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ABSTRACT

Mammalian antimicrobial peptides, including cathelicidins and defensins, play an important role in host defense via direct antimicrobial activity as well as immune regulation. The cathelin-related antimicrobial peptide (mCRAMP) is the only identified mouse cathelicidin and the orthologue of human LL-37. We show that all mouse B cell subsets, including follicular, marginal zone, B1a, and B1b cells, as well as CD4+ and CD8+ T cells produce mCRAMP directly ex vivo. In addition, mCRAMP-deficient B cells produced less IgG1 antibody in vitro in response to CD40L or LPS plus IL-4 when compared to WT B cells. The addition of recombinant mCRAMP at the time of mCRAMP-deficient B cell activation restored the level of IgG1 production to WT levels. mCRAMP-deficiency had no effect on proliferation, survival, or class switch recombination, but resulted in a decrease in the amount of IgG1 mRNA. Surprisingly, mCRAMP-deficient mice immunized with TNP-OVA absorbed in Alum resulted in an enhanced TNP-specific IgG1 response when compared to WT B6 mice. ELISpot and PCR analysis revealed increased numbers of TNP-specific IgG1-secreting splenic B cells and increased CD4+ T cell IL-4 expression in mCRAMP-deficient mice. Taken together, B cells express and respond to mCRAMP positively in vitro while in vivo mCRAMP

appears to also indirectly regulate B cell antibody production through regulation of T cell cytokine production.

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INTRODUCTION

Antimicrobial Peptides

Mammals have co-existed with a myriad of microorganisms on our planet for over millions of years, though individuals seldom get infected by those pathogens. One reason is that mammals had developed a physical barrier of skin and epithelia that is impermeable to most pathogens. However, in case of wound and diseases, pathogens can breach these defenses resulting in infection and diseases. Innate immunity is the first line of host defense against pathogen invasion, is constitutively present, and responds rapidly to a variety of microorganism infections. The adaptive immune system acts as a second line of defense, and is characterized by a delayed but highly effective antigen-specific response. The adaptive immune system is also characterized by immunological memory, which affords rapid protection against re-exposure to the same pathogen (1-2).

Antimicrobial substances are important components of innate immunity, comprising microbicidal chemicals (e.g., hydrogen peroxide, nitric oxide, etc.) and various host gene-encoded antimicrobial peptides (AMPs). AMPs are widely expressed and conserved in organisms ranging from plants (3), invertebrate (4) to mammals (5). Their continuous expression and rapid induction at mammal's skin epithelial surfaces provide effective protection against a broad spectrum of pathogens including bacteria, viruses, fungi and parasites (5-10).

As early as the 1960s, people found that lysozyme had bactericidal activity to-

ward gram-positive bacteria (11), which was revealed later to be attributed to AMPs. At the time of writing this thesis, there are 1548 AMPs that have been identified or predicted from various species and are described in the Antimicrobial Peptide Data base (http://aps.unmc.edu/AP/about.php). In addition to the extensively-studied capacity of AMP-mediated directly killing of microbes or inhibition of their growth, it is increasingly appreciated that AMPs are also immunomodulatory molecules. Previous studies reported that AMPs regulate production of chemokines and cytokines, promote cell migration to the site of infection, and modulate cell differentiation and activation (5), suggesting that these peptides play an important role in both innate and adaptive immunity.

Cathelicidins and defensins are two mammalian AMP gene families that have attracted most of the attention. They share some common characteristics such as being short (less than 100 amino acids) and containing cationic amino acids, which allows for interaction with negatively charged bacteria lipid membrane structures and contribute to the bacterial killing activity. Although their amino acid sequences are highly variable, most mature AMPs will adopt one of the following structures: α-helix, β-sheet, extended structure or loop structure (5).

Defensins are characterized by six highly conserved cysteine residues that form three pairs of intramolecular disulfide bonds. There are three subfamilies of defensins: α-defensins, whose six cysteines are linked 1-6, 2-4 and 3-5, β-defensins, linked 1-5, 2-4 and 3-6. θ-defensins are circular without a free N- or C-terminus (5, 7, 9). Defensins are expressed in neutrophils, monocytes, macrophages, NK cells, B cells, γδ T cells, dendrit-

ic cells, keratinocytes and epithelial cells, in which they are constitutively expressed or inducible in response to cytokine, growth factors, LPS activation or pathogen infection (9). Defensins are translated into inactive precursor proteins, which are stored in granules of neutrophils (12) or lamellar bodies of epithelial cells (13). Upon activation, these precursor proteins are released and activated following proteolytic processing during or after secretion (14).

Cathelicidins constitute another major family of AMPs and are characterized by a highly conserved N-terminal cathelin domain, a signal sequence and a variable C-terminal microbicidal domain (14). Cathelicidins have been found in many mammalian species including human, mice, monkey, rats, rabbits, guinea pigs, pigs, cattle, sheep, goats and horses (10). Some species express multiple types of cathelicidins, however, human and mouse generate only one cathelicidin, named LL-37 (15) and mCRAMP (16), respectively. Here we will focus on these two cathelicidins and discuss their expression, regulation, antimicrobial activity and immunomodulatory activity.

Human and Mouse Cathelicidins

Cathelicidin Genes, Transcription and Regulation

Multiple cathelicidin genes have been identified in domesticated mammals such as the pig, cow, and horse, which provide these animals with enhanced protection against microorganism infection (17). However, *Camp* is the only identified human cathelicidin gene that encodes an inactive precursor protein hCAP18 (15), while mouse *Camp* (orig-

inally named as *cnlp*) is the only identified cathelicidin gene that encodes mouse cathelin-related antimicrobial peptide (mCRAMP) (16). Human and mouse cathelicidin genes have been mapped to the same region on chromosome 9. Cathelicidin genes consist of 4 exons; the first three exons encode the signal sequence and the highly conserved cathelin-like domain, while the last exon encodes the cleavage site and the mature antimicrobial peptide (15, 18). Amino acid sequence comparison of hCAP18 with mCRAMP shows that their cathelin-like domains have 80% homology (16), suggesting that these domains maintain important biological functions. However, very few studies have been performed to investigate their function and the results from different species are opposite. The cathelin-like recombinant protein generated from hCAP18 demonstrates antimicrobial activity and inhibitory activity on the cysteine protease cathepsin L (19) while the cathelin-like domain from porcine cathelicidin protegrin-3 efficiently activate human cathepsin L (20), indicating that sequence variations and structural discrepancies between pig and human cathelin-like domain may modulate their biological activity.

The C-terminal regions of cathelicidins, which contain mature antimicrobial peptides, have no homology in amino acid sequences compared to each other. Similar to defensins, cathelicidins are also translated into a precursor protein, which are usually stored in the granules of neutrophils (21) or lamellar bodies of epithelial cells (22) and undergo proteolytic processing into mature peptide during or after secretion. For example, hCAP18 is cleaved by elastase (23) or proteinase 3 (24) to release its C-terminal mature antimicrobial peptide called LL-37, which consists of 37 amino acid residues and begins

with two leucine residues. Mouse mCRAMP precursor is processed by elastase-like serine protease (25) and liberates mature mCRAMP peptide containing 33 amino acid residues. Although their amino acid sequences demonstrate no homology, LL-37 and mCRAMP share a similar amphipathic α-helix structure. Based on the conservation of the cathelin-like domain it is likely that the cathelicidin family generated through gene duplication; and the divergence in mature peptide sequence indicates that they may have evolved to acquire specific activity in response to different environmental challenges (18).

hCAP18 has been reported to be expressed in epithelial cells (26), keratinocytes (27), neutrophils (48), monocytes, NK cells, B cells and γδT cells (28). Its physiological concentration varies dramatically depending on location, cell type and infection status. hCAP18/LL-37 is stored in neutrophil granules at a molar concentration as high as 40 μM or 630 μg/10^9 cells. Upon secretion into extracellular fluid, the level of hCAP18/LL-37 is estimated to be around 1.18μg/ml in plasma (21), 2.5μg/ml in gingival crevicular fluid (29), 0.3ng/ml in children urine (30), 86.5μg/ml in seminal plasma (31) and 1μM in crude sweat (32). mCRAMP transcripts were detected during embryogenesis as early as E12, the earliest stage of blood development, and in adult tissues including spleen, testis, colon, bone marrow, stomach, small intestine, heart, lung, and skeletal muscle (16). Further studies clarified its expression in various types of cells including keratinocytes (22), fibroblasts (33), neutrophils (16), macrophages (25), mast cells (34) and NK cells (35).

Due to their potential ability to disrupt lipid bilayers of host cell membranes and induce apoptosis at high concentration (36), cathelicidins expression is strictly and differentially regulated according to cell types and host developmental stage. The promoter of human *Camp* gene contains consensus vitamin D response element that mediate 1,25-dihydroxyvitamin D(3) [1,25(OH)(2)D(3)]-dependent gene expression. 1,25(OH)(2)D(3), the hormonal form of vitamin D(3), is an immune system modulator and induces protein expression such as TLR2 (37), TLR co-receptor CD14 (38). 1,25(OH)(2)D(3) signals through the vitamin D receptor, a ligand-stimulated transcription factor that recognizes specific vitamin D response elements (39). 1,25(OH)(2)D(3) induces human *Camp* transcription in isolated human keratinocytes, monocytes and neutrophils. Moreover, 1,25(OH)(2)D(3) increases hCAP18 protein production and secretion, and enhances LL-37 antimicrobial activity against pathogens including Pseudomonas aeruginosa (40). Oral intake of 1,25(OH)(2)D(3) in rickets patients for 4 weeks significantly increased hCAP18 expression in neutrophils compared to age-matched healthy controls without 1,25(OH)(2)D(3) (41). Skin is the major source for vitamin D(3) in human; and exposure to UVB is sufficient to induce production of vitamin D(3) and upregulates hCAP18 in human skin (42). The 1,25(OH)(2)D(3)-induced expression of hCAP18 can be further regulated by other molecules. For example, IL-17A enhances 1,25(OH)(2)D(3)-induced expression of hCAP18 in skin keratinocytes (43). In the human liver, biliary epithelial cells show intense immunoreactivity for hCAP18 and vitamin D receptor. In cultured biliary epithelial cells, chenodeoxycholic acid and ursodeoxy-

cholic acid induce LL-37 expression through 2 different nuclear receptors: the farnesoid X receptor and the vitamin D receptor, respectively; and the induction is enhanced by 1,25(OH)(2)D(3) (44). Furthermore, the actions of 1,25(OH)(2)D(3) on keratinocyte hCAP18 and CD14 expression are regulated by histone acetylation and require the steroid receptor coactivator 3 (SRC3), which mediates inherent histone acetyltransferase activity (38). However, the effect of calcipotriol, an analogue of Vitamin D(3), on hCAP18 expression is still debated; it is reported that topical treatment with calcipotriol enhances the upregulation of hCAP18/LL-37 expression during wounding healing in human skin in vivo (42). Another group reported that calcipotriol suppressed upregulated HBD-2 and LL-37 expression following UVB, LPS, and TNF-alpha stimulation (45). Thus 1,25(OH)(2)D(3) regulates hCAP18 expression independently or in collaboration with other molecules, revealing the potential of its application in treatment of opportunistic infections.

hCAP18 transcription is also regulated through cAMP-signaling pathway in epithelial cells. cAMP-response element-binding protein and activator protein-1 bind to the hCAP18 putative promoter in vitro. Additionally, transcriptional complexes containing CREB, AP-1, and hCAP18 upstream regulatory sequences are formed within epithelial cells, which are required for inducible hCAP18 expression in these cells (46). In addition to Vitamin D and cAMP, hCAP18 expression can also be regulated through TLR signaling pathway. Mycobacterial infection induced the expression and production of LL-37 in epithelial cells (47-48), alveolar macrophages (49), neutrophils (50), and mono-

cyte-derived dendritic cells (51). hCAP18 can be induced by stimulation through TLR-2, TLR-4, and TLR-9 signaling pathway (49) and by UVB irradiation, LPS, and TNF-α stimulation (45).

