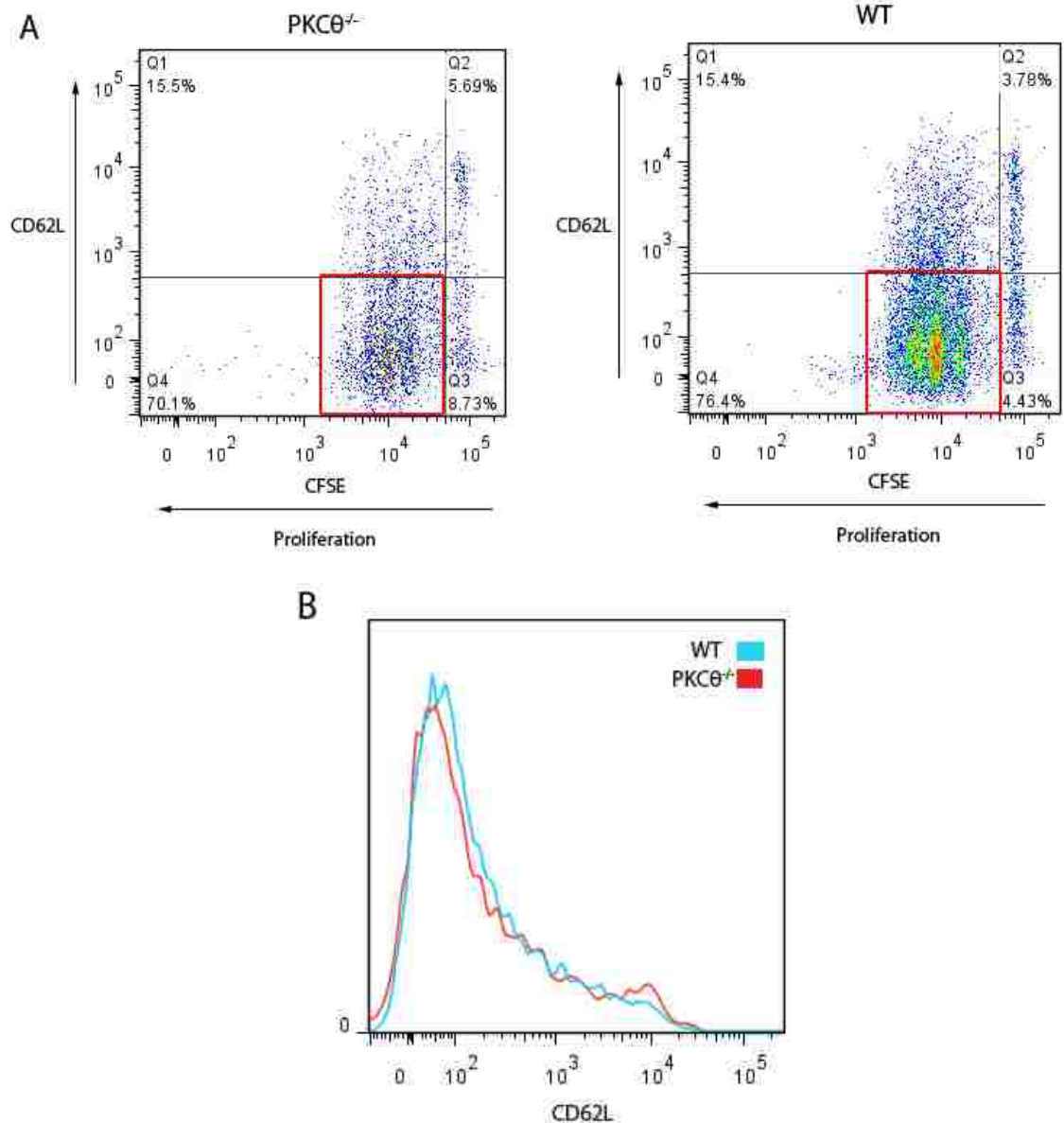


Figure 12. PKCθ^{-/-} T cells downregulate CD62L like WT T cells. Naive T cells isolated from lymph nodes were stained with 5μM CFSE and activated with α-CD3 and α-CD28 for 3 days before analysis. Activated T cells were stained with fluorescent α-CD62L prior to analysis with flow cytometry. (A) Decreased CFSE intensity indicates cells that have proliferated. The areas indicated with the red square show cells that have proliferated and have downregulated CD62L. WT and PKCθ^{-/-} T cells had similar percentages of T cells that had proliferated and downregulated CD62L. (B) Histogram comparing CD44 intensity.

videos, tracking them for 5-7 minutes of continuous unobstructed adhesion. We found that unlike the CARMA1^{-/-} T cells, there was no difference between activated WT and PKCθ^{-/-} T cells in average speed, maximum speed, or chemotactic index (Figure 13). We also compared the motility of naïve WT and PKCθ^{-/-} T cells, and we again saw no difference between the in vitro motility of WT and PKCθ^{-/-} T cells (Figure 14). These data show that unlike CARMA1, PKCθ does not affect the motility of activated T cells on ICAM-1 in vitro.

PKCθ does not affect T cell dependence on CCR7 signaling for activated T cell motility in vitro

Naïve T cells are dependent on CCL21 signaling through CCR7 for motility in vitro on ICAM-1, while activated T cells are less dependent. Our previous results have shown that activated WT T cells have a partial dependence on CCL21/CCR7 signaling for motility on ICAM-1, and while CARMA1^{-/-} T cells also have a partial dependence on CCR7 signaling for T cell motility, they also likely utilize other chemokine receptors to affect motility. We have also shown that PKCθ does not play a role in the motility of naïve or activated T cells on ICAM-1 in vitro. This lead us to investigate the role of PKCθ in CCR7 mediated migration. We activated WT and PKCθ^{-/-} T cells as previously described and 7 to 9 days post activation isolated the live cells using Ficoll. To test the role of CCL21 we placed activated WT and PKCθ^{-/-} T cells on ICAM-1 coated dishes in the absence of CCL21. We found that the PKCθ^{-/-} T cells responded similarly to WT T cells, and the speed of the WT and PKCθ^{-/-} T cells was similar in the absence of CCL21 (Figure 15A). The average speed of WT T cells in the absence of CCL21 was

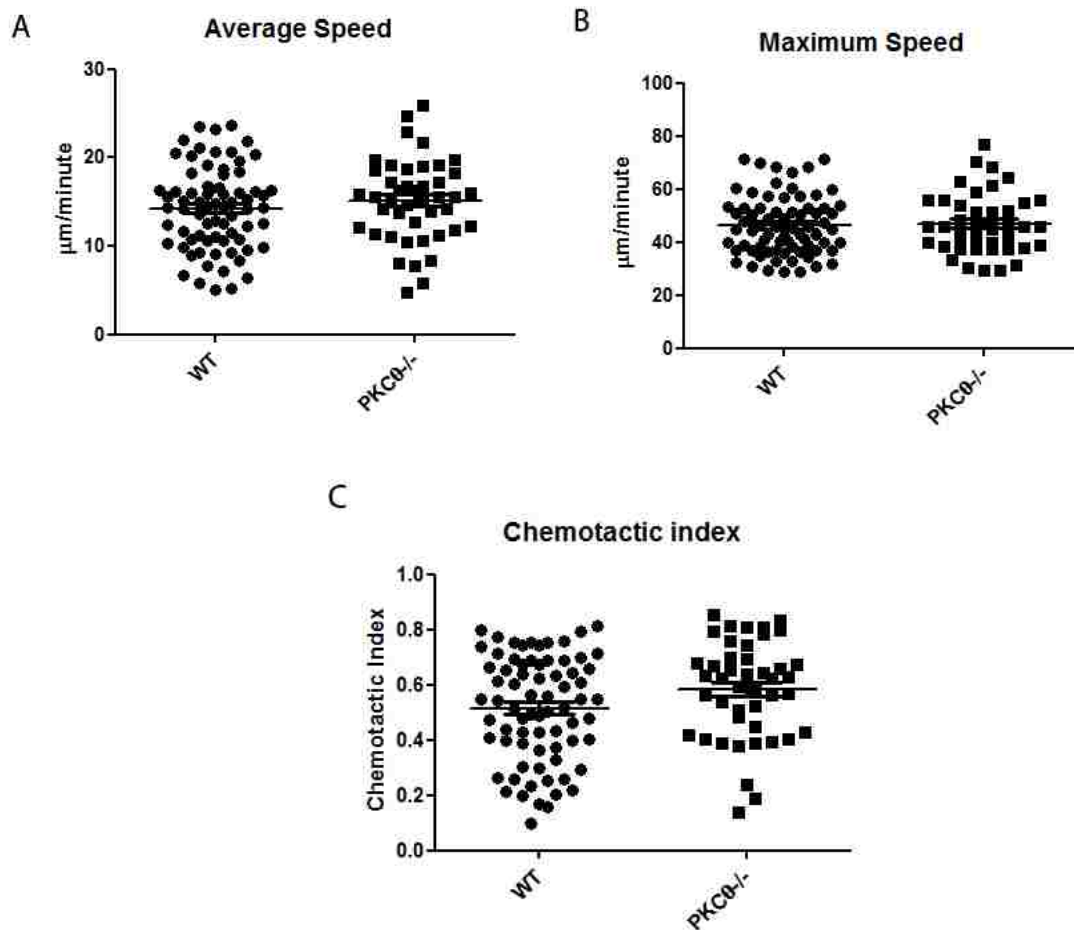


Figure 13. Activated PKC $\theta^{-/-}$ T cells migrate on ICAM-1 with similar speed and chemotactic index as WT T cells. WT and PKC $\theta^{-/-}$ T cells were activated with α -CD3 and α -CD28 7-9 days prior to the in vitro migration assay. T cells were suspended in media with 300ng/ml CCL21 and placed on dishes coated with ICAM-1. T cells were imaged in a 37°C 5% CO₂ humidified chamber starting 15 minutes after being placed in the chamber, taking time-lapse images in 2 second increments for up to 10 minutes. Videos of activated WT and PKC $\theta^{-/-}$ T cells were analyzed and individual cells tracked for 5-7 minute intervals. Average speed (A), Maximum speed (B), and Chemotactic index (C) were assessed; no significant difference was seen in any of the parameters assessed.