The promoter region of mouse *Camp* gene contains several regulatory motifs. A NF-κB binding site is present at position -282 in the promoter region of mouse *Camp*. LPS enhances mCRAMP expression in bone marrow-derived mast cells from BALB/c mice, but not C57BL/6 mice, through the NF-κB pathway, which is transient peaking at 2 hours, followed by a rapid decrease in amount (52). Like LL-37, mCRAMP is also regulated through certain TLR signaling pathway. Lipoteichoic acid (LTA) and IL-4 induce mCRAMP expression in mast cells, with IL-4 demonstrating the most potent induction (34, 53). However, only peptidoglycan, not LPS, induces expression of mCRAMP in corneal fibroblasts in a time and dose dependent manner, which is blocked by anti-TLR-2 antibody treatment (33). These data suggest that TLR-2 and TLR-4 signaling pathways may be involved in the regulation of mCRAMP transcription

To fit with the different requirement against bacterial infection in childhood, cathelicidin expression is developmentally regulated during human and mouse lifetime. Newborns have an immature cellular immune system that may cause increased susceptibility to invasive bacterial infections. As part of the effort to prevent pathogen invasion, mCRAMP transcripts is expressed during embryogenesis as early as E12, the earliest stage of blood development (16). Cathelicidin expression in the skin from embryonic and newborn mice, as well as human newborn foreskin, is 10-fold to 100-fold greater than in

adult skin, which is thought to provide first-line protection against infection during neonatal period. LL-37 and hBD-2 act synergistically to efficiently kill group B *Streptococcus*, an important neonatal pathogen (54). Meanwhile, intestinal mCRAMP synthesis was restricted to the neonatal period and gradually disappeared two weeks after birth, which is accompanied by increased stem cell proliferation and epithelial cell migration along the crypt-villus axis. The strictly controlled expression of mCRAMP in intestine facilitates protection against bacterial infection in neonatal period as well as bacterial colonization and establishment of gut homeostasis in adulthood. mCRAMP-deficiency leads to a defect in controlling intestinal bacterial growth of the newborn enteric pathogen *Listeria monocytogenes* (55). On the contrary, hCAP18 expression in neonate neutrophils is significantly lower than in adults, and its expression in monocytes is not significantly different between neonates and adults (41), indicating that epithelial cells may be the main source of cathelicidins during neonatal life. These studies demonstrate that the expression of cathelicidins in human and mouse are highly regulated to provide fast, effective and on-demand protection.

Mature Peptide Structure and Post-translational Processing

Although mature LL-37 and mCRAMP demonstrate no homology in their amino acid sequence, they share similar amphipathic α-helix structure. The structure of LL-37 in SDS micelles is composed of a curved amphipathic helix-bend-helix motif spanning residues 2–31 followed by a disordered C-terminal tail. The helical bend is located be-

tween residues Gly-14 and Glu-16 (56). The structure of LL-37 in dodecylphosphocholine micelles suggested that it is attracted to the surface of the micelle with the hydrophilic side exposed to the water and the hydrophobic side interact with the micelle hydrocarbon region (57). Similarly, mCRAMP peptide consists of two α-helices from Leu4 to Lys10 and from Gly16 to Leu33, which are connected by a flexible region from Gly11 to Gly16. The region from Gly16 to Leu33 creates amphipathic a-helices which can span the lipid bilayers and maintain antibacterial activity (16, 58-59).

The eccrine gland is one of the major cutaneous appendages that secret sweat. hCAP18 mRNA and protein are detected in the eccrine structures of normal human skin, in which they are localized to both the eccrine gland and sweat ductal epithelial cells (32). After secretion onto the skin surface in sweat, hCAP18 is processed by a serine protease into LL-37, which can be further processed into multiple shorter antimicrobial peptides such as KR-20, RK-31 and KS-30 (named according to their length and N-terminal amino acid residues). These peptides show enhanced antimicrobial action, acquiring lower minimal inhibitory concentrations for skin pathogens such as *Staphylococcus aureus* and *Candida albicans*. However, further processing of LL-37 decreases its ability to stimulate IL-8 release from cultured keratinocytes (60), suggesting that intact LL-37 is required for its immunoregulatory activity. The smallest antibacterial peptide KR-12 corresponding to residues 18–29 of LL-37 displays selective toxic effect on bacteria but not human cells (56), indicating the potential benefits of truncated peptide as natural antibiotics specific against pathogens infection.

Proteinase-3, another type of serine proteinase, processes hCAP18 to mature LL-37 in cystic fibrosis (CF) BAL fluid. However, the lung fluid from CF patients exhibits no detectable bacterial killing activity, since endogenous LL-37 bind to glycosaminoglycans, protecting LL-37 from proteolysis as well as inactivating its antimicrobial activity (61). LL-37 also induces degranulation in purified lung mast cells. As a consequence, LL-37 is degraded by the released beta-tryptase and loses functional capabilities of intact LL-37, including mast cell degranulation, bactericidal activity, and LPS neutralization. Meanwhile, platelet-derived chemokine CXCL4 can destabilize active tetrameric beta-tryptase and protect LL-37 from degradation (62).

The serine proteases stratum corneum tryptic enzyme (SCTE, kallikrein 5) and stratum corneum chymotryptic protease (SCCE, kallikrein 7) also control activation of the human cathelicidin precursor protein hCAP18 in skin and influence further processing to smaller peptides. In SPINK5-deficient mice that lack the serine protease inhibitor LEKTI, epidermal extracts of these animals show a significant increase in antimicrobial activity compared with controls, suggesting that mouse serine protease is also involved in mCRAMP activation (63).

In mice, mCRAMP precursor protein is processed by macrophage elastase-like proteases and liberates mature 33-a.a. mCRAMP peptides (25). Besides regular mCRAMP, mast cells are able to process cathelicidin into a unique 28-a.a. peptide (IGE24) that is distinct from those found in keratinocytes and neutrophils, which has antibacterial activity and kills group A *Streptococcus* intracellularly (53).

Antimicrobial Activity

It is highly appreciated that cathelicidins are by nature antibiotics with a broad spectrum of activity against gram-positive, gram-negative bacteria, viruses, fungi and parasites (5, 10, 14, 64); they have existed for millions of years and provided protection to both invertebrates and vertebrates.

LL-37 is constitutively expressed on epithelial surfaces, and increases during infection and would healing process. Extensive studies have shown that LL-37 exhibits effective bactericidal activity against multiple types of bacteria including *Escherichia coli* (65-69), *Pseudomonas aeruginosa* (68, 70-73), *Klebsiella pneumoniae* (68) and *Staphylococcus aureus* (69, 73-74), et al. LL-37 is a positively charged amphipathic molecule, which performs its bacteria killing activity by interacting with negatively charged bacterial molecules and insertion into the membrane, followed by pore formation and membrane disruption (6). However, physiological concentrations of LL-37 as described previously may not be high enough to kill pathogen directly in certain situations. In addition to its direct antimicrobial activity, LL-37 can inhibit the formation of *Pseudomonas aeruginosa* biofilms in vitro at a concentration of 0.5 μg/ml, which is closer to its physiologically meaningful concentration and far below that required to direct killing or growth inhibitory activity (MIC=64μg/ml). At this concentration LL-37 can decrease bacterial cells attachment, stimulate twitching motility, and influence Las and Rhl quorum sensing systems, resulting in the down-regulation of essential genes and interruption of biofilm formation (72).

Mouse mCRAMP has also been extensively studied for its bactericidal activity. Its expression is critical for the protective function of many types of cells in innate immunity, such as epithelial cells, mast cells, macrophages and neutrophils. mCRAMP is continuously expressed in skin and colon surface epithelial cells and up-regulated in the blood-brain barrier endothelial cells and meninges cells after *Neisseria meningitides* infection. mCRAMP-deficient mice are more susceptible to necrotic skin infection of group A *Streptococcus* (75), to oral infection of *Citrobacter rodentium* (76), to meningitides infection of *Neisseria meningitidis* (77), as well as to urinary tract and kidney infection of *E. coli* (30). mCRAMP is also expressed in murine mast cells, which is inducible by LPS or LTA. mCRAMP-deficient mast cells demonstrated a 50% reduction in their ability to kill group A *Streptococcus* (34). Macrophages express and upregulate mCRAMP after infection by the intracellular pathogen *Salmonella typhimurium*, and this up-regulation depends on reactive oxygen intermediates. Intracellular elastase-like serine protease is required to process precursor protein and release mature mCRAMP, which impairs *Salmonella* cell division and results in long filamentous bacteria. (25). mCRAMP remains intracellular during polymorphonuclear neutrophil exudation from blood and is secreted upon PMA stimulation. mCRAMP is recruited to phagolysosomes in infected neutrophils and exhibit intracellular anitibacterial activity against *S. aureus.* Later in infection, neutrophils produced neutrophil extracellular trap (NET); and association of mCRAMP with NET or DNA diminishes its antimicrobial activity againt *S. aureus* (78). Its protective function is even more significant in cornea since it is not accessible to im-

munological competent cells, mCRAMP-deficient mice show increased susceptibility to *Pseudomonas aeruginosa* (PA) meditated keratitis. mCRAMP-deficient mice show significantly defects of PA clearance in the cornea and display an increased number of infiltrating neutrophils and significantly up-regulated levels of cytokines compared to the WT mice (79). Furthermore, exogenous mCRAMP administration protects mice from *Bacillus anthracis* spore-induced death by recruiting more neutrophils to the site of infection, which reduces spore burden in Mac-1+ cells and increases spore clearance (80). These data suggest that mCRAMP is an important bactericidal factor in innate host defense.

Cathelicidins are positively charged and can potentially interact with any negatively charged molecules such as phospholipids in cell membranes. Accordingly, the ability of cathelicidins to distinguish between target and host cells is very important. Previous studies suggested that this process is likely based on several parameters including lipid net charge, packing density, the capability to form intermolecular H-bonds, lipid molecular shape (81), and lipid membrane component. For example, acidic phospholipids in target cell membrane cause enhanced association with the LL-37; meanwhile, high content of cholesterol and sphingomyelin in the peptide-secreting host cells attenuate LL-37 interaction with the outer surface of the plasma membrane. Further studies suggested that LL-37 may exert its antimicrobial effects by compromising the membrane barrier properties of the target microbes by a mechanism involving cytotoxic oligomers, similarly to other peptides forming amyloid-like fibers in the presence of acidic phospholipids (82). By these means cathelicidin peptides can effectively kill bacteria while keep-

ing host cells intact. However, high concentration of LL-37 still induces host cell apoptosis (36), emphasizing the importance of strict regulation of its expression.

Receptors and Immunomodulatory Activity

In addition to the direct antimicrobial killing properties against a variety of organisms, it is increasingly appreciated that cathelicidins are also immunomodulatory. The mechanism by which cathelicidins modulate immune trafficking and function is not completely understood, though a number of potential receptors have been suggested for the human cathelicidin LL-37 including formyl-peptide receptors like 1 (FPRL1) (83-86), epidermal growth factor receptor (EGFR) (87-90), CXCR2 (91), $P2X_7$ (84, 91-93) and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (94). Fewer studies had been done with the mouse mCRAMP receptor, and mFPR2 is the only one so far identified (95). Besides its direct binding with specific receptors in host cells, cathelicidins can also interfere with TLR signaling pathway, regulate cytokines expression and modulate inflammatory response (96-99).

FPRL1 belongs to the family of G protein-coupled receptors, which were originally identified by their ability to bind N-formyl peptides such as N-formylmethionine produced by the degradation of either bacterial or host cells (100). LL-37 stimulates IMR90 human fibroblast through FPRL1 and induces ERKs activation, p47 phosphorylation and translocation, as well as NADPH oxidase activation (86). LL-37 suppresses neutrophil apoptosis in a dose dependent manner (0.01-5μg/ml) via the activation of

FPRL1 and $P2X_7$ and induces phosphorylation of ERK-1/2, expression of Bcl-xL and inhibition of caspase 3 activity (84). Meanwhile, the signal through FPRL1 to MAPK pathway could be inhibitory. LL-37 specifically inhibits serum amyloid A-induced IL-8 production in transcriptional and post-transcriptional levels through FPRL1 by inhibition of ERK and p38 MAPK pathway and SAA-stimulated neutrophil chemotactic migration (101). In addition to modulation of MAPK pathway, LL-37 also induces calcium mobilization in human monocytes and functions as a chemoattractant for neutrophils, T lymphocytes (83) and mast cells (102), which can be blocked by pertussis toxin, an inhibitor for FPRL1.

CXCR2 is a member of the family of G protein-linked chemokine receptors that are known as seven transmembrane proteins, which specifically binds and responds to cytokines of the CXC chemokine family (103). LL-37 may act as a functional ligand for CXCR2 and induce receptor down-regulation, intracellular calcium mobilization and cell migration of human neutrophils; this effect is blocked by pretreatment of cells with selective CXCR2 antagonist (91). LL-37 also induced the release of pruritogenic mediators from mast cells, including histamine, IL-31, IL-2, IL-4, IL-6, GM-CSF, nerve growth factor, PGE2, and leukotriene C4. The LL-37-mediated IL-31 production and release is dependent on G-protein, PI3K and MAPK pathway, although it is unknown whether the induction is through FPRL1 or CXCR2 (104).

EGFR is a receptor tyrosine kinase, which belongs to the ErbB family (105). LL-37 activates airway epithelial cells through MAPK/ERK pathway and increases IL-8

release; the activation is suppressed by inhibitors to MAPK/ERK and EGFR metalloproteinase, as well as blocking antibodies to EGFR and EGFR-ligand (87). LL-37 induces keratinocyte migration via transactivation of EGFR as well as induction of FPRL1 (88) and promotes wound healing responses. By in vivo adenoviral transfer into excisional wounds in ob/ob mice, LL-37 significantly improves re-epithelialization and granulation tissue formation (89). The wound healing-enhancing activities of LL-37 indicate its potential therapeutic application in human skin diseases.

$P2X_7$ receptors are membrane ion channels which open in response to the binding of extracellular ATP and allow permeation by both small cations and large dyes (106-107). Stimulation of $P2X_7$ receptor with LL-37 leads to cell membrane pore formation, a transient ATP release, caspase-1 activation (93), calcium influx, and ethidium bromide uptake (92). LPS-primed human monocytes initiate cleavage and secretion of mature IL-1β upon LL-37 stimulation via the $P2X_7$ receptor, which can be inhibited by blockage of $P2X_7$ with oxidized ATP (93). The structural properties of LL-37 are critical for its capacity to activate through $P2X_7$ receptor, since LL-37-induced fibroblast cell proliferation requires a strong helix-forming propensity of LL-37 in aqueous solution (92).

GAPDH is well known as one of the key enzymes involved in glucose metabolism. It catalyzes the sixth step of glycolysis and breaks down glucose for energy and carbon molecules (108). Besides this long established metabolic function, recent studies suggested that GAPDH is also involved in several non-metabolic processes, including

regulation of H2B transcription (109), sensing of NO induced stress (110), and initiation of apoptosis (111-112). Previous studies have established that LL-37 executes its anti-infective function by inducing chemokines (e.g., CXCL-1/Gro-α) and anti-inflammatory cytokine (e.g. IL-10) expression; these functions are modulated through the MAPK p38 pathway (99). GAPDH was shown to directly bind to LL-37 in macrophages and monocytes; gene silencing of GAPDH causes defect in LL-37 anti-infective function, resulting in impaired p38 MAPK signaling, decreased downstream chemokine and cytokine transcription, as well as reduced cytokine production (94).

In addition to activating cells through specific receptors in host cells, LL-37 can also modulate TLR signaling pathway, regulate cytokines expression and inflammatory response. The effect of LL-37 on cell activation depends on TLR, cell types and co-stimulatory molecules. Positively charged LL-37 can directly bind to negative charged LPS, a ligand of toll-like receptor 4. LL-37 increases the levels of TLR4 mRNA and protein from mast cells, and induces the release of IL-4, IL-5 and IL-1β (113). However, when acting synergically with LPS, LL-37 displays inhibitory activity on cell activation and cytokines production. LL-37 inhibited TLR4-mediated induction of human peripheral dendritic cell and release of IL-6, IL-8, IL-10, GM-CSF and TNF-α, this inhibition was associated with an alteration of cell membrane receptor mobility and structure (114). The incubation with LL-37 significantly decreased the release of pro-inflammatory cytokines (such as IL-1β, IL-6, IL-8, and TNF-α) from human neutrophils (73) and peripheral blood mononuclear cells following stimulation with TLR4 or TLR2/1 agonists. The

mid-region of LL-37, comprising amino acids 13–31, acts as the active domain which can bind to LPS and modulate TLR4 responses (115). Meanwhile, LL-37 at lower and physiologically relevant concentrations (2-3μg/ml) alters epithelial cell responses to pro-inflammatory cytokines in vivo. Acting synergistically with the TLR2/1 agonist PAM3CSK4, TLR3 agonist polyI:C or TLR5 agonist flagellin plus IL-1β, LL-37 induces transcription and the release of both IL-8 and IL-6 from bronchial epithelial cells and keratinocytes (97).

Although many receptors have been suggested for human LL-37, only one receptor had been reported for mCRAMP, which is mouse formyl peptide receptor 2 (mFPR2). mCRAMP can induce migration of monocytes, neutrophils, macrophages, and peripheral blood leukocytes, initiate calcium flux and activate MAPK pathway in monocytes. Exogenous mCRAMP can act as an immune adjuvant and enhance both humoral and cellular ovalbumin-specific immune responses in WT C57BL/6 mice (95).

Like LL-37, positively charged mCRAMP can also directly bind to LPS and regulate TLR4 signaling pathway. mCRAMP inhibits LPS activation and blocks LPS down-regulation of TLR4 expression on dendritic cells. mCRAMP suppresses TLR4- but not TLR2-mediated induction of dendritic cell maturation and cytokine release, and the inhibition was correlated with an alteration of cell membrane receptor mobility and structure (114). mCRAMP-deficient peritoneal neutrophils releases significantly more TNF-α after LPS stimulation but shows decreased antimicrobial activity as compared to neutrophils from WT mice (73). However, the regulatory activities of exogenous and

endogenous mCRAMP were shown to be different in certain studies. Exogenous mCRAMP inhibits p38 and ERK phosphorylation, TNF-α expression, MyD88 synthesis and MyD88/IRAK-4 association in murine macrophages activated by LPS, LTA or flagellin. However studies with mCRAMP-deficient murine macrophages suggested that endogenous mCRAMP does not inhibit MAPKs activation and cytokine expression upon these TLR ligands activation. Furthermore, mCRAMP deficiency does not alter susceptibility of mice to lethal LPS challenge. These results suggest that the origin and concentration of mCRAMP peptide and the prior activation status of the cell is critical for the immunomodulatory effects of mCRAMP (116). In this study we will take advantage of mCRAMP-deficient mice and investigate the effect of endogenous mCRAMP on B cell antibody production.

In addition to exogenous ligand LPS, TLR4 has an endogenous ligand called small fragment hyaluronan (HA), which is released following injury and is also a CD44 ligand. mCRAMP inhibits HA-induced MIP-2 released from mouse bone marrow derived macrophages in a CD44 dependent manner, but is independent of Gi protein or EGFR signaling pathway. mCRAMP-deficiency leads to a large increase in ear swelling, cell infiltration, and MIP-2 expression in a skin inflammation model induced by repeated application of 2,4-dinitrofluorobenzene to mice ears. These results suggest that cathelicidin has anti-inflammatory activity in skin that may be mediated in part by inhibition of HA-mediated processes (117).

Cathelicidins Expression in Skin Diseases and Cancers

LL-37 has been implicated in a wide variety of inflammatory skin diseases including psoriasis, atopic dermatitis (AD), and rosacea, in which LL-37 has dual functions, acting as an innate antibiotic and as an adaptive immunomodulator.

Acne rosacea is an inflammatory skin disease that affects 3% of the US population over 30 years of age. Individuals with rosacea express abnormally high levels of cathelicidin in their facial skin. Some active forms of hCAP18 are unique in that they are absent in normal skin, which is a result of a post-translational processing correlated with an abnormal increase in stratum corneum tryptic enzyme (SCTE, a serine protease of the kallikrein family) in the granular, cornified, or basal layer of the epidermis. In mice, mCRAMP-deficiency attenuates inflammation in the skin following SCTE injection, suggesting that SCTE induces skin inflammation through enhancement of cathelicidin processing (118). Patients with atopic eczema also exhibit enhanced expression of LL-37 in lesional skin compared with nonlesional skin, suggesting the role of LL-37 in the process of re-epithelialization (119).

AD and psoriasis are the two most common chronic skin diseases. Patients with AD, but not psoriasis, suffer from frequent skin infections. There is abundant LL-37 and HBD-2 presented in the superficial epidermis of all patients with psoriasis. However, these peptides were significantly decreased in acute and chronic lesions from AD patients. A deficiency in the expression of antimicrobial peptides may account for the susceptibility of patients with atopic dermatitis to skin infection with *S. aureus* (120) and dissemi-

nated herpes simplex virus (HSV) (121). Notably, the mechanism to upregulate hCAP18 following vitamin D treatment was functional in lesional AD (122). Besides increased susceptibility of infections, AD is associated with elevated skin production of Th2 cytokines (IL-4 and IL-13) and low levels of proinflammatory cytokines (TNF-α, IFN-γ, and IL-1β) (123). Interestingly, neutralizing antibodies to IL-10 augments the production of TNF-α and IFN-γ by peripheral blood mononuclear cell from AD patients, suggesting that increased levels of IL-10 may contribute to the LL-37 deficiency in AD patients by reducing cytokines that enhance LL-37 expression (124).

Since these small peptides have effective bacterial killing activity, people began to test the possibility of applying them by gene therapy. It had been reported that LL-37 delivered via adenoviral vectors/CMV-LL37 is able to clear chronic infections such as occur in osteomyelitis resulting from trauma or an infected foreign body in rodent models (125). However, increased expression of LL-37 is also observed in a variety of cancers, which promotes cancer cell proliferation, migration and metastatic phenotype. In this circumstance the application of LL-37 in gene therapy needs to be further evaluated.

Previous studies have shown that expression of hCAP-18/LL-37 was localized to immune and granulosa cells of normal ovarian tissue and up-regulated in ovarian cancers. LL-37 mediates migration of multipotent mesenchymal stromal cells (MSCs) to tumors likely through FPRL1 signaling pathway. LL-37-treated MSCs secret increased levels of cytokines and pro-angiogenic factors compared with controls, including IL-1 receptor antagonist, IL-6, IL-10, CCL5, VEGF, and matrix metalloproteinase-2 (126-127). Human

lung and breast cancer cells also express the LL-37/hCAP-18 mRNA and proteins. Immunohistochemistry of lung cancer tissues showed that the peptide is expressed mostly in adenocarcinoma and squamous cell carcinoma. LL-37/hCAP-18, acting as growth factor, enhances proliferation and migration of cancer cells through phosphorylation of the EGFR and activation of MAPKs (128-129).

Contrasting to these findings, a recent study showed that mCRAMP expression is readily detected in tumor-infiltrating NK1.1(+) cells in mice. mCRAMP-deficiency causes impaired cytotoxic activity toward tumor target, leads to faster tumor growth than WT controls in xenograft tumor mouse models, indicating that mCRAMP is required for NK-cell mediated antitumor effect (35). TLR ligands, such as CpG oligodeoxynucleotides (CpG-ODN), have been tested as a potential approach for the control of ovarian tumors. Addition of LL-37 to the CpG-ODN treatment enhanced proliferation and activation of NK cells in the peritoneal cavity. It generated significantly better therapeutic antitumor effects and enhanced survival in murine ovarian tumor-bearing mice compared with treatment with CpG-ODN or LL-37 alone. (130).

Bacteria Resistance to AMPs

Although extensive studies have shown that cathelicidins can effectively kill invading pathogens, the potential of their antimicrobial therapy could be impaired by the emergence of resistant bacterial strains. Pathogenic bacteria have evolved many mechanisms to evade the immune system, such as expressing AMP-binding polysaccharides or

proteins, producing proteinase to degrade AMPs or down-regulating AMP expression.

Neisseria meningitidis responds to sublethal doses of LL-37 and upregulates two capsule genes, siaC and siaD, which further result in upregulation of capsule biosynthesis. The bacterial endotoxin lipooligosaccharide and the polysaccharide capsule prevent LL-37 from reaching the bacterial membrane, as more LL-37 reaches the bacterial membrane on both lipooligosaccharide-deficient and capsule-deficient mutants whereas both mutants are also more susceptible to LL-37 killing than the wild-type strain (131).

Staphylococcus aureus produces two major proteinases: glutamylendopeptidase (V8 protease) and metalloproteinase (aureolysin). The V8 proteinase only hydrolyzes the Glu16-Phe17 peptide bond and keeps its antimicrobial activity. On the contrary, aureolysin cleaves LL-37 between the Arg19-Ile20, Arg23-Ile24, and Leu31-Val32 peptide bonds, which instantly aborting its antibacterial activity. Certain *S. aureus* strains that produce significant amount of aureolysin are less susceptible to LL-37 than strains possessing no aureolysin activity (74). *Porphyromonas gingivalis*, the major pathogen associated with periodontitis, was also capable of degrading LL-37 by utilizing arginine-specific gingipains. Saliva collected from volunteers with a healthy periodontium protected LL-37 from proteolysis by *P. gingivalis* (65).

Pathogenic *Neisseria* may gain a survival advantage in the female genital tract by down-regulating LL-37 expression. *Neisseria gonorrhoeae* is a human pathogen which preferentially attaches to and invades epithelial cells of the genital tract. LL-37 displays in vitro killing activity against *N. gonorrhoeae*. However, *Neisseria* infection

down-regulates the transcript and peptide levels of LL-37, which is most prominent with pathogenic strains. Non-pathogenic strains such as *Neisseria lactamica* and *E. coli* only exhibit moderate suppressive effects. Heat-killed *N. gonorrhoeae* lost its suppression on LL-37 expression, suggesting live bacteria is required for the effects (132).