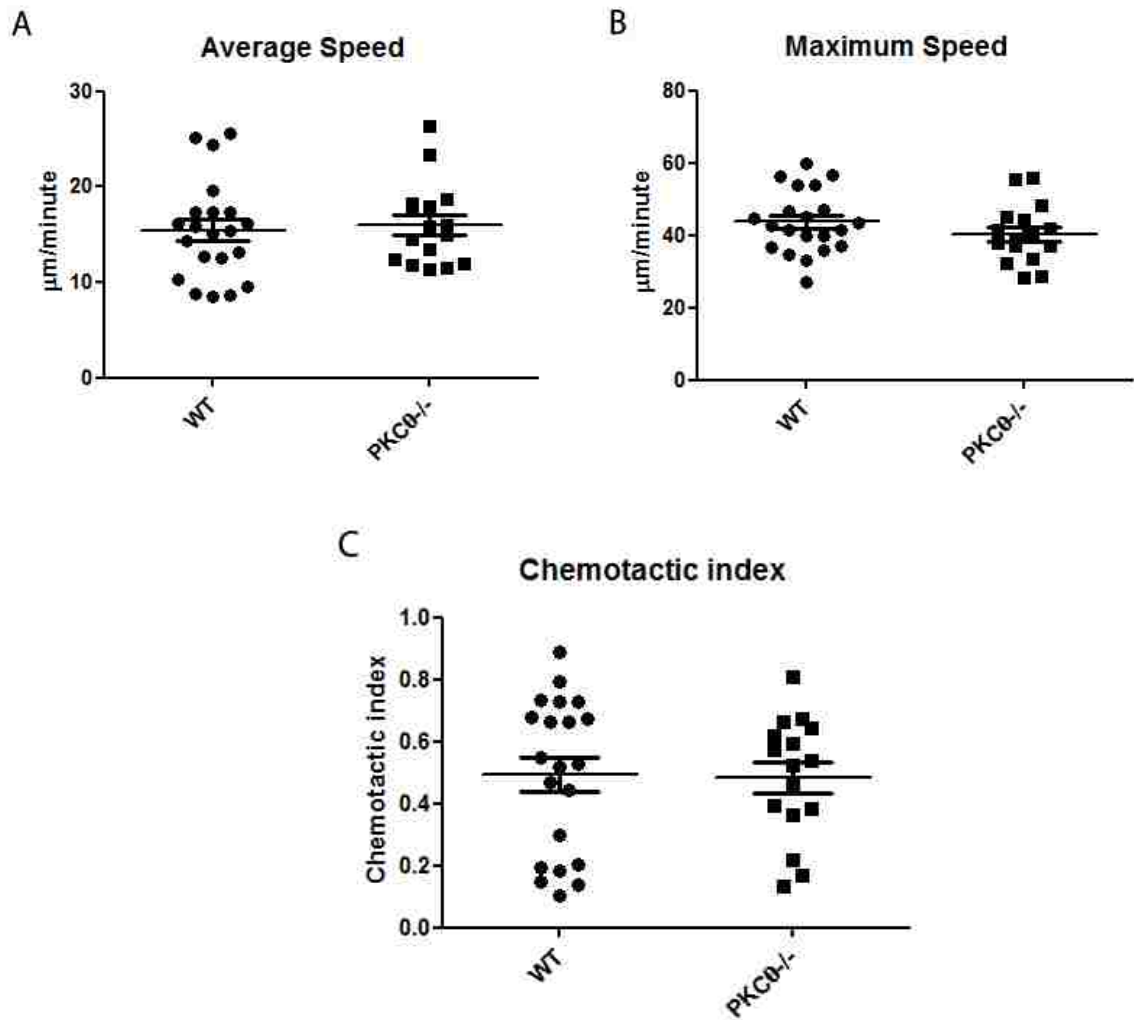


Figure 14. Naïve PKCθ^{-/-} T cells migrate on ICAM-1 with similar speed and chemotactic index as WT T cells. Naïve WT and PKCθ^{-/-} T cells were isolated and suspended in media with 300ng/ml CCL21 and placed on dishes coated with ICAM-1. T cells were imaged in a 37°C 5% CO₂ humidified chamber starting 1 hour after being placed in the chamber, taking time-lapse images in 2 second increments for up to 10 minutes. Videos of activated WT and PKCθ^{-/-} T cells were analyzed and individual cells tracked for 5-7 minute intervals. Average speed (A), maximum speed (B), and chemotactic index (C) were determined; no significant difference was seen in the parameters assessed.

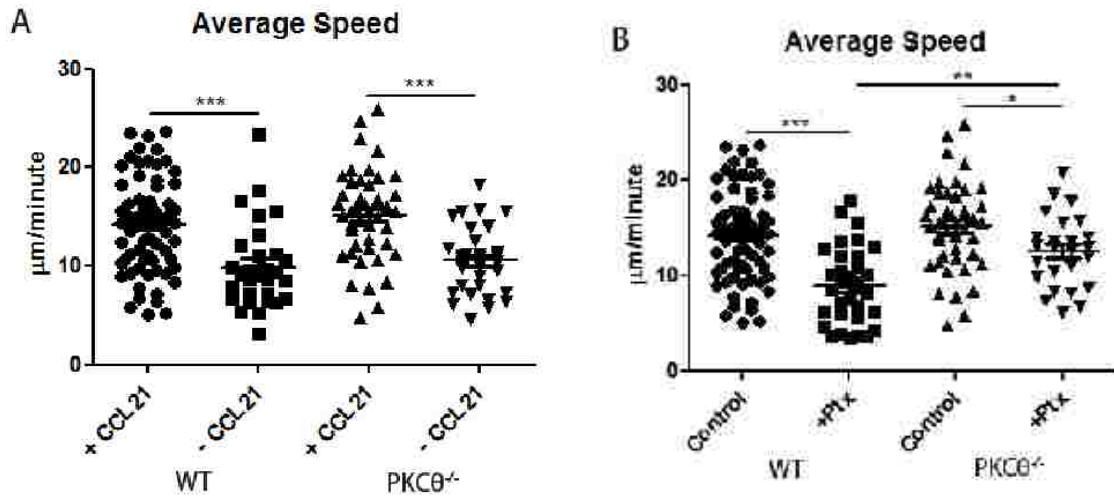


Figure 15. Activated WT and PKC $\theta^{-/-}$ T cells are partially dependent on CCL21 and CCR7 signaling for migration on ICAM-1. WT and PKC $\theta^{-/-}$ T cells were activated with α -CD3 and α -CD28 7-9 days prior to the in vitro migration assay. T cells were imaged on dishes coated with ICAM-1 in a 37°C 5% CO₂ humidified chamber starting 15 minutes after being placed in the chamber, taking time-lapse images in 2 second increments for up to 10 minutes. Videos of activated WT and PKC $\theta^{-/-}$ T cells were analyzed and individual cells tracked for 5-7 minute intervals. (A) WT and PKC $\theta^{-/-}$ T cells were imaged either in media with 300ng/ml CCL21 (+CCL21) or in media with no CCL21 (-CCL21). Both WT and PKC $\theta^{-/-}$ T cells had slower average speeds in the absence of CCL21. (B) WT and PKC $\theta^{-/-}$ T cells were either treated with 0.5 μ g/ml pertussis toxin (+Ptx) or imaged without treatment (Control). Both conditions were imaged in media with 300ng/ml CCL21. WT and PKC $\theta^{-/-}$ T cells both moved with a slower average speed following Ptx treatment than the untreated T cells. *: p=0.0182, **: p=0.0014, ***: p<0.0001. Differences were considered statistically significant with p<0.05.

9.9 $\mu\text{m}/\text{min}$, down from 14.2 $\mu\text{m}/\text{min}$ when CCL21 was present, and the average speed of PKC $\theta^{-/-}$ T cells in the absence of CCL21 was 10.5 $\mu\text{m}/\text{min}$ compared to 15.1 $\mu\text{m}/\text{min}$ with CCL21 (Figure 15A). These results indicate that activated WT and PKC $\theta^{-/-}$ T cells are both dependent on CCL21 for motility to a similar degree.

To test that these effects are through chemokine receptors, we treated the WT and PKC $\theta^{-/-}$ T cells with pertussis toxin (Ptx) to inhibit all GPCRs, including CCR7, and imaged the treated cells in the presence of CCL21. We saw a similar decrease in average speed in the Ptx treated WT T cells as in the untreated cells without CCL21, the Ptx treated WT T cells migrated at 8.9 $\mu\text{m}/\text{min}$, down from 14.2 $\mu\text{m}/\text{min}$ in the untreated WT T cells (Figure 15B), suggesting that most of the motility we observed was due to CCR7. We saw that the PKC $\theta^{-/-}$ T cells responded similarly to Ptx treatment as the WT T cells; we saw a decrease in average speed in the Ptx treated cells to 12.5 $\mu\text{m}/\text{min}$ compared to 15.1 $\mu\text{m}/\text{min}$ in the untreated T cells (Figure 15B). However, unlike in the T cells without CCL21, we found that the speed of the PKC $\theta^{-/-}$ T cells did not decrease as much as the WT T cells following Ptx treatment, and that they moved significantly faster, and the Ptx treated PKC $\theta^{-/-}$ T cells moved slightly faster than the PKC $\theta^{-/-}$ T cells with no CCL21, although the difference was not significant (Figure 15B). These data show that PKC $\theta^{-/-}$ T cells respond to CCL21 signaling through CCR7 similarly to WT T cells.

Together these data show that activated WT and PKC $\theta^{-/-}$ T cells are both partially dependent on CCL21 signaling through CCR7 for motility, and CCR7 is likely the major chemokine receptor inducing motility in both WT and PKC $\theta^{-/-}$ T cells because they

respond similarly to both lack of CCL21 and Ptx treatment. The PKC θ ^{-/-} T cells treated with Ptx moved slightly faster than those with no CCL21, but this may be due to inefficient treatment with Ptx as Ptx treatment should reduce the speed of the T cells at least as much as the lack of CCL21 does due to blocking of CCR7.

PKC θ regulates the localization of ERM proteins to the uropod

We have shown in the data above that PKC θ does not play a role in regulating CD44 and CD62L expression as well as in vitro motility. However, the localization of proteins in a polarized T cell is very important for motility and PKC θ is already known to interact with proteins that localize to the uropod. Specifically, PKC θ has been shown to phosphorylate moesin directly (Pietromonaco, Simons, Altman, & Elias, 1998). As moesin is the most abundant ERM protein found in T cells, and we have already shown that CARMA1 plays a role in localizing ERM proteins to the uropod, we investigated whether PKC θ also plays a role in the localization of ERM proteins.

To do this we isolated T cells from WT and PKC θ ^{-/-} mice on nylon wool and used immunofluorescence as previously described to analyze the localization of moesin and p-ERM in the cells after CCL21 treatment. We again saw the typical migratory phenotype upon CCL21 treatment, with a leading edge and the uropod (Figure 16A,B). We found that like CARMA1^{-/-} T cells, PKC θ ^{-/-} T cells showed less polarization of moesin to the uropod than WT T cells; only 31% localized compared to 55% (Figure 16C).

Additionally, we found that in the PKC θ ^{-/-} T cells there was also less polarization of p-ERM to the uropod with only 40% polarized compared to 70% in WT T cells (Figure