Our Hypothesis

Abnormal expression of human cathelicidin LL-37 has been detected in human skin diseases and cancers; and many studies have clearly shown that those patients with down-regulated LL-37 expression, as well as *Camp* -/- mice, have increased susceptibility to pathogen infections because of the defect in innate hose defense system. Meanwhile, how cathelicidins regulate adaptive immunity, particularly antibody production as in our interest, is still under debate.Since Previous studies showed that exogenous mCRAMP can enhance antibody response in C57BL/6 mice; we hypothesized that endogenous mCRAMP, expressed by B cells as well as other cells, regulates antibody production in respond to antigen challenge.

Experimental Rationale

We tested this hypothesis in a mouse model by addressing the following three questions: i) Do B cell subsets express mCRAMP and does mCRAMP expression change upon activation? ii) Does mCRAMP directly act on B cells and regulate antibody production? iii) Do mCRAMP-deficient mice respond to antigen challenge differently from

WT mice?

Although several types of human lymphocytes including B and γδT cells had been shown to express LL-37 (28), it is not known whether mouse B and T cells express mCRAMP. Due to the lack of specific anti-mCRAMP antibody, we use RT-PCR to detect mCRAMP transcripts and our preliminary data suggested that mCRAMP mRNA is expressed in both splenic B and T cells. Further studies showed mCRAMP mRNA expression in all B cells subset tested as well as CD4+ and CD8+ T cells. mCRAMP expression is specifically regulated in certain type of cells by TLR ligands such as LPS, LTR or peptidoglycan and by cytokines such as IL-4 (53) and IFN-γ (28). Based on previous reports that mouse B cells express TLRs, IL-4 and IFN-γ receptors (133-135), we predicted that mCRAMP expression is regulated in these B cells through TLR or cytokine signaling pathway. Since IL-4 and IFN-γ are important cytokines in adaptive immunity that caninduce isotype switch in B cells, we also tested their effect on mCRAMP expression in combination with CD40L or LPS. Defects in the B cell development may affect antibody production; however the only data with respect to lymphocytes in mCRAMP-deficient mice was obtained from total blood leukocytes by microscope examination and FACS analysis of the forward scatter and side scatter signal. These limited studies suggested that the proportions of leukocytes, monocytes and lymphocytes were not significantly different between WT and mCRAMP-deficient mice (75). In our studies examination of proportion and absolute number of lymphocyte subsets, including FO, MZ B cells, CD4+ and CD8+ T cells in spleen as well as B1a, B1b, B2 cells in peritoneal cavity by FACS

analysis, showed that mCRAMP-deficiency has no effect on the development of B and T cell subsets.

After showing that B and T cells constitutively express mCRAMP mRNA and that it is up-regulated in B cells after activation, we pursued the answer to the second question: does mCRAMP act directly on B cells to regulate antibody production? There are few papers in the literature that have studied the role of LL-37 in regulating the magnitude of the adaptive immune antibody response and the results are controversial. LL-37 at a concentration of 20μg/ml was shown to decrease IgM and IgG2a production in mouse splenic B cells activated by LPS plus IFN-γ in vitro, primarily through inhibition of cell activation and proliferation (136). However, another study demonstrated that LL-37 at 6μg/ml raises the sensitivity of human peripheral B cells to the CpG motifs in bacterial DNA, enhancing B cell activation and increasing IgM and IgG production (137). These data suggest that LL-37 acts directly on B cells and modulates antibody production; however it appears that the origin of B cells (human vs. mouse), cathelicidin concentration and activation agents determine whether the effect of LL-37 results in activation or inhibition of functions. However, no studies have been done to investigate the direct effect of mouse mCRAMP on purified splenic B cells. Since mCRAMP and LL-37 share similar structure and electronic charge, and regulate cell functions through similar receptor and signaling pathway, we predicted that mCRAMP directly regulates splenic B cell antibody production. Because previous studies showed that exogenous and endogenous mCRAMP function differently on macrophage activation and cytokine production, we

took the advantage of mCRMAP-deficient B cells and study the effect of endogenous mCRAMP on B cell antibody production. Another benefit of using WT and mCRAMP-deficient B cells is that mCRAMP concentration in this situation is more physiologically relevant, which may be a factor in modulating the antibody production as described above.

In the last part of the study we tried to answer the question: do mCRAMP-deficient mice respond to antigen challenge differently from WT mice? Previous studies accomplished by Kurosaka et al. suggested that exogenous mCRAMP acts as an immune adjuvant and enhances antibody production in WT mice (95). In this study they used WT mice and exogenous mCRAMP, which may not be representative of the physiological mCRAMP concentration and may induce effects quite different from those dependent on endogenous mCRAMP. Another caveat on the use of exogenous cathelicidin is that increased LL-37 is detected in cancer cells and may contribute to cancer metastasis, indicating the use of exogenous cathelicidins need careful consideration (128-129). Additionally, i.p. injection of exogenous mCRAMP induces cell migration to peritoneal cavity (80), which may not occur in normal immunization situation and could lead to potential artifacts. Another caveat on the use of exogenous cathelicidin is that increased LL-37 is detected in cancer cells and may contribute to cancer metastasis, indicating the use of exogenous cathelicidins need careful consideration. Finally Kurosaka et al. investigated only T-dependent response to OVA-protein in the presence or absence of exogenous LL-37, and did not test the T-independent immune response. In our study we

compared WT vs. mCRAMP-deficient mice and investigated the effect of endogenous mCRAMP on antibody production to both T-independent antigen (TNP-LPS and R36A) and T-dependent antigen (TNP-OVA plus Alum).

By answering these three questions, we obtained more detailed understanding of the immunoregulatory function of this small but multifunctional peptide in adaptive immunity and provided clues for its future clinical application.

MATERIALS AND METHODS

Animals - C57BL/6 (B6) mice were purchased from the Jackson Laboratory. Camp-deficient 129/SVJ mice (*Camp -/-,* KO) were a generous gift from Dr. Richard L. Gallo (University of California, San Diego, CA). *Camp* -/- mice were backcrossed to B6 mice for 10 generations. *Camp* -/- mice were identified by PCR analysis as described previously (75). All mice were maintained under pathogen-free conditions and under approved animal protocols from the Institutional Animal Care and Use Committee at the University of Alabama at Birmingham.

Peptide preparation and storage - mCRAMP peptide (ISRLAGLLRKGGEKIGEKLKKIGQKIKNFFQKLVPQPE) was synthesized by Alpha Diagnostic Int. (San Antonio, TX). Lyophilized peptides were resuspended in 0.01% acetic acid to generate 100μM working stocks, which were stored at -80°C until time of use.

B cell purification and activation – B cell purification and activated was performed as described previously (138). Purified splenic B cells were obtained using a CD43 magnetic bead depletion strategy following the manufacturer's instructions (Miltenyi Biotec). B cells (5×10^4) were cultured in 96-well flat-bottom plates in 200μl of complete medium consisting of RPMI 1640 plus L-Glutamine (GIBCO) supplemented

with 10% heat-inactivated FBS (HyClone), 50μM 2-mercaptoethanol (Sigma-Aldrich), 100U/ml penicillin and 100U/ml streptomycin (Cellgro). Cultures were incubated at 37°C in a humidified incubator containing 10% (v/v) CO_2. B cells were stimulated with 20μg/ml LPS (Sigma-Aldrich), 1ng/ml recombinant mouse IL-4 (eBioscience), CD40L-expressing Sf9 cells (a kind gift from Dr. Virginia Sanders, The Ohio State University) at a B cell to Sf9 ratio of 10:1. Culture supernatants were collected and stored at -80°C until further analysis.

Flow cytometry and cell sorting – Flow cytometry and cell sorting was performed as described previously (139). FITC-labeled anti-γ1, anti-CD23, anti-Mac 1; PE-labeled anti-CD5, anti-Mac 1; and APC-labeled anti-B220 antibodies were purchased from BD Pharmingen. Anti-CD21 (clone 7G6) antibody was purchased from BD Pharmingen and labeled with PE in our lab. Cy5-labeled goat anti-mouse IgM antibody was purchased from Jackson ImmunoResearch. FcR blocker Ab93 was generated in our laboratory. All FACS analyses were performed on a FACSCalibur (BD Biosciences) and analyzed using FlowJo software (Tree Star). B and T cell subsets were sorted using a FACSAria flow cytometer (BD Biosciences) from the spleen or peritoneal cavity. MZ ($B220^+$ $CD21^{high}$ $CD23^-$), FO ($B220^+$ $CD21^{int}$ $CD23^+$), CD4+, and CD8+ T cells were purified from naive WT B6 spleens with final purities ranging from 95–99%. B1a ($CD5^+$ Mac-1^+ $B220^{int}$), B1b ($CD5^-$ Mac-1^+ $B220^{int}$), B2 ($CD5^-$ Mac-1^- $B220^{high}$) and T cells ($CD5^+$ $B220^-$) were sorted from the peritoneal cavity lavage with purities ranging

from 95–99%.

Immunization - 7- to 9-wk-old female mice were immunized i.v. with 1×10^8 heat-killed *Streptococcus pneumonia* (R36A) or i.p. with 100μg TNP-LPS (Biosearch Technologies). Serum was collected on day 0 before immunization and days 3, 7, 14 after immunization. Additional mice were immunized i.p. or s.c. with 100μg TNP-OVA (Biosearch Technologies) absorbed in 4mg alum (Sigma-Aldrich) on day 0 and day 21. Serum was collected on day 0 before immunization and days 7, 14, 21, 28, and 35.

ELISA - Total immunoglobulin levels were determined by ELISA, as described in detail previously (140). Briefly, B cell culture supernatants were collected on days 2 - 7 after activation and stored at -80°C until use. Total IgM, IgG3, or IgG1 were captured by plate-bound goat anti-mouse IgM or IgG (Southern Biotechnology Associates) and detected with alkaline phosphatase-conjugated goat anti-mouse IgM, IgG3 or IgG1 (Southern Biotechnology Associates). A standard curve was prepared using known quantities of BH8 (anti-PC IgM, generated in our lab) or anti-TNP Ab (IgG1, eBioscience). To measure specific anti-PC or anti-TNP Abs concentration, plates were coated with PC-BSA or TNP-BSA and antibodies were detected by alkaline phosphatase-conjugated goat anti-mouse IgM, IgG3 or IgG1. p-Nitrophenyl phosphate (Sigma-Aldrich) was added, and color development was determined on a Titertek Multiskan Plus reader (Labsystems, ICN Biomedicals) at 405nm.

ELISPOT - 96-well high binding plates were coated with goat anti-mouse IgG or TNP-BSA and single cell splenic suspensions were prepared 7 days after primary or secondary TNP-OVA/Alum immunization. 1×10^6 total splenocytes were seeded in each well containing 100µl RPMI followed by a 1: 3 serial dilution. Cells were incubated at 37°C for 24hrs before being lysed with PBS containing 0.05% Tween 20. Alkaline phosphatase-conjugated goat anti-mouse IgG1 was added and spots visualized by 5-bromo-4-chloro-3-indolyl phosphate (Sigma-Aldrich) and counted under a dissection microscope. Spots were then dissolved in 50µl DMSO and absorbance of each well was measure with a spectrophotometer at 650nm.

Reverse Transcriptase PCR – RT-PCR was performed as described previously (139). Briefly, total RNA was isolated using TRIzol reagent according to the manufacturer's instructions (Invitrogen). Purified mRNA was dissolved in DEPC-treated water and quantified by measuring the absorbance at 260nm using NanoDrop 1000 (Thermo Scientific). All mRNA samples were treated with DNase I (Sigma-Aldrich) before RT-PCR. 1µg RNA/sample was used with the Cloned AMV First-Strand cDNA Synthesis Kit (Invitrogen). For PCR, 3µl of cDNA was added to a master mix containing 20mM Tris-HCl (pH 8.4), 50mM KCl, 0.2mM each dNTP, 1.5mM MgCl2, 0.5µM primer mix and 1U recombinant Taq DNA Polymerase (Invitrogen) for a final reaction volume of 25µl. Cycling protocol: 94°C for 3 minutes; then 35 cycles of 94°C denaturing for 45s, 58°C annealing for 30s, 72°C extending for 1min; followed by 72°C extending for addi-

tional 10 mins. The following primer pairs were used: β-actin: 5'-TACAGCTTCACCACCACAGC-3' and 5'-AAGGAAGGCTGGAAAAGAGC-3'; Iγ1: 5'-CATCCTATCACGGGAGATTGGG-3' and 5'-ATCCTCGGGGCTCAGGTTT G-3'; mature IgG1: 5'-TATGGACTACTGGGGTCAAG-3' and 5'-CCTGGGCA CAATTTTCTTGT-3'; Camp: 5'-CGAGCTGTGGATGACTTCAA-3' and 5'-CAGGCTC GTTACAGCTGATG-3'; CD19: 5'- GGAGGCAATGTTGTGCTGC-3' and 5'- ACAATC ACTAGCAAGATGCCC-3'; CD3e: 5'- ATGCGGTGGAA-CACTTTCTGG -3' and 5'- GCACGTCAACTCTACACTGGT -3'; IL-4: 5'-ACCACAGAGAGTGAGCTCG-3' and 5'-ATGGTGGCTCAGTACTACG-3'. 10 µl of the PCR reaction were visualized on a 1.5% agarose gel containing 0.5 µg/ml ethidium bromide. Pictures were taken using MultiImage Light Cabinet (Alpha Innotech) and the densities of gene-specific bands were measured by ImageJ (http://rsbweb.nih.gov/ij/index.html). Actin was used as internal control and the concentration of actin cDNA in each sample was used to normalize the gene-specific product concentrations.

IgG1 mRNA stability assay - On day 4 following CD40L/IL-4 activation of resting B cells, 20µg/ml actinomycin D (Sigma-Aldrich) was added to the cell cultures to inhibit further mRNA transcription. Cells were collected and stored in TRIzol reagent every 2 hours following the addition of actinomycin D for a total time course of 12 hours. Total RNA was isolated and the level of mature IgG1 mRNA was quantified by

semi-quantitative RT-PCR.

In vitro activation and differentiation of naïve CD4+ T cells - Purified splenic naïve CD4+ T cells were obtained using two step negative selections followed by a CD62L positive magnetic bead selection following the manufacturer's instructions (Miltenyi Biotec). Purified naïve CD4+ T cells (0.5×10^6 cells/ml) were stimulated with 2μg/ml plate-bound anti-CD3 (eBioscience) and 2μg/ml anti-CD28 (eBioscience). Cells were cultured in 96-well flat-bottom plates in 200μl of RPMI as described earlier with 1ng/ml recombinant mouse IL-4, 5μg/ml anti-IL-12 antibody (eBioscience), in the presence or absence of 100-1000ng/ml mCRAMP peptide. Cells were incubated at 37°C for 4 days before RT-PCR analysis.

Cell cycle Analysis by Propidium Iodide (PI) Staining - Purified splenic B cells were activated by 20μg/ml LPS plus 1ng/ml recombinant mouse IL-4 as described early. 1×10^6 cells were collected everyday from day 2 to day 4, washed twice with PBS, pelleted and resuspended in 1ml PBS in 15 ml polypropylene tube. 3ml cold absolute ethanol was added dropwise while vortexing the tube gently. Cells were stored at -20°C for at least 1 day until PI staining. Cells were pelleted, washed once with cold PBS and resuspended in 500μl PBS staining solution containing 0.1% (V/V) Triton X-100 (Sigma), 0.2mg/ml DNAse-free RNAse A (Sigma) and 20μg/ml PI (Sigma). Staining mixes were incubated at 37°C for 15 minutes and data was acquired on FACS Caliber.

Calcium flux - Calcium flux assay was done in purified splenic B cells using fluo 4 following the manufacturer's instructions (Molecular Probes). Briefly, splenic B cells were purified as described above, washed once with CLM buffer (PBS containing 5% FCS, 2mM $CaCl_2$ and 2mM $MgCl_2$) and resuspended in CLM buffer at a concentration of 10^7 cell/ml. Fluo 4 working solution was prepared by mixing Fluo 4 stock solution (1mM) with Plutonic solution (10% V/V) at a ratio 1:1. 1ml splenic B cells were loaded with 2µl fluo 4 working solution and incubated in 37°C water bath for 30 minutes followed by washing with CLM buffer twice. Cells were incubated at 37°C for 5 minutes before activated by CRAMP peptide or PMA as positive control. Data was acquired on BD LSRII.

Chemotaxis assay - Cell migration was assessed using transwell (5.6mm diameter, 5.0µM pore size, Corning) as previously described (95). 10^6 total splenic cells were seeded in the transwell insert in 100µl chemotaxis medium (RPMI 1640 supplied with 1% BSA). 600µl chemotaxis medium with increased concentration of CRAMP peptide was added into the outside compartment. The culture plate was incubated in 37°C incubator for 3 hours and cells that migrated from transwell inserts to the outside compartments were enumerated and analyzed by FACS staining.

OVA-induced asthma and bronchoalveolar lavage (BAL) - WT and CRAMP KO mice were sensitized by i.p. injection of 100µg TNP-OVA absorbed in 4mg alum on day

1 and 21. On day 28, 29, 30, mice were given 50μg TNP-OVA (in 50 μl PBS) through i.t. challenge every day. 24 hours after last challenge, mice were sacrificed and BAL was performed. Cells were pelleted by centrifuge; supernatant was saved for cytokine and antibody ELISA test, meanwhile BAL cells were counted, half of BAL cells will be used for FACS staining, and the rest was saved in TRIzol reagent for cytokine RT-PCR analysis. Whole lung will be embedded in OCT compound, frozen at -80°C, sectioned and stained with H&E.

Statistics - Statistical comparisons were performed using Prism 4.0 software (GraphPad). Data with three or more groups were analyzed by a one-way ANOVA followed by post hoc analysis, while data with two groups were analyzed by a two-tailed unpaired t test to determine whether an overall statistically significant change existed. Statistically significant results were determined by a p value of *<0.05, ** <0.01, ***<0.001.

RESULTS

B and T cells express mCRAMP.

The human cathelicidin LL-37 has been more highly characterized for expression and function than its mouse homolog mCRAMP. For example, LL-37 is expressed in neutrophils, epithelial cells, mast cells, B cells, NK cells, and γδ T cells [reviewed in (5, 141)], while the detailed expression of mCRAMP is less well known. Mature mouse splenic B cells are divided into two main subsets, marginal zone (MZ) and follicular (FO) B cells. To determine if splenic B and T cells express mCRAMP, splenocytes from B6 mice were sort-purified to obtain MZ ($B220^{+}$, $CD21^{hi}$, $CD23^{low}$) B cells, FO ($B220^{+}$, $CD21^{int}$, $CD23^{+}$) B cells, CD4+, and CD8+ T cells. In addition, total peritoneal lavage cells were sort-purified to obtain B1a ($CD5^{+}$ $Mac\text{-}1^{+}$ $B220^{int}$), B1b ($CD5^{-}$ $Mac\text{-}1^{+}$ $B220^{int}$), B2 ($CD5^{-}$ $Mac\text{-}1^{-}$ $B220^{high}$) and T cells ($CD5^{+}$ $B220^{-}$). Post-sort analysis revealed greater than 95% purity of each B and T cell population. Total RNA was isolated from each sort-purified cell population and RT-PCR was performed for the expression of *Camp*, CD19, CD3e, and actin (Fig. 1A). All B and T cell subsets tested expressed *Camp* mRNA directly ex vivo. To determine if B cells regulate the expression of *Camp* following activation, total CD43-negative splenic B cells were sort-purified and activated with either CD40L or LPS, in the presence or absence of IL-4 or IFN-γ. CD40L, IL-4, IFN-γ, but not LPS, induced an increase in *Camp* mRNA (Fig. 1B). Taken together, all B and T cells tested expressed *Camp* mRNA directly ex vivo and B

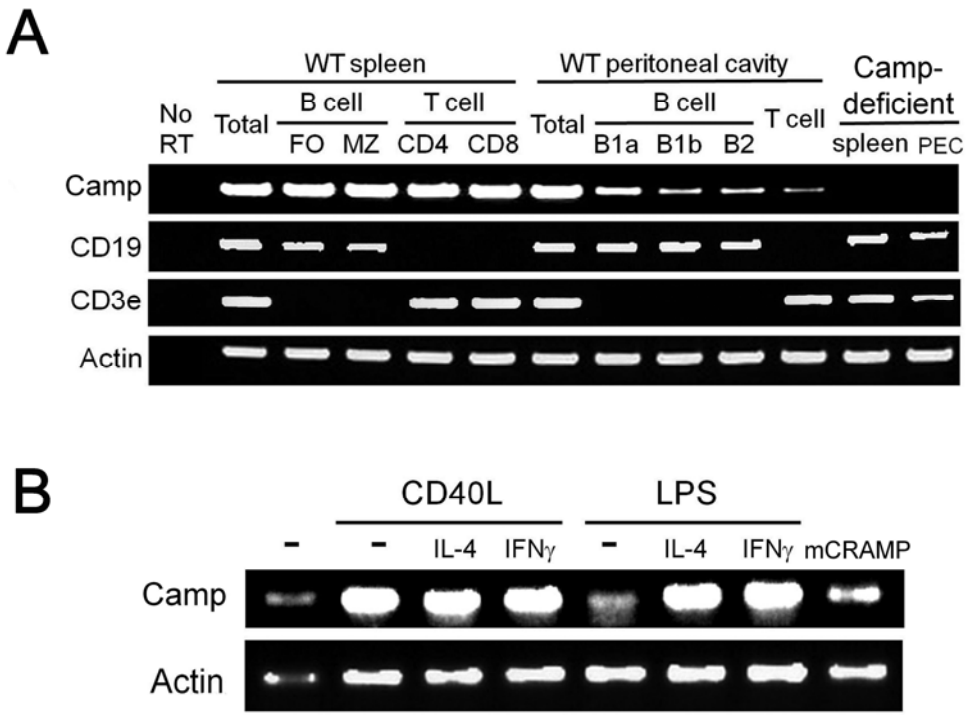

Figure 1: *B and T cell subsets express Camp.* Marginal zone (MZ), follicular (FO) B cells, $CD4^+$, and $CD8^+$ T cells were sort-purified from C57BL/6 spleens while B1a, B1b, B2, and T cells were sort-purified from peritoneal cavity washes. Total spleen and peritoneal cavity wash were also collected from *Camp* -/- (KO) mice. Total RNA was isolated and analyzed using (A) RT-PCR analysis for the mRNA level of *Camp*, CD19, CD3e, and actin. (B) Sort-purified $CD43^-$ B cells were activated in vitro for 24 hrs with the indicated stimuli and the level of *Camp* and actin mRNA was measured by RT-PCR. One representative gel from 3 independent experiments shown.

cells rapidly upregulated *Camp* expression following specific cell activation.

Camp-deficiency has no effect on mature B and T cell subset development

Blood lymphocytes from *Camp*-/- mice had been studied previously using microscope examination and FACS analysis, suggesting that the proportions of leukocytes, monocytes and lymphocytes were not significantly changed compared to those from WT mice (75). To determine whether mature B and T cell subset development from spleen and peritoneal cavity was affected by *Camp*-deficiency, total splenic cells were enumerated and the absolute numbers were similar between WT and *Camp*-/- mice (Fig. 2A). The percentages of FO, MZ B cells, CD4+ and CD8+ T cells in the spleen (Fig. 2B) as well as B1a, B1b, B2 cells in the peritoneal cavity lavage (Fig. 2C) were determined by FACS staining and there was no significant difference between WT and *Camp* -/- mice. Taken together, these data suggested that *Camp*-deficiency has no effect on the development of B and T cell subsets tested here.

mCRAMP has no effect on LPS activation of splenic B cells

Exogenous mCRAMP had been shown to inhibit LPS activation of macrophages and reduce cytokine expression. However, endogenous mCRAMP does not possess these activities. Since we used LPS as antigen in both in vitro activation and in vivo immunization, we tested whether mCRAMP regulate LPS activation of B cells. $CD43^-$ splenic B cells from WT and *Camp* -/- mice were sort purified and activated by LPS in the absence

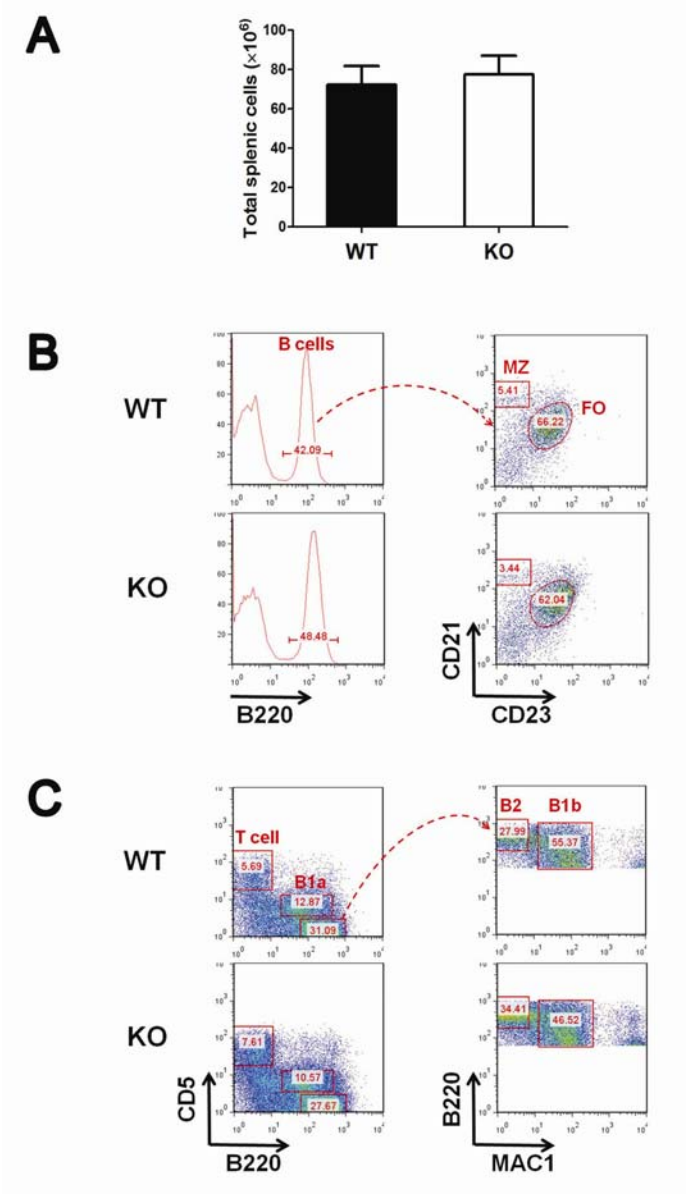

Figure 2: *CRAMP-deficiency does not affect mature B and T cell subset development.* Total splenic cells from WT and *Camp* -/- mice were enumerated directly ex vivo (A). The percentage of FO, MZ B cells, CD4+ and CD8+ T cells in the spleen (B) as well as B1a, B1b, B2 cells in the peritoneal cavity lavage (C) were determined by FACS staining.