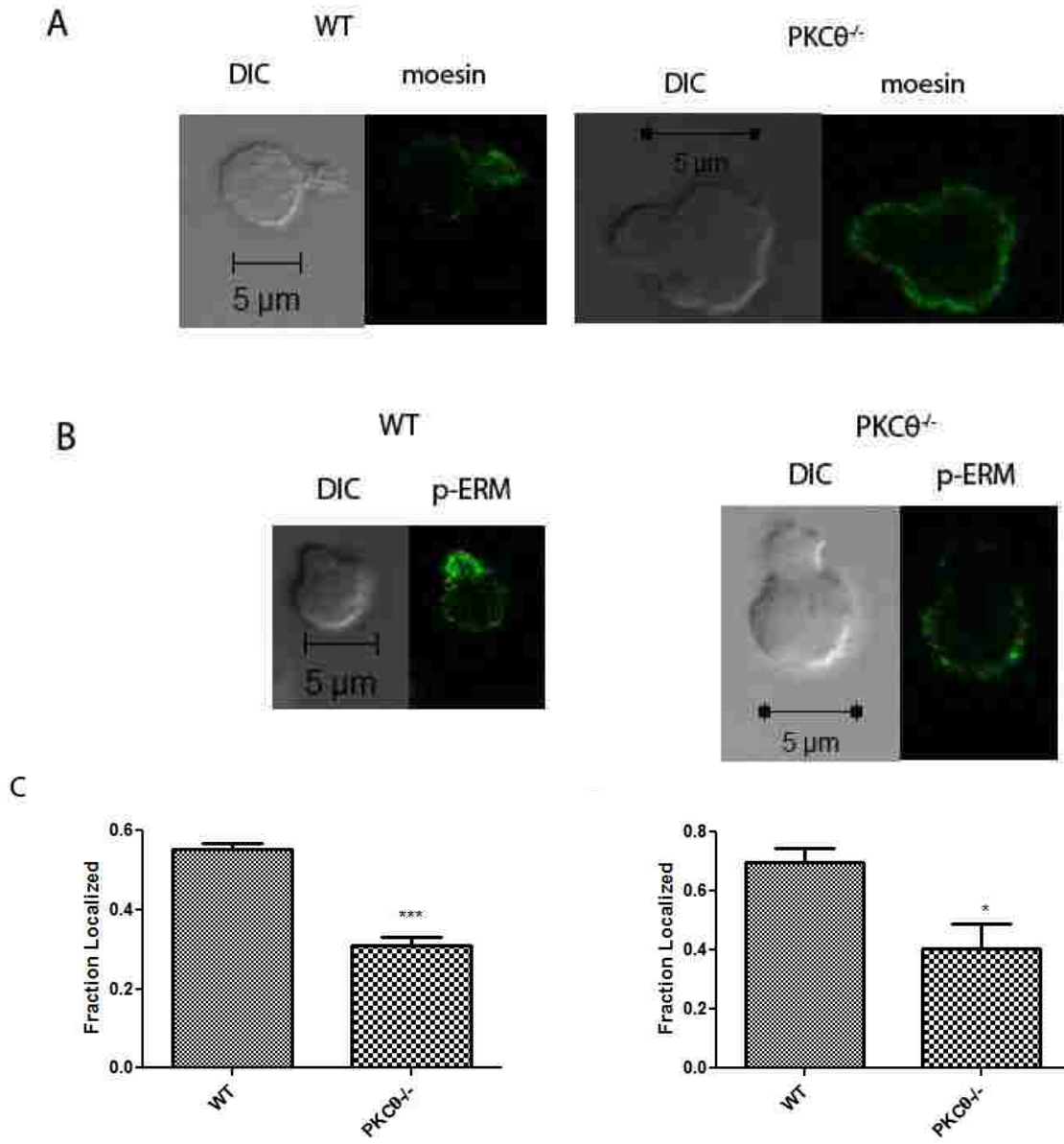


Figure 16. Localization of moesin and p-ERM in WT and PKC $\theta^{-/-}$ T cells. WT and PKC $\theta^{-/-}$ T cells were treated with 300 ng/ml CCL21 and placed on coverslips coated in poly-L-lysine and the T cells were fixed and labeled with antibodies to detect localization of moesin and p-ERM. . (A) The localization of moesin in WT and PKC $\theta^{-/-}$ T cells; the uropod is indicated with an arrow. Moesin is localized to the uropod in the WT T cell while it is evenly distributed around the PKC $\theta^{-/-}$ T cell. (B) The localization of p-ERM in WT and PKC $\theta^{-/-}$ T cells; the uropod is indicated with arrow. The WT T cell shows localization of p-ERM to the uropod, while the PKC $\theta^{-/-}$ T cell shows the p-ERM distributed more toward the leading edge. (C&D) Quantification of the localization of moesin (C) and p-ERM (D) to the uropod. Significantly fewer PKC $\theta^{-/-}$ T cells had moesin and p-ERM localized to the uropod than WT T cells. *** indicates $p=0.0002$, * indicates $p=0.0233$. Differences were considered statistically significant with $p<0.05$.

16D). These data show that PKC θ does play a role in regulating the localization of ERM proteins to the uropod, and that it affects the localization of both phosphorylated and non-phosphorylated forms of these proteins.

Summary

In this chapter our data show that PKC θ does not play a role in regulating many aspects of T cell migration including expression of the migration markers CD44 and CD62L, T cell motility on ICAM-1, and CCR7 induced motility. However, PKC θ does play a role in the localization of proteins that are involved in T cell migration, the ERM proteins, in both an active and inactive form. The effect of PKC θ on T cell migration contrasts with that of CARMA1, showing that while these two molecules coordinately regulate TCR induced NF- κ B activation, the two diverge in regulation of T cell migration downstream of chemokine receptors. These data also indicate for the first time that the role of CARMA1 in regulating T cell migration is likely to be in a PKC θ -independent manner.

3. The role of PKC θ in *Francisella tularensis* infection

The role of PKC θ in infection

The requirement for PKC θ in immune responses is not entirely understood. As discussed in the introduction, it has been shown to be important for both Th17 and Th2 responses, and sometimes for Th1 and cytotoxic T cell responses. An early study showed that NF- κ B was involved in the production of cytokines in the EAE model of multiple sclerosis, including cytokines important for Th1 (IL-2 and IFN γ) and Th2 (IL-4 and IL-10) responses (Hilliard, Samoilova, Liu, Rostami, & Chen, 1999). Since PKC θ is involved in NF- κ B activation, defective NF- κ B downstream of PKC θ -deficiency may explain why PKC θ deficient mice also show decreased production of these specific cytokines.

One type of response that PKC θ has been shown to be important for is Th1/Th17 responses. PKC θ deficient mice were protected in models of chronic intestinal inflammation and experimental autoimmune encephalomyelitis (EAE), both of which are characterized by Th1 and Th17 induced inflammation, with reduced expression of IFN γ and IL-17 (Anderson et al., 2006; Tan et al., 2006). Another study showed that PKC θ was required for development of experimental autoimmune myocarditis (EAM), which also requires a Th17 response, and that there was reduced differentiation of Th17 cells in the absence of PKC θ (Marsland et al., 2007). These data indicate that PKC θ plays a role in the differentiation of Th17 cells and in the production of Th1 and Th17 cytokines.

PKC θ has also been shown to be required for a Th2 response. A study looking at the

immune response to infection by the helminth *Nippostrongylus brasiliensis* and airway hyperresponsiveness to allergen, both which require a Th2 response, found that PKC θ ^{-/-} mice were unable to generate a Th2 response to either, but that addition of exogenous IL-2 to PKC θ ^{-/-} T cells was able to induce Th2 development (Marsland et al., 2004). PKC θ was also shown to be required for an effective response against *Toxoplasma gondii*, a parasite infection that requires a Th2 response to control (Nishanth et al., 2010). These data show that PKC θ is required for Th2 responses, and this requirement is likely partly due to the role of PKC θ in production of IL-2, which is an important cytokine for Th2 development.

Another role for PKC θ in immune responses in some cases is in regulation of adhesion molecule expression. PKC θ -deficiency was shown to protect mice from *Plasmodium berghei* ANKA (PbA) induced cerebral malaria in two studies. A 2010 study showed that there was reduced Th1 cytokine (IFN γ and TNF α) production and reduced upregulation of LFA-1 in response to PbA infection in PKC θ ^{-/-} mice (Ohayon et al., 2010). A 2011 study showed recruitment of activated T cells to the brain was reduced and the upregulation of ICAM-1 was reduced in PKC θ -deficient mice (Fauconnier et al., 2011). LFA-1 was also not upregulated in response to MOG in one of the studies using EAE as a model (Tan et al., 2006). These data indicate that PKC θ may not only be involved in T cell differentiation in response to infection, but also in the regulation of adhesion molecule expression to regulate T cell response to infection.

On the other hand, PKC θ does not seem to be required for some Th1 and cytotoxic T cell

responses. It was found that the PKC $\theta^{-/-}$ mice were able to generate an effective immune response to *Leishmania major*, which requires a Th1 response (Marsland et al., 2004). In another study, PKC θ was not required for clearance of viral infection caused by murine gammaherpesvirus 68 (MHV-68), which requires a T cell response, and T cell expansion and migration to the site of infection, particularly CD8⁺ T cells, was the same in both PKC $\theta^{-/-}$ and WT mice (Giannoni et al., 2005). A 2005 study demonstrated that T cells may get signals from other sources in the absence of PKC θ . The authors showed that PKC θ was not necessary for antiviral CD8⁺ T cell responses to multiple viruses, including influenza and vaccinia virus and that this was because the T cells could get activation signals from TLR activated dendritic cells in the absence of PKC θ (Marsland et al., 2005). These data show that for some T cell responses PKC θ is not required, and this may be due to having other signals that can fulfill the role of PKC θ in its absence.

In summary, PKC θ was shown to be involved in Th17 and Th2 differentiation, as well as in the production of multiple Th1, Th2, and Th17 cytokines. PKC θ is also involved in the regulation of expression of some adhesion molecules, particularly LFA-1, and so PKC θ -deficiency affects the migration of T cells to infection sites in some cases.

However, PKC θ is not required for some immune responses, including some Th1 and cytotoxic T cell responses, and in some cases this is due to T cells receiving activation signals from other sources when PKC θ is absent.

The role of T cells in *F. tularensis* infection

The role of PKC θ in *Francisella tularensis* infection has not been studied previously. We

know that T cells are very important in *F. tularensis* infection because mice lacking T cells are unable to clear the infection and eventually succumb (K L Elkins et al., 1996). There are 4 T cell populations that can play a role in clearing an *F. tularensis* infection: CD4⁺ T cells, CD8⁺ T cells, CD4⁻ CD8⁻ NK1.1⁻ TCRαβ⁺ Thy1.2⁺ or “DN” T cells, and γδ⁺ T cells. Either CD4⁺ or CD8⁺ T cells are individually able to clear infection (Yee et al., 1996), while DN T cells alone are able to control an *F. tularensis* infection for months with a chronic infection but are unable to clear it (Cowley et al., 2005). A γδ⁺ T cell population has been shown to increase in humans when exposed to *F. tularensis* antigen (Poquet et al., 1998). All four of these T cell populations, CD4⁺, CD8⁺, DN, and γδ⁺, produce the cytokines IFNγ and TNFα which are important for helping to control the growth of *F. tularensis* in macrophages (Cowley & Elkins, 2011).

While the T cell populations involved in immunity to *F. tularensis* have been investigated, little is known about specific molecules that might regulate immunity to tularensis. So we investigated whether PKCθ might play a role. Because the T cell response is critical for clearing *F. tularensis* infection, and because PKCθ is critical for T cell proliferation and survival, we hypothesized that PKCθ would be important in the immune response to infection with *F. tularensis*.

PKCθ deficiency protects against lethal *Francisella tularensis* infection

We wanted to investigate whether PKCθ was important for immunity against *F. tularensis* infection. To do this, we assessed whether there was a difference in survival between WT and PKCθ^{-/-} mice following infection with *F. tularensis*. For our studies, we used the live

vaccine strain (LVS), which is very infectious and deadly in mice but not in humans. We infected age and sex matched WT and PKC $\theta^{-/-}$ mice intranasally (IN) with 1×10^5 cfu of LVS and determined survival of infected mice. Surprisingly, we found that the PKC $\theta^{-/-}$ mice had better survivability than the WT mice. The WT mice started dying around day 7 or 8 post infection and all died within less than two weeks (Figure 17A). The PKC $\theta^{-/-}$ mice had much better survival, only 66% died and the surviving mice living for more than 40 days (Figure 17A). The trend remained when we infected WT and PKC $\theta^{-/-}$ mice with a 10-fold lower dose of LVS (1×10^4 cfu); 60% of the WT mice died while only 15% of the PKC $\theta^{-/-}$ mice died (Figure 17B). To control for possible differences between the inbred WT (C57Bl/6) and homozygous C57Bl/6.PKC $\theta^{-/-}$ strains, we back bred C57Bl/6.PKC $\theta^{-/-}$ mice to the WT C57Bl/6 and generated PKC $\theta^{+/+}$ (WT), PKC $\theta^{+/-}$ (het), and PKC $\theta^{-/-}$ mice. We infected littermates that were sex and age matched WT (PKC $\theta^{+/+}$), PKC $\theta^{+/-}$, and PKC $\theta^{-/-}$ mice with LVS, and found that while the PKC $\theta^{-/-}$ still showed increased survival in two separate infections with 1×10^5 cfu (0% and 45% died) and the WT still nearly all died (100% and 86% died). The survival of the heterozygous PKC $\theta^{+/-}$ mice was less than the PKC $\theta^{-/-}$ mice and greater than WT in all experiments done (Figure 17C,D). This shows that the partial loss of PKC θ in the heterozygous PKC $\theta^{+/-}$ mice also offers some protection. These results show that PKC θ -deficiency confers protection against *F. tularensis*.