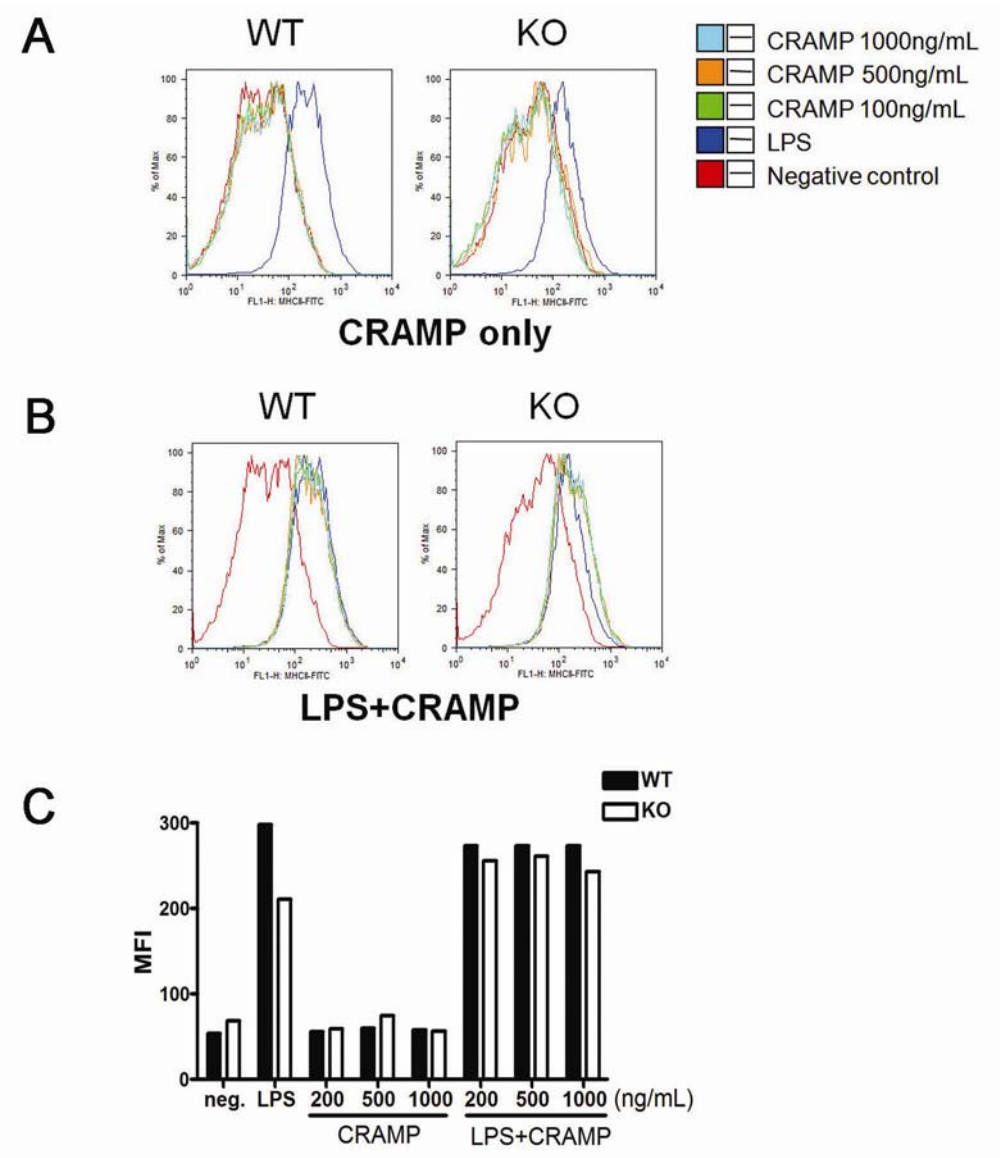

Figure 3: *mCRAMP has no effect on LPS activation of splenic B cells.* CD43^{-} splenic B cells from WT and *Camp* -/- mice were sort purified and activated by mCRAMP (A) or LPS in the absence or presence of exogenous mCRAMP (B). 24 hours later cell surface expression of MHCII was determined by FACS staining. Median fluorescence intensity was calculated by Flowjo (C).

or presence of exogenous mCRAMP. 24 hours later cell activation status was analyzed by FACS staining of MHCII, CD80 and CD86. Exogenous mCRAMP did not upregulate MHCII expression on B cells (Fig. 3A). When co-administrated with LPS, neither endogenous nor exogenous mCRAMP altered LPS-induced MHCII expression on B cells (Fig. 3B and 3C). Similar results were obtained for CD80 and CD86 expression. These data suggest that within the concentration we tested, exogenous mCRAMP as well as endogenous mCRAMP has no effect on LPS activation of B cells.

Camp-deficient B cells produce less IgG1 antibody in vitro.

mCRAMP and its human homolog LL-37 have many known activities including direct microbial killing and a variety of immunomodulatory functions. However, mCRAMP's role during an antibody response to a T cell independent and T cell dependent antigens has not been investigated. Since B and T cells express Camp and Camp is rapidly upregulated following activation of the B cell, the possibility exists that mCRAMP directly regulate B cells in antibody responses. Furthermore, since LPS induces switching to IgG3 (138) and IL-4 is required for CSR to IgG1 (142-143), respectively, we hypothesized that mCRAMP upregulation following activation with these factors might affect the level of antibody produced. Resting splenic B cells were sort-purified from C57BL/6 and *Camp* -/- mice and activated in vitro in the presence of LPS, LPS/IL-4 or CD40L/IL-4. Figure 4 shows that WT and *Camp* -/- B cells produce similar amounts of IgM (Fig. 4A) and IgG3 (Fig. 4B) in response to LPS stimulation.

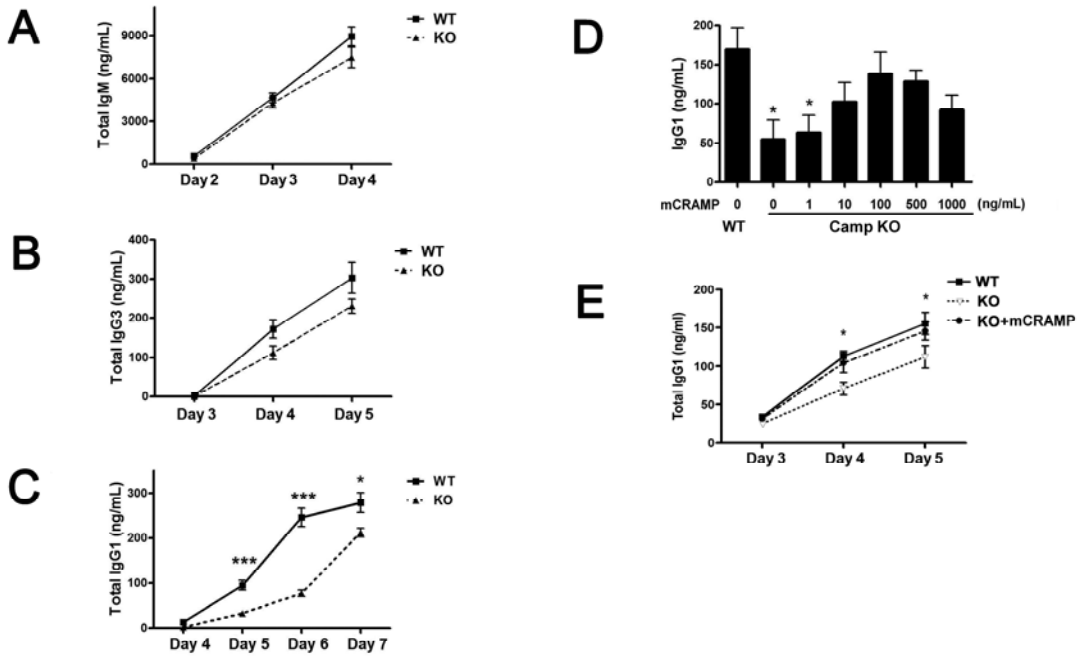

Figure 4: *Camp-deficient B cells produce less IgG1 antibody in vitro.* Resting CD43$^-$ WT and *Camp* -/- (KO) B cells were sort-purified and cultured in the presence of LPS for 5 days and cell culture supernatant was collected daily and analyzed by ELISA for the level of (A) IgM and (B) IgG3 antibody. B cells were also cultured in the presence of LPS and IL-4 for 7 days and cell culture supernatant was analyzed on days 4-7 for (C) IgG1 antibody. (D) *Camp* -/- B cells were cultured with LPS and IL-4, in the presence or absence of an increasing concentration of exogenous mCRAMP peptide, and the level of IgG1 antibody was measured on day 6. B cells were also cultured in the presence of CD40L and IL-4 for 5 days in the presence or absence of mCRAMP and cell culture supernatant was analyzed for (E) IgG1 antibody. One representative graph is shown from 3 independent experiments. Error bars represent the SD. * = $p<0.05$, *** = $p<0.001$

In contrast, *Camp* -/- B cells produced significantly less IgG1 in response to either LPS/IL-4 (Fig. 4C) or CD40L/IL-4 (Fig. 4E) when compared to WT B cells. In order to show that mCRAMP directly mediated these effects in vitro, increasing concentrations of recombinant mCRAMP peptide was added to *Camp* -/- B cell cultures and the level of IgG1 production returned to WT levels (Fig. 4D and 4E). Taking together these results show *Camp* -/- B cells produced similar amounts of IgM and IgG3 in response to LPS, but significantly less IgG1 in response to LPS/IL-4 and CD40L/IL-4, an effect that was reversed with the addition of exogenous mCRAMP peptide.

B cell proliferation, survival, and isotype switching are unaffected by Camp-deficiency.