PKC θ regulates the T cell populations present in infected tissue following *F. tularensis* infection

To determine why the PKC $\theta^{-/-}$ mice survived longer than the WT mice, we infected WT

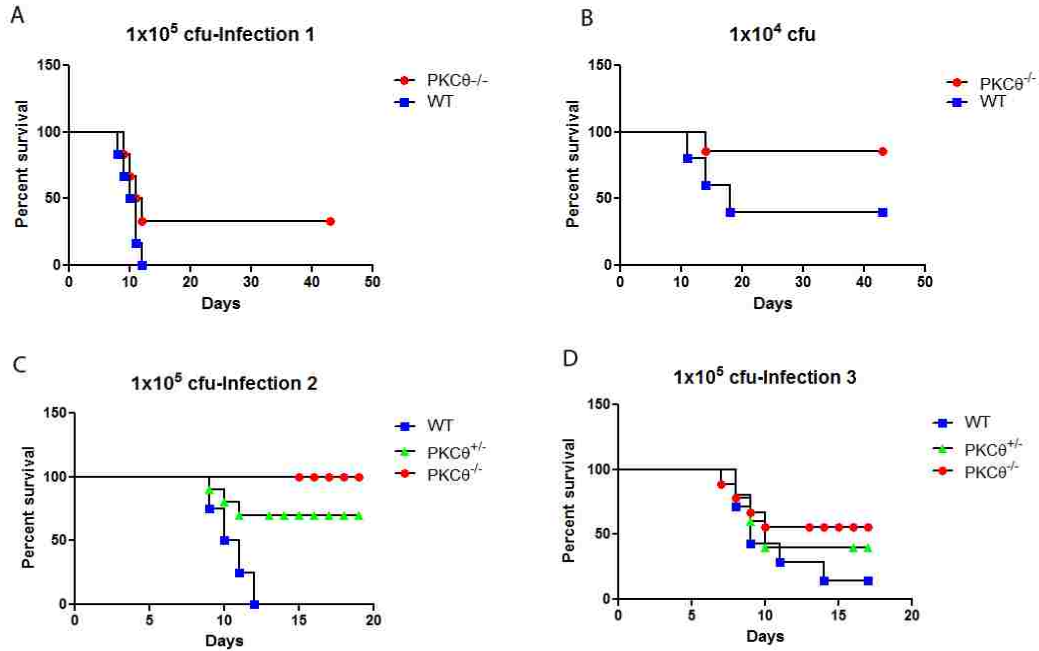


Figure 17. PKC θ -deficiency confers protection against *F. tularensis*. (A,B) WT and PKC θ ^{-/-} mice were infected IN with 1x10⁵ cfu or 1x10⁴ cfu of LVS and survival was tracked for up to 40 days. PKC θ ^{-/-} mice had better survival following inoculation with 1x10⁵ cfu and a higher percentage of PKC θ ^{-/-} mice survived inoculation with 1x10⁴ cfu. (C,D) WT, PKC θ ^{+/-}, and PKC θ ^{-/-} mice from a WT x PKC θ ^{-/-} backbreeding were infected with 1x10⁵ cfu of LVS and survival was tracked for 3 weeks. WT and PKC θ ^{-/-} mice showed the same trend as before and the heterozygous PKC θ ^{+/-} mice were partially protected from LVS infection.

mice and PKC $\theta^{-/-}$ mice IN with 1×10^5 cfu of LVS, and then harvested organs from infected mice 7 days post infection to determine the effect of PKC θ on immune cell infiltration during infection. To ask whether increased survival in PKC $\theta^{-/-}$ mice was a result of enhanced immune cell infiltration during infection, we determined the extent of immune cell infiltration into organs of infected mice at day 7. Day 7 is just prior to the time the WT mice usually started to die, and the day 7 time point allowed us to quantify immune cells in infected organs just before the infected mice succumb to infection.

Because the *F. tularensis* bacteria were introduced IN, the organ expected to be the main site of infection was the lung. Thus we harvested the lungs as well as the mediastinal lymph nodes, which are the draining lymph nodes from the lung (designated DLN). We also harvested the liver and spleen, as others have reported that there is infection in the liver and spleen as well following pulmonary infection (J Wayne Conlan, Chen, Shen, Webb, & KuoLee, 2003). As a control, we determined immune cell composition in the inguinal lymph nodes as non-draining lymph nodes (NDLN) that should not be infected.

We harvested DLNs, NDLNs, spleen, liver, and lung from infected WT and PKC $\theta^{-/-}$ mice and analyzed the cells from each organ using flow cytometry. We assessed total T cells by gating on CD3 $^{+}$, then gated on CD4 and CD8 to assess CD4 $^{+}$, CD8 $^{+}$, and CD4 $^{-}$ CD8 $^{-}$ T cell sub-populations. We were unable to get results from the DLN due to the fact that the DLN in the WT mice were very small and so we were either unable to find them, or unable to get many cells from them (data not shown). This is likely due to the mice having a very severe infection, and the infected tissue, including the lungs and DLN, becoming necrotic. We found that in the lungs, there was not a significant difference in

the total number of T cells between WT and PKC $\theta^{-/-}$ (data not shown), but of the total T cells in the lungs, a significantly smaller percentage were CD4⁺ T cells in lungs from the PKC $\theta^{-/-}$ mice (51%) than in lungs from the WT mice (59%) (Figure 18A). Interestingly, there was no significant difference in the percentage of T cells that were CD8⁺ in the lungs of infected PKC $\theta^{-/-}$ mice compared to the lungs of infected WT mice (25% in WT versus 22% in PKC $\theta^{-/-}$) (Figure 18B), but there was a significantly higher percentage of CD4⁻CD8⁻ T cells in the lungs of PKC $\theta^{-/-}$ mice (25% of the T cells from PKC $\theta^{-/-}$ were CD4⁻ CD8⁻, versus 15% in the lungs of WT mice) (Figure 18C).

The results in the liver were somewhat different than the lungs. Unlike in the lungs, we saw about 4-fold fewer T cells in the livers of infected PKC $\theta^{-/-}$ mice compared to the livers of infected WT mice, (6×10^5 T cells in PKC $\theta^{-/-}$ compared to 2×10^6 T cells in WT). However, like in the lungs, there was a smaller percentage of CD4⁺ T cells in the livers of infected PKC $\theta^{-/-}$ mice compared to the livers of infected WT mice (47% PKC $\theta^{-/-}$ compared to 62% WT) (Figure 19A). In contrast, unlike the lung, there were more CD8⁺ T cells in the livers of infected PKC $\theta^{-/-}$ mice than in the livers of infected WT mice (28% PKC $\theta^{-/-}$ compared to 22% WT) (Figure 19B). Again, there was a higher percentage of CD4⁻ CD8⁻ T cells in the livers of infected PKC $\theta^{-/-}$ mice (24%) compared to the livers of infected WT mice (15%) (Figure 19C).

In the spleen we saw similar results to what we saw in the lung. There was no significant difference in total number of T cells (data not shown) or percentage of T cells that are CD8⁺ (35% CD8⁺ T cells in the spleens of infected PKC $\theta^{-/-}$ mice compared to 31% CD8⁺

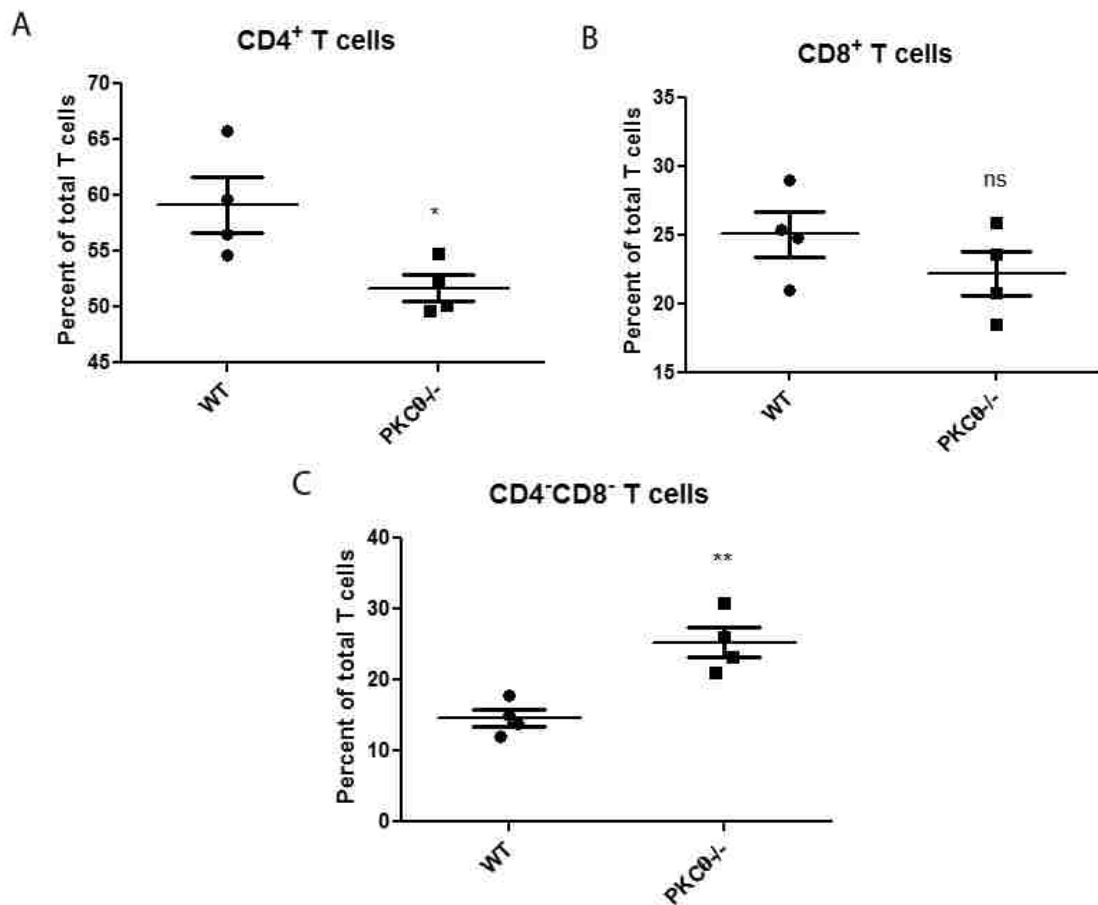


Figure 18. PKCθ^{-/-} mice infected with LVS have different T cell populations in the lungs than infected WT mice. WT and PKCθ^{-/-} mice were infected with 1x10⁵ cfu of LVS and then the lungs were harvested from the mice 7 days post infection and the T cell composition in the lungs was determined with flow cytometry. The lungs of infected PKCθ^{-/-} mice had a smaller percentage of CD4⁺ T cells (A) and a larger percentage of CD4⁻CD8⁻ T cells (B) than the lungs of infected WT mice. There was no significant difference in CD8⁺ T cells. *: p=0.0337, **: p=0.0045, ns: not significant. Differences were considered statistically significant with p<0.05.