The mechanism by which *Camp*-deficient B cells produce less IgG1 in comparison to WT B cells could be explained by a number of factors including differences in proliferation, survival, and CSR. To determine the mechanism by which *Camp* -/- B cells produces less IgG1, resting B cells were sort-purified and activated as described above. Cell cycle analysis was performed on days 2-4 following activation and no difference was determined when comparing the number of cells in G_0/G_1, S, or G_2/M phases (Fig. 5A). The total live cell number in each culture was counted on Day 5 and no difference between the WT and *Camp* -/- B cells was determined (Fig. 5B). The percentage of surface IgG1 positive cells on day 5 in CD40L/IL-4 and LPS/IL-4 activated cultures was also measured by FACS analysis (Fig. 6A), showing no difference between WT

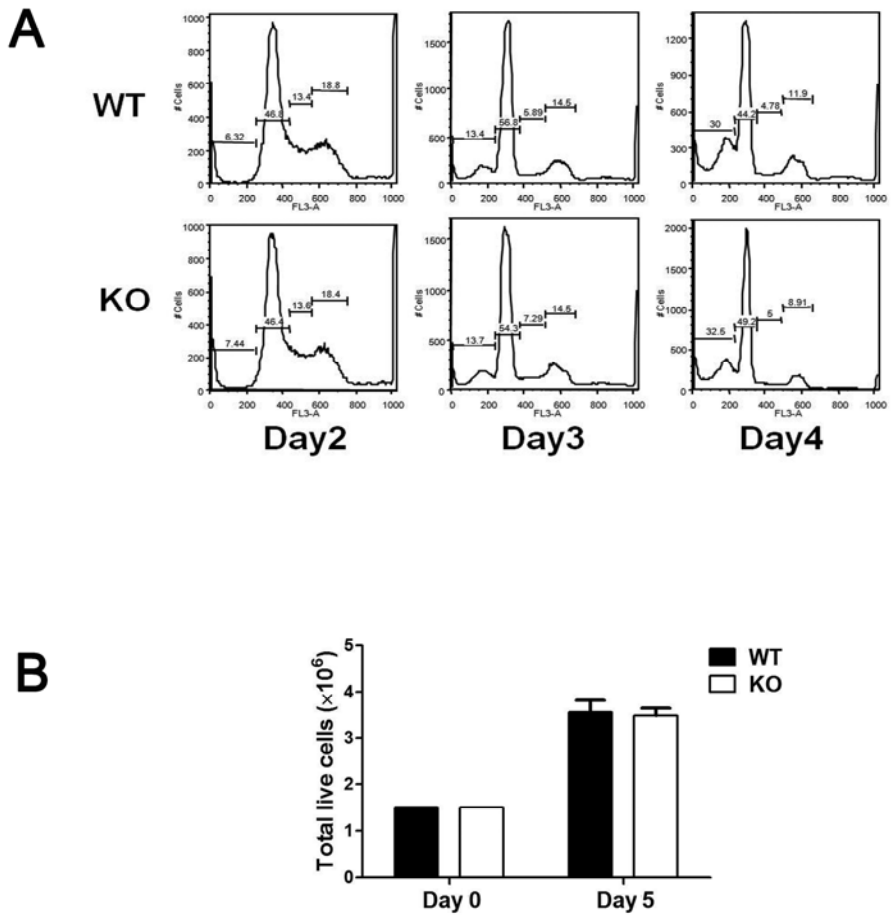

Figure 5: *Camp-deficiency does not affect B cell proliferation and survival.* Resting CD43$^-$ WT and *Camp* -/- (KO) B cells were sort-purified and cultured with LPS in the presence of IL-4. (A) Cells were collected everyday from day 2 to day 4 and fixed in cold ethanol followed by PI cell cycle staining. (B) Total live B cells were enumerated on Day 0 and Day 5 following LPS/IL-4 activation.

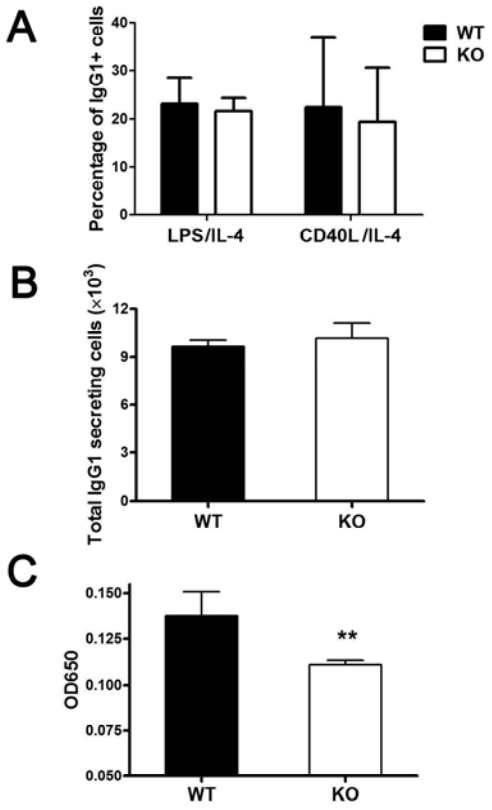

Figure 6: *Camp-deficiency does not affect B cell isotype switching.* WT and *Camp* -/- (KO) B cells were sort-purified and cultured with indicated stimuli. (A) The percentage of surface IgG1+ B cells in LPS/IL-4 and CD40L/IL-4-activated cultures were determined using flow cytometry. (B) The number of IgG1-secreting B cells was determined using ELISpot analysis on LPS/IL-4-activated cells. (C) The IgG1 spots developed from the ELISpot procedure were dissolved using DMSO and the optical density was determined at 650nm. One representative graph is shown from 3 independent experiments. Error bars represent the SD. ** = $p<0.01$.

and *Camp* -/- B cells. Finally, ELISpot experiments were performed on day 5 B cell cultures and spots were enumerated to determine the number of IgG1-secreting B cells. Total spot counts were equivalent between the WT and *Camp* -/- B cells (Fig. 6B), suggesting that CSR is not affected. However, the spot size from WT B cells was larger than *Camp* -/- B cells spots, which was quantified when the spots were dissolved with DMSO and the absorbance measured at 650nM (Fig. 6C). Taken together, these results suggest that the differences in IgG1 production between WT and *Camp* -/- B cells is not due to defects in proliferation, cell cycle progression, survival, or CSR, but is due to increased IgG1 production per cell.

Camp-deficient B cells produce less IgG1 mRNA.

Differences in IgG1 production between WT and *Camp* -/- B cells could be explained if there was a change in class switch recombination to IgG1. A linear relationship has been shown between the amount of sterile Iγ1 transcript and CSR (144). Alternatively, the amount of IgG1 mRNA production could be increased in the WT cells compared to *Camp* -/- cells. Therefore, to determine the amount of Iγ1 and IgG1 mRNA in WT and *Camp* -/- B cells, cells were sort-purified and activated as described earlier and total RNA was isolated on Days 2, 3 and 4. Semi-quantitative RT-PCR showed no significant difference in the levels of Iγ1 transcript over the time course analyzed (Fig. 7A), suggesting no change in CSR. In contrast, the level of IgG1 mRNA was significantly higher in the WT compared to *Camp* -/- B cells (Fig. 7B), suggesting

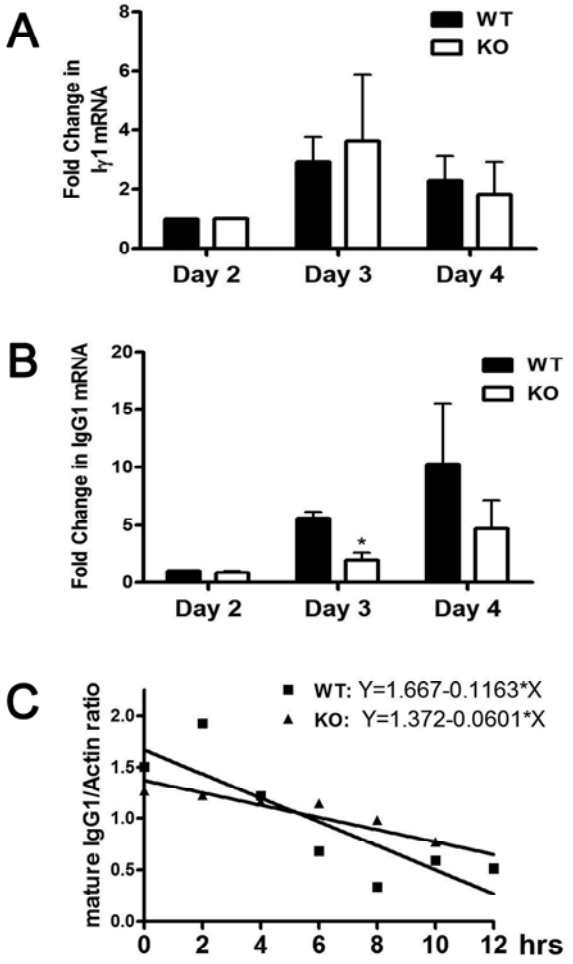

Figure 7: *Camp-deficient B cells produce less IgG1 mRNA.* Resting CD43$^-$ splenic WT and *Camp* -/- (KO) B cells were sort-purified and activated with LPS and IL-4. RT-PCR analysis was performed on Day 2, 3, and 4 after activation for the level of (A) Iγ1 and (B) IgG1 mRNA. Actinomycin D was added to day 5 WT and *Camp* -/- B cell cultures and total RNA was collected every 2 hours for 12 hours. RT-PCR was performed for the level of (C) IgG1 mRNA at each time point and regression analysis was applied to estimate the stability of the mRNA transcript. One representative graph is shown from 3 independent experiments. Error bars represent the SD. * = $p<0.05$.

that mCRAMP was increasing either the rate or stability of the IgG1 mRNA. To determine the stability of the IgG1 mRNA, actinomycin D was added to the B cell cultures and total RNA was collected every two hours for 12 total hours. The stability of the IgG1 mRNA did not differ significantly between the WT and *Camp* -/- B cells (Fig. 7C). Thus, it appears that mCRAMP production by B cells increases the amount of IgG1 produced per cell by increasing the rate of IgG1 mRNA production, without affecting CSR or the stability of the mRNA.

Camp-deficient mice produce more IgG1 in response to a T-dependent antigen.

The antibody responses to TI-1, TI-2, and T-dependent antigens have not been investigated in *Camp* -/- mice to date. Our data presented in figure 4 and others in the literature show that AMPs is able to regulate some antibody responses either positively or negatively (95, 136-137). Therefore, to investigate the antibody response in vivo to these three characteristic antigens, B6 WT and *Camp* -/- mice were immunized with either TNP-LPS, S. *pneumonia* (R36A), or TNP-OVA absorbed in Alum. IgM and IgG3 antibodies against TNP and PC was determined using ELISA and the data show no difference in the level of antibody produced between WT and *Camp* -/- mice (Fig. 8A-D), a finding similar to our LPS activated B cells in vitro. Mice were also immunized i.p. and s.c. with TNP-OVA absorbed in alum on day 0 and day 21 and the level of serum antibody was measured. The level of TNP-specific IgG1 was significantly higher in the *Camp* -/- mice following the second i.p. immunization (Fig. 8E) and the first and second

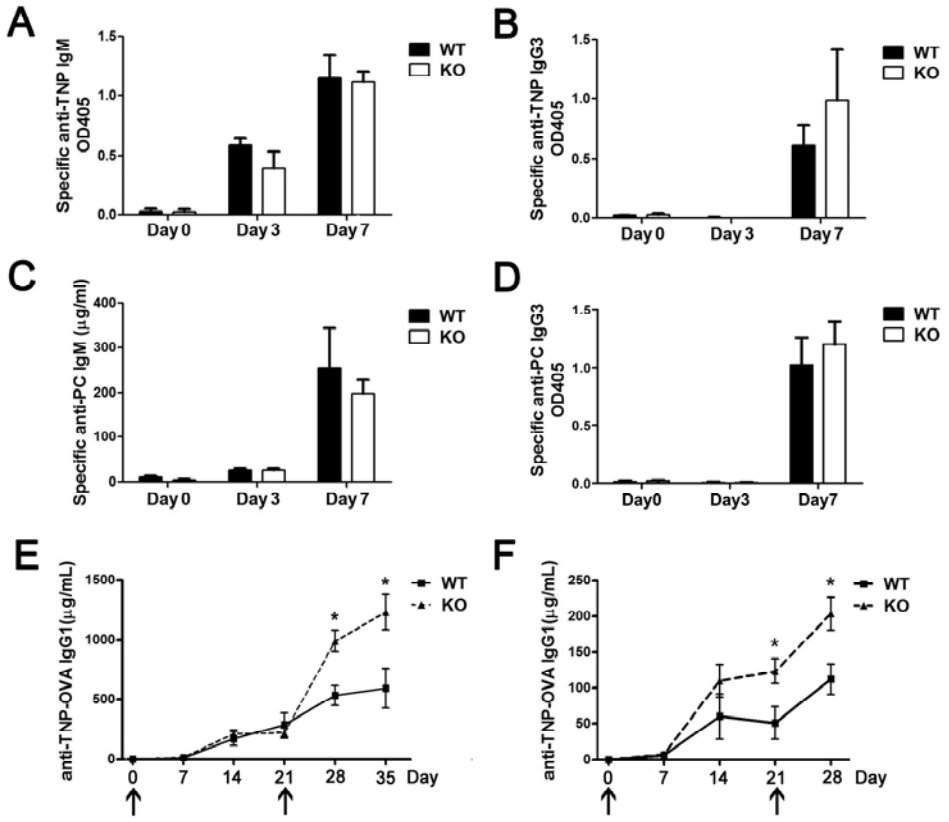

Figure 8: *Camp-deficient mice produce more IgG1 in response to a T cell-dependent antigen.* WT and *Camp* -/- (KO) mice were immunized with TNP-LPS or S. pneumoniae (R36A) on day 0 and the level of serum antibody was measured using ELISA. The level of (A) TNP-specific IgM and (B) IgG3 as well as (C) PC-specific IgM and (D) IgG3 were measured on day 0, 3, and 7. WT and *Camp* -/- (KO) mice were immunized with TNP-OVA/alum on day 0 and 21. The amount of TNP-OVA-specific IgG1 was measured following (E) i.p. or (F) s.c. immunizations. One representative graph is shown from 3 independent experiments. ↑ = time of injection. Error bars represent the SD. * = $p<0.05$.

s.c. immunizations (Fig. 8F), a result that is in contrast to the CD40L/IL-4 activated IgG1 responses in vitro. Taken together, WT and *Camp* -/- mice have the same antibody responses to TI-1 and TI-2 antigens, but *Camp* -/- mice produce significantly more antigen specific IgG1 antibody in response to a T-dependent antigen.