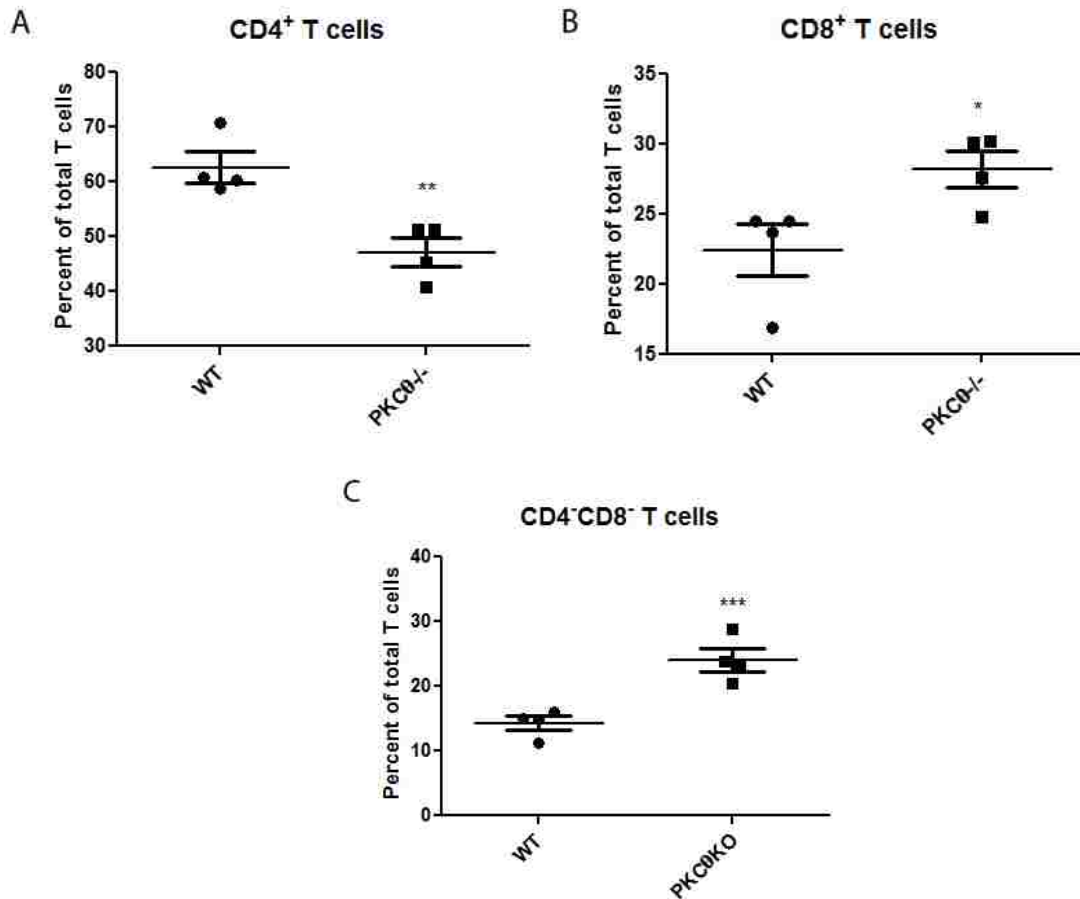


Figure 19. PKC $\theta^{-/-}$ mice infected with LVS have different T cell populations in the liver than infected WT mice. WT and PKC $\theta^{-/-}$ mice were infected with 1×10^5 cfu of LVS and then the livers were harvested from the mice 7 days post infection and the T cell composition in the livers was determined with flow cytometry. The livers of infected PKC $\theta^{-/-}$ mice had a smaller percentage of CD4⁺ T cells (A), and greater percentages of CD8⁺ T cells (B) and CD4⁻CD8⁻ T cells (C). *:p=0.0420, **:p=0.0063, ***:p=0.0031. Differences were considered statistically significant with p<0.05.

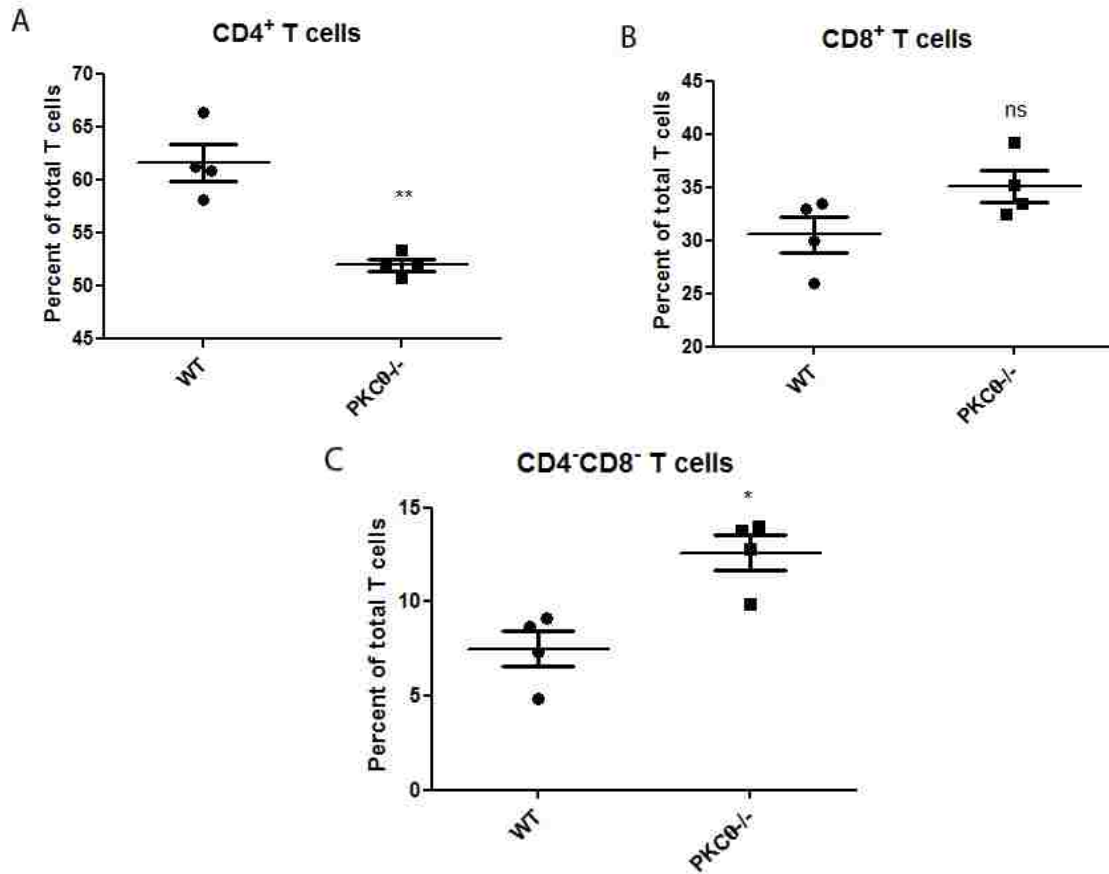


Figure 20. PKC $\theta^{-/-}$ mice infected with LVS have different T cell populations in the spleen than infected WT mice. WT and PKC $\theta^{-/-}$ mice were infected with 1×10^5 cfu of LVS and then the spleens were harvested from the mice 7 days post infection and the T cell composition in the spleens was determined with flow cytometry. The spleens of infected PKC $\theta^{-/-}$ mice had a smaller percentage of CD4⁺ T cells (A), no significant difference in percentage of CD8⁺ T cells (B), and a higher percentage of CD4⁺CD8⁻ T cells (C) than the spleens of infected WT mice. *: $p=0.0095$, **: $p=0.0017$, ns: not significant. Differences were considered statistically significant with $p < 0.05$.

T cells in the spleens of infected WT mice) (Figure 20B). But there was a smaller percentage of CD4⁺ T cells in the spleens of infected PKCθ^{-/-} mice compared to the spleens of infected WT mice (52% PKCθ^{-/-} versus 62% WT) (Figure 20A). There was a higher percentage of CD4⁻ CD8⁻ T cells in the spleens of infected PKCθ^{-/-} mice compared to spleens of the infected WT mice (12.5% PKCθ^{-/-} versus 7.5% WT) (Figure 20C). In contrast to the lung, liver, and spleen, we found no difference in total T cells or percentages of T cell subpopulations in the NDLNs of infected PKCθ^{-/-} mice compared to the NDLNs of infected WT mice (37% CD4⁺ T cells PKCθ^{-/-} versus 38% CD4⁺ T cells WT, 55% CD8⁺ T cells PKCθ^{-/-} versus 56% CD8⁺ T cells WT, 8% CD4⁻ CD8⁻ T cells PKCθ^{-/-} versus 5% CD4⁻ CD8⁻ T cells WT).

These results show that the T cell populations in the lungs, liver, and spleen upon infection are different in PKCθ^{-/-} mice infected with *F. tularensis* compared to infected WT mice. We saw that a decreased T cells were CD4⁺ T cells and an increased percentage of T cells were CD4⁻ CD8⁻ T cells in the lungs, livers and spleens of the infected PKCθ^{-/-} mice compared to the WT mice. These data suggest that differential T cell populations may contribute to the protection seen in PKCθ^{-/-} mice.

Activated PKCθ^{-/-} T cells have an increased population of CD4⁻ CD8⁻ T cells

The increased population of CD4⁻ CD8⁻ T cells present in the lungs, liver, and spleen of the PKCθ^{-/-} mice suggests that the presence of CD4⁻ CD8⁻ T cells may contribute to protection against *F. tularensis*. There are at least two possible mechanisms by which PKCθ may lead to the increased number of CD4⁻ CD8⁻ T cells. First, CD4⁻ CD8⁻ T cells

may come from increased migration to the sites of infection in response to LVS. Second, the PKC $\theta^{-/-}$ mice may generate more CD4 $^{-}$ CD8 $^{-}$ T cells, resulting in increased numbers we see in infected tissues. To ask if PKC θ affects generation of CD4 $^{-}$ CD8 $^{-}$ T cells, we purified WT and PKC $\theta^{-/-}$ T cells from uninfected mice and activated the T cells in vitro using anti-CD3 and anti-CD28 as described in Fig. 1. At 7 days after activation we looked at the expression of CD4 and CD8 via flow cytometry. We found that nearly all of the activated WT T cells were either CD4 $^{+}$ or CD8 $^{+}$ and very few were either double positive or double negative T cells (60% CD4 $^{+}$, 35% CD8 $^{+}$, 3% CD4 $^{-}$ CD8 $^{-}$, and 2% CD4 $^{+}$ CD8 $^{+}$) (Figure 21A). In contrast, in activated PKC $\theta^{-/-}$ T cells while there were still large populations of single positive CD4 $^{+}$ and CD8 $^{+}$ T cells (22% CD4 $^{+}$, 56% CD8 $^{+}$), there was also a significant population of CD4 $^{-}$ CD8 $^{-}$ T cells present that was not found in the activated WT T cells (19% CD4 $^{-}$ CD8 $^{-}$) (Figure 21A).

One possibility for the increased numbers of DN T cells could be due to a T cell development effect, with PKC $\theta^{-/-}$ mice having more DN T cells in the naïve cell pool before activation. To test for this possibility, we also analyzed naïve WT and PKC $\theta^{-/-}$ T cell repertoires for CD4 and CD8 via flow cytometry, and found that there was no difference in the percentage of CD4 $^{+}$, CD8 $^{+}$ and CD4 $^{-}$ CD8 $^{-}$ T cell populations between WT and PKC $\theta^{-/-}$ mice (Figure 21B). These data indicate that upon activation PKC $\theta^{-/-}$ T cells are able to generate a greater population of CD4 $^{-}$ CD8 $^{-}$ T cells, suggesting that this may be part of the reason for increased numbers of this population of T cells in LVS infected organs.

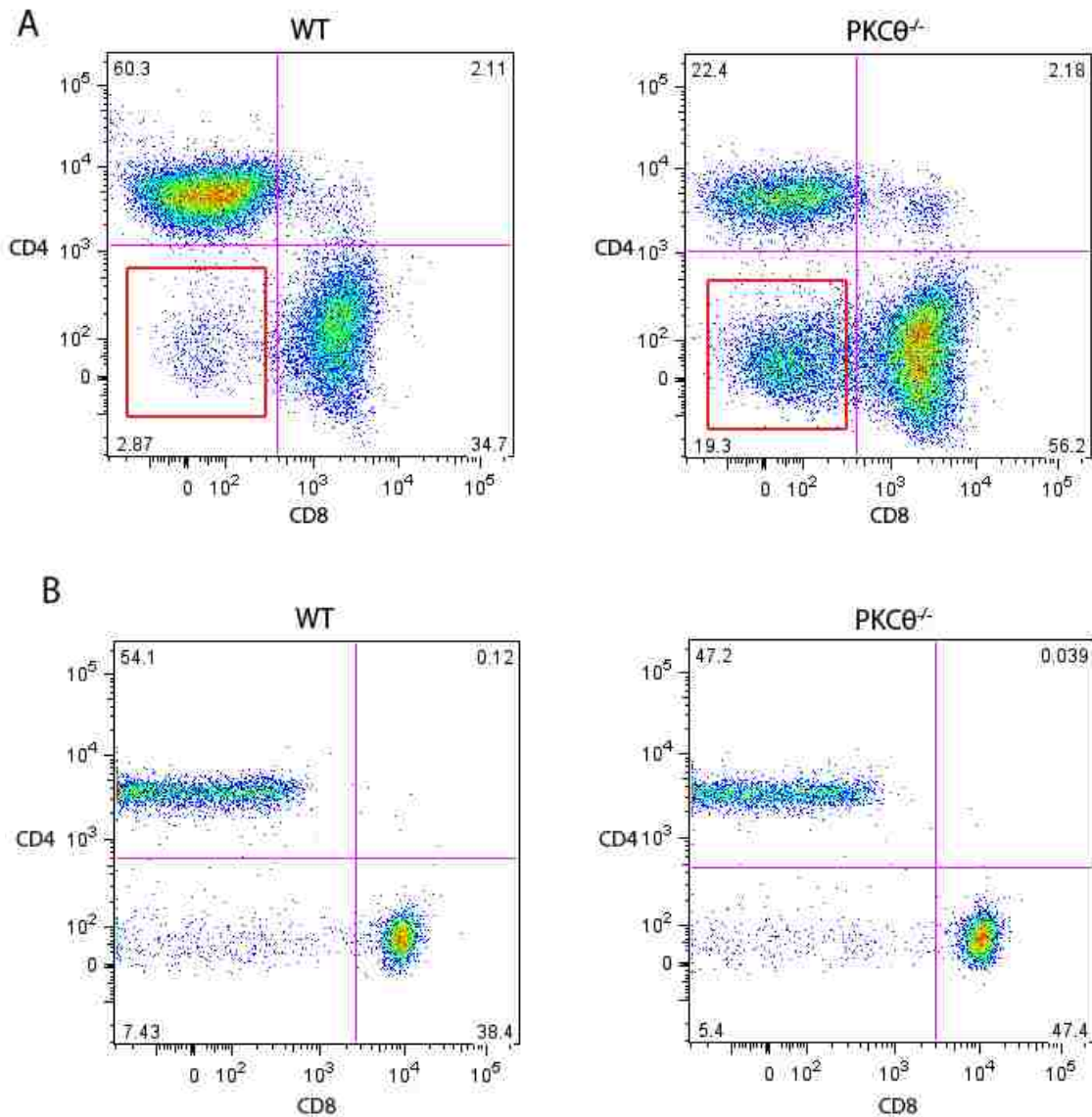


Figure 21. Activated PKCθ^{-/-} T cells have an increased population of CD4⁻ CD8⁺ T cells. (A) Naïve T cells were isolated from WT and PKCθ^{-/-} mice and activated with α-CD3 and α-CD28 for 7 days before analysis. T cells were stained with fluorescent α-CD4 and α-CD8 prior to analysis with flow cytometry. Population in the red square indicates the CD4⁻ CD8⁺ T cells. CD4⁻ CD8⁺ T cells made up 19% of the PKCθ^{-/-} T cells, while the CD4⁻ CD8⁺ T cells made up less than 3% of the WT T cells. (B) Naïve T cells were isolated from WT and PKCθ^{-/-} mice and stained with fluorescent α-CD4 and α-CD8 prior to analysis with flow cytometry. No difference was seen between the T cell populations of PKCθ^{-/-} T cells compared to WT T cells.

Summary

In summary, we found that PKC $\theta^{-/-}$ mice are protected against infection with *F. tularensis*, and that infected PKC $\theta^{-/-}$ mice have increased populations of CD4 $^{-}$ CD8 $^{-}$ T cells and decreased populations of CD4 $^{+}$ T cells in the lungs, liver, and spleen compared to infected WT mice. We also found that upon activation, PKC $\theta^{-/-}$ T cells have an increased population of CD4 $^{-}$ CD8 $^{-}$ T cells, indicating a potential source for the increased numbers of CD4 $^{-}$ CD8 $^{-}$ T cells seen in the infected organs.

DISCUSSION

1. The roles of PKC θ and CARMA1 in T cell migration

T cells are an important part of the adaptive immune system, and perform multiple functions, including directly killing infected cells and helping to activate other immune effectors. In order to properly function, T cells must be able to migrate throughout the body, first naïve T cells must scan the lymph nodes for their cognate antigen, then once activated, T cells must migrate to the site of infection where they can perform their effector function. T cells use multiple cell surface molecules to migrate, including selectins, chemokine receptors, and integrins. These molecules and their roles in T cell migration have been well studied, but the intracellular signaling involved in regulating T cell migration is not well understood. In this study we investigated two proteins that are well established in signaling downstream of TCR activation, CARMA1 and PKC θ , to determine their roles in T cell migration.

Downstream of TCR signaling, PKC θ plays a role in multiple signaling pathways, including activation of AP-1 and NFAT, but PKC θ 's role in NF- κ B activation appears to be the most important (Altman et al., 2000). Both CARMA1 and PKC θ are known to be part of the NF- κ B signaling pathway, with PKC θ directly phosphorylating CARMA1, and eventually leading to NF- κ B activation (Blonska & Lin, 2009). CARMA1 is constitutively at the plasma membrane, associated with lipid rafts, and upon TCR activation associates with the TCR in the immune synapse (IS) (Gaide et al., 2002). PKC θ is normally in the cytosol, but is recruited to the IS as well upon TCR activation (Villalba, 2004). CARMA1 is phosphorylated at several different sites, but the

phosphorylation in the linker region at S552 and S635 by PKC θ is the most important and this phosphorylation is required to recruit other signaling components (R. Matsumoto et al., 2005; Thome, Charton, Pelzer, & Hailfinger, 2010). Phosphorylated CARMA1 can then bind to BCL10 and MALT1, forming the CBM complex. The CBM complex helps with the degradation through ubiquitination of NEMO (IKK γ), the regulatory subunit of the I κ B kinase (IKK) complex (Smith-Garvin, Koretzky, & Jordan, 2009). PKC θ can also interact directly with the IKK complex and may be involved in bringing the IKK to the CBM (M S Hayden, West, & Ghosh, 2006) The IKK complex can then phosphorylate I κ B, leading to its degradation and activating NF- κ B (M S Hayden et al., 2006). NF- κ B then translocates into the nucleus and acts as a transcription factor, regulating the transcription of genes involved in apoptosis, proliferation, IL-2 production, and T helper cell differentiation (Matthew S Hayden & Ghosh, 2011).

Because they are both involved in this crucial pathway, the effects of CARMA1 deficiency and PKC θ deficiency are similar when looking at T cell activation, including reduced proliferation and IL-2 production. CARMA1 deficient T cells were unable to activate IKK following CD3/CD28 costimulation, and had deficient IL-2 production (Wang et al., 2002). Another study also shows poor IL-2 production in CARMA1 deficient T cells, as well as impaired proliferation as a result of the lack of IL-2 (Egawa, Albrecht, Favier, & Sunshine, 2003). PKC θ deficient T cells also have a defect in NF- κ B activation and decreased IL-2 production and proliferation (Sun et al., 2000).

While the roles for PKC θ and CARMA1 in response to T cell receptor activation are well

studied, their roles in other T cell functions are unknown. For example, not much is known about the signaling pathways involved in T cell migration. Several studies offer possible connections of PKC θ to regulation of T cell migration. One study showed that a motile phenotype was induced in T cells when PKCs were inhibited (Southern et al., 1995). Another study showed that PKC θ is involved in activation of Rap1, which leads to increased adhesion of LFA-1 on T cells downstream of TCR activation (Letschka et al., 2008). PKC θ was also found to phosphorylate CD43, which associates with ERM proteins in polarized T cells and promotes migration (Cannon et al., 2011). Because CARMA1 is directly downstream of PKC θ it seemed a likely candidate for a role in regulating T cell migration as well. This led us to investigate the roles of PKC θ and CARMA1 in various aspects of T cell migration.

Chemokine receptor signaling is important for T cell migration, activating pathways that lead to uropod and leading edge formation, polarization of the cell, and integrin activation (Kinashi, 2005). Much less is known about the signaling downstream of different chemokine receptors than is known about the signaling pathways upon TCR activation, but several signaling components have been shown to be common to both TCR and chemokine receptor activation. Rap1 and 2 are activated downstream of both the TCR and chemokine receptors, and lead to integrin activation (Abram & Lowell, 2009). Signaling through the chemokine receptor CXCR4 was shown to lead to activation of ERK1 and 2, PI3K, and NF- κ B (Ganju et al., 1998). PI3K is activated downstream of both the TCR and chemokine receptors, regulating migration and T cell activation (Ward, 2004). The PI3K subunit p110 γ was shown to be important for

chemotactic responses to CCL19 and CCL21 (which bind to CCR7), and CXCL12 (which binds CXCR4) (Reif et al., 2004). Yet another shared pathway between TCR signaling and chemokine receptor signaling is via activation of LFA-1. Both chemokine receptors and TCR signaling contribute to the redistribution of the LFA-1 to allow for rapid adhesion when there is not a high density of ICAM for the LFA-1 to bind to (Constantin et al., 2000).

Pathways traditionally considered exclusively downstream of TCR or chemokine receptors have recently been shown to cooperate as well. Migration of T cells induced by CXCL12 (ligand to CXCR4) was reduced in mice lacking functional ZAP-70, which is also involved in signaling downstream of the TCR (Ottoson, Pribila, Chan, & Shimizu, 2001). Later it was shown that CXCR4 actually associates with the TCR to activate ZAP-70 (Kumar et al., 2006). Recently a study demonstrated that short-term TCR activation increased the migration of T cells toward CCL19 and CCL21, indicating that the signaling through the TCR and CCR7 can cooperate to control the migration of the T cells (Schaeuble, Hauser, Singer, Groettrup, & Legler, 2011).

We now identify a novel role for CARMA1 and PKC θ in regulating T cell migration. However we found that CARMA1 and PKC θ do not regulate the same aspects of T cell migration. We have shown that CARMA1 plays a role in regulating many aspects of T cell migration, including cell surface expression of motility markers, speed of migrating T cells in vitro, signaling through CCR7 to induce motility, and ERM protein localization to the uropod. On the other hand, we showed that PKC θ does not play a role in

regulating the expression of motility markers, migrating T cell speed, or CCR7 signaling in T cell motility, but does regulate the localization of ERM proteins. These results suggest that many of the ways that CARMA1 regulates T cell migration are likely to occur independently of PKC θ . Our findings show that while both TCR induced NF- κ B signaling and T cell migration involve PKC θ and CARMA1, the mechanism by which PKC θ and CARMA1 regulate these two pathways is fundamentally different.

Our data shows that CARMA1 can regulate CD62L downregulation upon T cell activation. CD62L expression is downregulated by first proteolytic cleavage and then by gene transcription downregulation (Chao, Jensen, & Dailey, 1997). However, the signaling pathway that leads to this downregulation is not fully understood. The transcription factor KLF2 is known to directly activate the promoter of CD62L (Bai, Hu, Yeung, & Chen, 2007; Sebzda, Zou, Lee, Wang, & Kahn, 2008). PI3K has been shown to be involved in the shedding of CD62L from the surface of the T cells upon TCR activation (Sinclair et al., 2008). Sinclair et al. also showed that PI3K is also involved in suppressing transcription of the CD62L gene, through mTOR. This inhibition is through suppression of KLF2 (Finlay & Cantrell, 2010). Our results show that CARMA1 is involved in this pathway. CARMA1 interacts with Akt downstream of TCR signaling to activate NF- κ B (Thome et al., 2010). Since Akt is part of the signaling pathway to activate mTOR CARMA1 may regulate CD62L expression via Akt and KLF2. We found that PKC θ deficiency does not result in any defects in CD62L downregulation, showing that PKC θ phosphorylation of CARMA1 is likely not to play a role in CARMA1-dependent CD62L regulation.

We also find that CARMA1 is involved in CD44 upregulation as well as CD62L downregulation. CD44 upregulation is induced by TCR activation and the increased level of CD44 is sustained on effector T cells and memory T cells (Baaten, Tinoco, Chen, & Bradley, 2012). NF- κ B is a known transcription factor for CD44 (Hinz et al., 2002), and since we know CARMA1 deficient T cells have poor NF- κ B activation, that is the likely explanation for the inability of CARMA1^{-/-} T cells to upregulate CD44. However, PKC θ deficient T cells also have impaired NF- κ B signaling, but we did not see a deficiency in the upregulation of CD44 in the PKC θ ^{-/-} T cells. This suggests that there may be another signaling pathway in which CARMA1 but not PKC θ is involved that also regulates CD44 expression.

CD44 is involved in trafficking to inflamed sites, and CD62L is responsible for homing of T cells to the lymph nodes, so concomitant downregulation of CD62L and upregulation of CD44 allow activated T cells to leave lymph nodes and go to sites of inflammation. T cells that remain CD62L^{hi} and CD44^{lo} after activation may have difficulty trafficking to other tissues and remain circulating through the secondary lymphoid tissues. However, because CARMA1^{-/-} T cells have defective activation and effector function, they would likely be unable to efficiently clear pathogens at a site of infection even if they trafficked normally. Thus, our results show that T cell migration and effector function are jointly controlled by CARMA1, resulting in defective T cell responses.

Because CARMA1^{-/-} T cells have impaired activation, and we have shown they have defective regulation of the expression of motility markers, we hypothesized that CARMA1^{-/-} T cells would show defective motility compared with WT T cells. To our surprise, CARMA1^{-/-} T cells moved at a higher velocity than WT T cells (Fig 4). These results indicate that CARMA1 actually acts as a negative regulator to control the speed of activated T cells when they are migrating as defects in CARMA1 result in higher motility. One possible mechanism by which CARMA1 might hinder the motility of T cells is through regulation of the actin or microtubule networks within the T cell. This could be through localization of the actin and microtubules or associated proteins. ERM proteins are actin regulatory proteins that can bind to actin through their C-terminal domains and to membrane bound proteins through their N-terminal domains (Arpin, Chirivino, Naba, & Zwaenepoel, 2011). ERM proteins must be activated before they can bind actin or other proteins; in T cells PKC θ has been shown to phosphorylate and activate moesin (Pietromonaco et al., 1998). ERM proteins have been shown localize to the uropod in motile cells, and to bind to CD44, CD43, and ICAM-2, as well as ICAM-1 and ICAM-3 (Serrador et al., 1997; Yonemura et al., 1998). The association of CD43 with ERM proteins is important for phosphorylation of CD43 and T cell migration (Cannon et al., 2011). ERM proteins also regulate membrane tension in lymphocytes; constitutively active ezrin slowed migration and increased the membrane tension in T cells (Liu et al., 2012).

Our results show that CARMA1 is involved in regulating the localization of ERM proteins in motile T cells; fewer of the CARMA1^{-/-} T cells had moesin localized to the

uropod. Defective localization could decrease the membrane tension in the CARMA1^{-/-} T cells and lead to faster migration. However, we also saw defective localization in the PKCθ^{-/-} T cells but we did not see increased migration speed in PKCθ^{-/-} T cells. Furthermore, we observed defective ERM localization in naïve PKCθ^{-/-} and CARMA1^{-/-} T cells, where there was no difference in migration speed between WT and PKCθ^{-/-} or CARMA1^{-/-} T cells. Therefore it is unlikely that mislocalization of ERM proteins alone is the cause of the increased speed in the CARMA1^{-/-} T cells. One potential mechanism for the enhanced motility seen in CARMA1^{-/-} T cells is via regulation of myosin-IIA (myoIIA), a motor protein important for cell motility (Even-ram et al., 2007). T cells have been described to move along a substrate in two ways: “walking” that is dependent mainly on myoIIA, and “sliding” that is mainly dependent on integrins; it was shown that making the myoIIA dependent movement dominant lead to the T cells moving faster (Jacobelli, Bennett, Pandurangi, Tooley, & Krummel, 2009). Therefore one potential explanation for enhanced motility of CARMA1^{-/-} T cells is through greater reliance on myoIIA, leading to more rapid motility seen in CARMA1^{-/-} T cells.

Our results show that CARMA1 regulates T cell motility in a 2D environment. The physiological consequences of the faster migration in CARMA1^{-/-} T cells in vitro are not completely clear. We coated dishes with ICAM-1 and placed the T cells on this coated surface in the presence of CCL21, so our experiments only looked at one adhesion molecule and one chemokine in a 2D environment. This differs from the lymph node environment in vivo in which there is a 3D network of cells and many different molecules involved. Different signaling pathways are also required for 3D vs. 2D environments. It

was shown that inhibition of LIM-kinase 1 by MEK, which then allows cofilin, a protein involved in actin remodeling, to be dephosphorylated, was required for optimal migration in 3D environments but not 2D environments (Klemke, Kramer, Konstandin, Wabnitz, & Samstag, 2010). However some molecules are known to be important for migration in both 2D and 3D environments. Myosin-IIA is not only important for 2D migration, it is also required for migration in 3D environments as myosin-IIA-deficient cells showed defects in interstitial migration and recirculation, as well as overadherence to HEVs (Jacobelli et al., 2010). Therefore if the difference we see in the CARMA1^{-/-} T cells is related to myoIIA, it is likely that this enhanced migration will be recapitulated in vivo in a 3D environment as well.

Integrins also play different roles in different environments. Integrins are important for strong adhesion of T cells to endothelial cells in blood vessels to allow the T cells to enter tissue, but in the absence of the shear forces caused by the blood flow, such as within lymph nodes, it was found that the integrins are not so strongly activated and are less adhesive (Woolf et al., 2007). Woolf et al. also showed that T cells lacking LFA-1 in WT mice, or WT T cells in ICAM-1 deficient mice, were still able to migrate within the lymph nodes, but they migrated more slowly than when LFA-1 and ICAM-1 were both functional. This indicates that LFA-1/ICAM-1 interactions are less important for migration within lymph nodes, but still play a role. In our in vitro migration assay we did not introduce a shear force to the cells, so the role of LFA-1 in our assay may be similar to that in vivo.

Our assay involved activated T cells, and it is possible that faster migration would be beneficial for getting Teff cells to the site of infection more quickly. However, the increased speed may also inhibit maximal T cell function in vivo. Increased migration speed might make it more difficult to detect and interact with antigen. Not a lot of studies have been done looking at CARMA1's role in infections, but enhanced migration by CARMA1^{-/-} T cells may compensate for defective activation.

It is known that naïve T cells do not adhere and migrate well on ICAM-1 without CCL21 signaling through CCR7 (Stachowiak et al., 2006) and we have seen this in our own experiments as well (data not shown). We have shown that activated T cells are not as strictly dependent on CCL21 as naïve T cells because activated T cells are still able to adhere to the ICAM-1 and migrate in the absence of CCL21. However, WT, CARMA1^{-/-}, and PKC θ ^{-/-} T cells all lose speed in the absence of CCL21 or GPCR signaling when T cells are inhibited with pertussis toxin. The WT T cells migrated at a similar speed in the absence of CCL21 as with GPCR inhibition, indicating that CCR7 is likely the main chemokine receptor used for activated T cell motility in WT T cells. Activated PKC θ ^{-/-} T cells also showed a similar reliance on CCR7 as the main chemokine receptor it uses for migration, as both absence of CCL21 and PTX treatment led to decreased motility.

The activated CARMA1^{-/-} T cells also had a similar decrease in speed with lack of CCL21 and with Ptx treatment, indicating a partial reliance on CCR7 signaling for motility. However, even with Ptx treatment, which inhibits all GPCR signaling through the Gi subunit, including all chemokine signaling, the CARMA1^{-/-} T cells migrated at a

greater speed than the similarly treated WT T cells. This indicates that the increased speed that we see in the activated CARMA1^{-/-} T cells is not due solely to chemokine signaling, and the speed of the T cells is likely increased by some other mechanism, such as defective regulation of cytoskeleton or associated proteins, as discussed above.

Our data show that CARMA1 regulates T cell migration in a PKCθ-independent manner in regulation of CD62L and CD44 expression and motility in a 2D environment.

However, we found that both CARMA1 and PKCθ can regulate the localization of ERM proteins. We saw reduced localization of ERM proteins in both PKCθ^{-/-} T cells and CARMA1^{-/-} T cells, which suggests that the localization of ERM proteins is mediated by a pathway involving both PKCθ and CARMA1. However we only saw significantly reduced localization of p-ERM in the PKCθ^{-/-} T cells and not in the CARMA1^{-/-} T cells.

One possible explanation is the fact that PKCθ directly phosphorylates ERM proteins and so in the absence of PKCθ there may be much less total p-ERM levels. While we saw defective localization of ERM proteins in naïve PKCθ^{-/-} and CARMA1^{-/-} T cells, we saw no difference in the migration of either naïve CARMA1^{-/-} or PKCθ^{-/-} T cells compared to WT. This result was surprising as we would expect an effect on migration because the ERM proteins are important for actin regulation. Our in vitro migration assay only looks at 2D migration on ICAM so it is possible that not all defects will be apparent. In a 3D environment, T cells have to move through tight spaces in order to get to tissue as well as migrate through tissues, and defective localization of ERM proteins could lead to a defect in cytoskeletal rearrangement that could make it more difficult to move through these spaces.

2. The role of PKC θ in immunity to *Francisella tularensis*

The effectiveness of a T cell response to an infection depends on multiple factors, including migration of T cells to sites of infection, proliferation of the T cells, and differentiation of the T cells into effector cells that are able to respond to the pathogen when they arrive at the infection. If T cells have defects in any of these functions, the effectiveness of the immune response may be reduced. However, the specific molecules required for an effective immune response to many infections is not well understood. PKC θ has been shown to be involved in signaling that leads to proliferation, as well as migration and differentiation, and is important to varying degrees in different types of infection. In this study we investigated the role of PKC θ in immunity to the intracellular bacteria *Francisella tularensis*.

Signaling downstream of TCR activation leads to activation of many transcription factors, including AP-1 and NF- κ B, to regulate proliferation, apoptosis, cytokine production, and T cell differentiation, which all contribute to mounting an effective immune response to a pathogen. Activated T cells must differentiate into effector T cells that are able to respond effectively to the infection. CD8⁺ T cells become cytotoxic T cells (CTLs) that can directly destroy an infected cell. CD4⁺ T cells are more diverse; they can differentiate into one of many lineages of T helper (Th) cells, each of which is effective against specific types of pathogens. Th lineages include Th1, Th2, and Th17 cells. Th1 cells produce IFN γ , and are important for defense against viruses and intracellular pathogens, while Th2 cells produce IL-4, IL-5, and IL-13, and are important for immunity to extracellular parasites and are responsible for allergic inflammatory

responses (Liao, Lin, & Leonard, 2011). Th17 cells produce IL-17A and IL-22 and they participate in immunity to bacteria and fungi, and they are also involved in autoimmune diseases (Liao et al., 2011). Th cell differentiation depends on many factors, with the major determinant being specific cytokines present during initial T cell activation. Th1 differentiation is largely induced by IL-12 and IFN γ , while Th2 differentiation is induced by IL-2 and IL-4, and Th17 differentiation is induced by TGF β , IL-6, and IL-21 (Zhu, Yamane, & Paul, 2010).

IL-2 plays an important role in T cell differentiation. IL-2 is involved in promoting CD8⁺ differentiation into effector and memory CTLs (Liao et al., 2011). IL-2 is also crucial for Th2 and Th1 differentiation; it activates STAT5 which increases IL-4 production (Cote-Sierra et al., 2004), and IL-2 also contributes to Th1 differentiation by inducing IFN γ production (Liao et al., 2011). As discussed earlier, PKC θ is involved in the signaling that leads to activation of AP-1 and NF- κ B, transcription factors that activate many genes, including the IL-2 gene. PKC θ deficient T cells have been shown to have reduced IL-2 production (Sun et al., 2000).

Multiple studies have shown that importance of PKC θ varies for different types of T cell responses. PKC θ is important for Th2 and Th17 responses, and is involved in IFN γ production, but is largely not required for Th1 or cytotoxic CD8⁺ T cell responses.

The role of PKC θ in *Francisella tularensis* infection was previously unknown, but most studies had shown that PKC θ was required for IFN γ production and Th1 immunity. *F.*

tularensis is an intracellular bacterium that infects macrophages primarily, and so a Th1 response is required to help kill infected cells and clear the infection. Thus, we had hypothesized that PKC θ would likely be required for an effective immune response against *F. tularensis*. However, we found that PKC θ was not only not required for immunity against *F. tularensis*, but in fact PKC θ deficiency actually allowed the mice to survive longer following a lethal dose of LVS for WT mice. We saw the same trend in a lower dose of LVS, with PKC $\theta^{-/-}$ mice showing higher survival than WT mice.

From our preliminary findings, we have some clues as to what may be leading to the increased survival in the PKC $\theta^{-/-}$ mice. As T cells have been previously shown to be critical to mediate immunity against *F. tularensis*, we analyzed different T cell types in infected WT and PKC $\theta^{-/-}$ mice. Specifically, we determined the number of CD4 $^{+}$, CD8 $^{+}$, and CD4 $^{-}$ CD8 $^{-}$ T cells in infected organs. We found that the percentage of T cells that were CD4 $^{+}$ was decreased in the lungs, liver, and spleen of infected PKC $\theta^{-/-}$ mice compared to infected WT mice, and that there was an increase in the percentage of CD4 $^{-}$ CD8 $^{-}$ T cells in the lungs, liver and spleen of infected PKC $\theta^{-/-}$ mice. These results suggest that one potential mechanism for protection in PKC $\theta^{-/-}$ mice against *F. tularensis* infection is an increase in CD4 $^{-}$ CD8 $^{-}$ T cells in the infected organs.

However, because we only looked at CD3, CD4, and CD8, we don't have more specific information on what kind of T cells the CD4 $^{-}$ CD8 $^{-}$ T cells are. We know that mice without CD4 $^{+}$ or CD8 $^{+}$ T cells are still able to control an infection with *F. tularensis* (J W Conlan, Sjöstedt, & North, 1994). Two likely candidates are either $\gamma\delta^{+}$ T cells or the

CD4⁻ CD8⁻ NK1.1⁻ TCR $\alpha\beta$ ⁺ Thy1.2⁺ “DN” T cells, which have both been shown to play a role in the immune response to *F. tularensis*. Our results showing that activated PKC θ ^{-/-} T cells have an increased population of CD4⁻ CD8⁻ T cells, and the increase in CD4⁻ CD8⁻ T cells in the infected organs of PKC θ ^{-/-} mice indicate that this population is potentially an important component contributing to the increased survival of the PKC θ ^{-/-} mice. One possibility is that the CD4⁻CD8⁻ population are $\gamma\delta$ T cells which would increase protection. Human $\gamma\delta$ ⁺ T cells have been shown to control *F. tularensis* growth in vitro, and expansion of human $\gamma\delta$ ⁺ T cells was shown to be triggered by phosphoantigens from *F. tularensis* in vivo (Poquet et al., 1998; Rowland et al., 2012). $\gamma\delta$ ⁺ T cells also have been shown to play an important role in other infections, and are able to respond to an infection both earlier and later than the $\alpha\beta$ ⁺ T cell response (Carding & Egan, 2002). In response to infection with *Listeria monocytogenes*, another intracellular bacterium, the $\gamma\delta$ ⁺ T cells were shown to respond early in the infection, at only 3 days after infection during the innate immune response, helping to activate NK cells and also to promote T helper differentiation (Ferrick et al., 1995; Ladel, Blum, & Kaufmann, 1996). In another study with *L. monocytogenes*, it was shown that $\gamma\delta$ ⁺ T cells play a role late in the infection, by helping to control inflammation and prevent excessive tissue damage (Fu et al., 1994). If the increased numbers of CD4⁻ CD8⁻ T cells we see in the organs of our LVS infected PKC θ ^{-/-} mice are $\gamma\delta$ ⁺ T cells, then a boost in the innate response and a reduction of later inflammation could help explain the survival of these mice over the WT mice.

Alternatively, the CD3⁺CD4⁻CD8⁻ T cell pool we see in the PKC θ ^{-/-} T cells may belong to

the unique and rare $CD4^- CD8^- NK1.1^- TCR\alpha\beta^+ Thy1.2^+$ “DN” T cells which have been shown to control *F. tularensis* growth in vitro and control infection in vivo (Cowley et al., 2005). DN T cells are a significant portion of the T cells found in the infected tissues, and produce $IFN\gamma$, IL-17A, and TNF- α which all help control the infection (Cowley & Elkins, 2003; Cowley et al., 2010). While they produce the same cytokines as the $CD4^+$ and $CD8^+$ T cells, DN T cells alone cannot completely clear the infection, so there must be a difference in their mechanisms for controlling infection with LVS (Yee et al., 1996). Thus, it is possible that in the infected $PKC\theta^{-/-}$ animals which survive, the prolonged survival is merely enhanced control of *F. tularensis* infection rather than actual clearance. While we saw survival up to 40 days of infection, which indicates long term survival, we have not yet analyzed whether the $PKC\theta^{-/-}$ mice might still harbor LVS after prolonged survival.

The mechanism by which these DN T cells develop and how they affect bacterial control remains to be determined. $PKC\theta$ is not required for T cell development in the thymus (Altman & Villalba, 2003), and naïve $PKC\theta^{-/-}$ T cells taken from $PKC\theta^{-/-}$ lymph nodes were found to have similar populations of $CD4^+$, $CD8^+$, and double positive ($CD4^+CD8^+$) and double negative ($CD4^-CD8^-$) T cell populations as WT T cells. Therefore it is likely that any change in the ratios of the different T cell populations occurs upon T cell activation and differentiation, and our results show that $PKC\theta^{-/-}$ T cells appear to develop an increased number of $CD4^-CD8^-$ T cells upon activation (Chapter 3, Figure 21). How $PKC\theta$ may contribute to the development of $CD4^-CD8^-$ T cells upon activation remains unknown.

With the increased CD4⁻CD8⁻ T cell population, we see a decrease in CD4⁺ T cells in infected PKCθ^{-/-} mice compared with infected WT mice. One possible explanation for this is that the CD4⁺ T cells lose the CD4 marker and become CD4⁻ CD8⁻ T cells, however, this does not seem likely as there is no evidence of this type of development occurring in other models. A second possible explanation is that the proliferation of the CD4⁺ T cells or migration of CD4⁺ T cells is decreased in the PKCθ^{-/-} mice. We know that PKCθ^{-/-} T cells have defects in proliferation following TCR activation as a result of PKCθ's role in NF-κB activation (Altman & Villalba, 2003), so it is likely that proliferation of CD4⁺ T cells was reduced in the infected PKCθ^{-/-} mice. Interestingly, we do not see any change in the ratios of the CD4⁺ and CD4⁻CD8⁻ T cell populations in the non-draining lymph nodes, only in the sites of infection, suggesting the CD4⁻ CD8⁻ T cells may be receiving signals to increase their proliferation within the infected organs.

Alternatively, it is also possible that there is a change in the rate of migration of the PKCθ^{-/-} T cells compared to WT in response to LVS infection. PKCθ^{-/-} T cells may have enhanced migration to infection sites, or may be less efficient at migration out of infection sites and thus, provide enhanced protection for longer at the infection site. In Part 1, we showed that polarized PKCθ^{-/-} T cells had mislocalization of ERM proteins. Because ERM proteins are important for regulation of the actin cytoskeleton, this could affect migration of the T cells. While we did not see any changes in migration of the PKCθ^{-/-} T cells compared to the WT T cells on ICAM-1 in vitro, the ERM localization defect may cause changes in migration of the T cells in vivo. We also saw no defect in

activated PKC θ ^{-/-} T cells of the cell surface expression of two migration markers, CD44 and CD62L, but others have seen defective upregulation of LFA-1 in response to an infection that resulted in reduced migration to the site of infection (Ohayon et al., 2010). It is also possible that PKC θ may regulate the expression of additional migration factors that can control the number of T cells at an inflammatory site, such as PSGL1 or other proteins that mediate T cell migration to inflammatory sites. Because we have seen differences in the sub-populations of T cells within the sites of infection between infected WT and PKC θ ^{-/-} mice, it may also be that PKC θ -deficiency affects the migration of some of these populations differently, in a way that is beneficial to the host.

We have shown that PKC θ deficiency protects mice from lethal LVS infection, and also show preliminary data that indicates that PKC θ deficiency increases the CD4⁺ CD8⁻ T cell population upon activation and in LVS infected organs, which may enhance immunity against *F. tularensis*. Further investigation of LVS infection in PKC θ ^{-/-} mice will be required to determine the precise role of PKC θ in T cell responses upon infection and the role of the CD4⁺ CD8⁻ T cell population in the response. Future studies would include determining the infiltration of immune cells at earlier time points to assess whether PKC θ affects T cell migration or activation. Production of cytokines should also be analyzed in the infected WT and PKC θ ^{-/-} mice to determine whether PKC θ might change cytokine profile to increase survival of the PKC θ ^{-/-} mice.

In conclusion, we have shown a novel role for CARMA1 in regulating T cell migration, and we have shown that CARMA1 does this in a PKC θ independent manner. Further

studies may help determine the pathways through which CARMA1 acts, and determine other molecules that may be involved in PKC θ independent signaling pathways. Future studies would include determining what CARMA1 may be acting on to regulate the speed of activated T cells, such as Bcl10 and MALT1, which have both been shown to be in a complex with CARMA1. Further investigation of the effects of CARMA1 and PKC θ on T cell migration in vivo would provide valuable insight into the potential effect of these proteins in protection against infection such as *Francisella tularensis*. Our studies show that specific signaling molecules regulate discrete T cell functions such as proliferation and migration, leading to differential effects on T cell immunity against pathogens.

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