Camp-deficient T cells produce more IL-4 and induce more isotype switching to IgG1.

The *in vivo* immunization experiments in figure 8 were very surprising to us since they showed an opposite result compared to the purified B cell data in vitro. One explanation for this could be that T cells are differentially regulated by mCRAMP. To determine if CD4+ T cell IL-4 production is affected by mCRAMP expression, 7 days following the second immunization with TNP-OVA/Alum, RT-PCR was performed to determine the level of IL-4 in total spleen. Figure 9A shows that *Camp* -/- spleens contain more IL-4 mRNA than WT spleens following a secondary immunization with a T-dependent antigen. Furthermore, since *Camp* -/- mice produce more IL-4 in response to a T-dependent antigen, we would predict that they would have more IgG1-secreting B cells as a result of increased CSR. WT and *Camp* -/- mice have the same number of IgG1 secreting cells 7 days after the first immunization (data not shown), but *Camp* -/- mice have increased IgG1-secreting B cells 7 days after the second immunization (Fig. 9B). To further determine if T cell cytokine levels are regulated by mCRAMP, we isolated naïve CD4+ T cells using magnetic bead sorting and activated them in vitro

w/anti-CD3, anti-CD28, rIL-4, and anti-IL-12 in the presence or absence of mCRAMP. Total RNA was collected on day 4 and RT-PCR was performed to determine the level of IL-4 mRNA produced. Figure 9C shows that *Camp* -/- T cells produce more IL-4 than WT T cells and the addition of exogenous mCRAMP brings the level back to WT. Taken together, these results suggest that *Camp* -/- mice produce more IgG1 antibody in response to a T-dependent antigen due to increased T cell IL-4 production and increased B cell CSR.

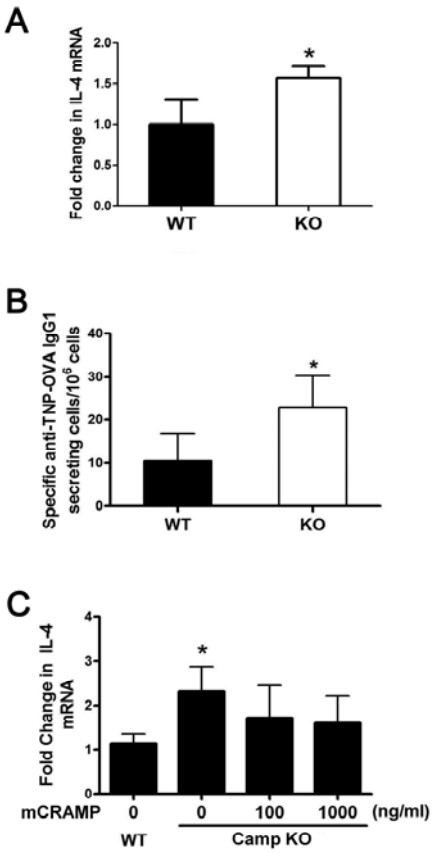

Figure 9: *Camp-deficient T cells produce more IL-4 and induce more isotype switching to IgG1.* Total splenic cells were isolated on day 7 following the second immunization with TNP-OVA/alum. (A) Total RNA was isolated and RT-PCR analysis was performed for the level of IL-4 and actin mRNA, graphed as fold change in IL-4 expression. (B) ELISpot analysis was performed on the splenic cells for anti-TNP-OVA IgG1 secreting cells. (C) WT and *Camp* -/- naive CD4+ T cells were sort-purified and activated in vitro with anti-CD3, -CD28, -IL-12, and rIL-4, in the presence or absence of mCRAMP peptide, for 4 days. Total RNA was collected and the level of IL-4 mRNA was quantified using RT-PCR. One representative graph is shown from 3 independent experiments. Error bars represent the SD. * = $p<0.05$

DISCUSSION

Analysis of AMPs have shown that their cellular expression is widespread and their functions are becoming more appreciated. Our data showing that mouse B and T cells are capable of expressing and responding to mCRAMP further add to this complexity. Specifically, all B and T cell subsets tested showed expression of Camp mRNA. Purified B cells activated in vitro produced more IgG1 antibody in the presence of mCRAMP, while purified T cells produced less IL-4 in the presence of mCRAMP. Immunization with a T-dependent antigen in vivo leads to less IgG1 antibody in WT mice when compared to Camp -/- mice. Thus, the level of antibody produced is regulated by mCRAMP, though its ability to either positively or negatively regulate the response depends on the concentration and cell type responding.

The ability to study the role of mCRAMP in host defense has been aided by the development of the Camp -/- mouse (32, 45). Using this model, we investigated the role of mCRAMP in regulating T-dependent antibody responses. Our data show that Camp -/- mice immunized with TNP-OVA produced more TNP-specific IgG1 antibody when compared to WT mice. In contrast, Kurosaka et al. showed that mCRAMP acts as an immune adjuvant and enhances T-dependent antibody production in WT mice (3). The most obvious difference in the experiment design, which may contribute to the opposing findings, is that we studied the endogenously produced mCRAMP by comparing

antibody responses in WT vs. Camp -/- mice, while Kurosaka et al. (3) added additional exogenous mCRAMP to WT mice. The administration of exogenous mCRAMP to a WT mouse that is also making mCRAMP in response to the immunization may or may not accurately model the role of mCRAMP during an antibody response. In support of this possibility, previous studies have demonstrated that exogenous and endogenous mCRAMP functions differently in macrophage activation (22). An alternative explanation is the specific identity of the cathelicidin peptides produced by the Camp gene. Prior work has shown that alternative proteolytic processing is possible for endogenously expressed cathelicidin peptides, and that this alternative processing can lead to different physiological effects (46). As the identity of the mature peptide produced by T and B cells is not known, it is possible that the exogenous synthetic mCRAMP administered in the Kurosaka paper is different in both identity and abundance than the endogenous mCRAMP released under normal conditions and absent in the Camp -/- mice. Taken together, the concentration, location, and cell types responding to mCRAMP exposure, and the sequence of mCRAMP in these settings, will affect the immunological response under observation, though it is clear that mCRAMP has the ability to regulate antibody production.

The role of AMPs in regulating the magnitude of the adaptive immune antibody response has not been investigated extensively and the results to date are contradictory. LL-37 at 20 ug/ml was shown to decrease IgM and IgG2a production from mouse splenic B cells activated with LPS and IFN-γ, primarily through inhibition of cell activation

and proliferation (23). In contrast, another study demonstrated that LL-37 at 6 ug/ml increased the sensitivity of human peripheral B cells to CpG, enhancing B cell activation and increasing IgM and IgG production (21). Our data using mCRAMP and purified mouse B cells agrees with Hurtado et al. (21) and shows an increase in IgG1 production. Two obvious differences that may account for the discrepancies seen are the use of LL-37 versus mCRAMP and mouse versus human B cells. In addition, another very important variable to consider is AMP concentration. Since it is nearly impossible to measure the physiological concentration within the splenic microenvironment where these responses are occurring, we titrated the mCRAMP concentration within our culture system ranging from 1ng/ml to 10μg/ml. Consistent with previous findings (47), our data showed that mCRAMP at the highest concentration we tested induced cell apoptosis, while moderate concentrations increased IgG1 production, and the lowest concentration showed no effect on IgG1 production. Taken together, the AMP concentration within the microenviroment of an immune response may partially dictate the positive or negative effect on antibody production.

mCRAMP acts directly on sort-purified B cells and T cells in vitro to enhance IgG1 and inhibit IL-4 production, respectively. However, mCRAMP has been shown to induce migration of human monocytes, neutrophils, macrophages, and mouse peripheral blood leukocytes through mouse formyl peptide receptor 2 (3). Therefore, the possibility exists that Camp -/- mice have altered cellular recruitment following an immunization. In our experiment model, we used alum as the immune adjuvant. Alum

induces an influx of eosinophils, monocytes, neutrophils, DCs, NK cells and NKT cells into the site of injection and leads to a strong antigen-specific Th2 response (48, 49). We observed no significant difference in the cellular influx being recruited to the peritoneal cavity after immunization between WT and Camp -/- mice (data not shown), suggesting that Camp -/- has no effect on cell recruitment following immunization. Alternatively, since Camp -/- mice show enhanced expression of mouse beta defensin 3 (mBD3) (50), which has chemotactic activity, this may compensate for the loss of mCRAMP. Taken together, the results suggest that mCRAMP directly acts on T and B cells to regulate the adaptive immune response.

Our in vitro and in vivo data show that T cells exposed to mCRAMP produce less IL-4. However, the possibility exists that other cell types are being affected by mCRAMP and are then affecting the T cells. LL-37 has been shown to drive mouse dendritic cell differentiation and enhance IL-6 and IL-12 production, while inhibiting IL-4 production. In addition, LL-37-exposed DCs increased IFN-γ production from T cells and polarize them to Th1 cells (51). Our in vitro data clearly show that mCRAMP directly acts on sort-purified T cells that were polarized to Th2 cells to decrease IL-4 production. However, in vivo we were able to determine that the T cells were producing less IL-4, but not whether it was a direct effect on the T cell or effects on other cell types like DCs. IL-4 is the critical cytokine for the IgG1 class switch (52), and its elevated expression in the Camp -/- spleen after secondary i.p. immunization is associated with an increased number of antigen-specific IgG1 secreting cells. These results suggest

that mCRAMP regulates antigen-specific IgG1 production in vivo by enhancing B cell antibody production and inhibiting T cell IL-4 expression. While appearing counter-productive, the microenvironment within the responding host that is generated following different types of insults will affect the magnitude of the immune response generated.

mCRAMP is an AMP that is beginning to be appreciated as a potent and important immunomodulatory molecule. We show that B and T cells express and respond to mCRAMP. Purified B cells clearly respond to mCRAMP and produce more IgG1 antibody in vitro while in contrast; in vivo more IgG1 antibody is produced in the Camp-deficient mice. While our data begin to elucidate the role of mCRAMP in the adaptive immune response, more information is needed to fully understand its role in the different microenvironments within the host. It is clear that the cell type producing and/or responding to mCRAMP will partially determine the effect observed. Additional studies are needed to fully understand the role of mCRAMP and other AMPs in the adaptive immune response.

CONCLUSION AND FUTURE PERSPECTIVES

Since the generation of *Camp* -/- mice by Nizet et al., extensive studies have been done to investigate their susceptibility to invading pathogens. However, little attention was paid to the effect of mCRAMP on antibody production. The only study reported by Kurosaka et al. was that exogenous mCRAMP acts as an immune adjuvant and enhance antibody response in WT C57BL/6 mice (95). Since endogenous and exogenous mCRAMP may function differently (116), we decided took the advantage of *Camp* -/- mice to study the effect of endogenous mCRAMP on antibody production. Our results showed that mouse B and T cells are capable of expressing and regulating mCRAMP expression upon certain activations. Purified splenic B cells activated in vitro produced more IgG1 antibody in the presence of mCRAMP, while purified T cells produced less IL-4 in the presence of mCRAMP. Meanwhile immunization with TNP-OVA/Alum, a T-dependent antigen, leads to less IgG1 antibody in WT mice when compared to *Camp* -/- mice. Thus, the level of antibody produced is regulated by mCRAMP, though its ability to either positively or negatively regulate the response depends on the concentration and cell type responding.

Camp -/- mice has been reported to be susceptible to a variety of pathogens (30, 75-77), however, most of these studies focus on innate immunity which takes effect shortly after infection. We have shown that *Camp* -/- mice exhibit enhanced IgG1 anti-

body response to T-dependent antigen challenge. The question now rises whether increased antigen-specific IgG1 production in *Camp* -/- mice contributes to the bacterial killing and growth control of pathogens? Since certain vaccines induce as low as two-fold increase in antigen-specific antibody production which is still associated with enhance opsonic activity (145), we predict that increased antigen-specific IgG1 production in *Camp* -/- mice contribute to the control of infections and diseases. To test this hypothesis, we propose the following experiments using *Bacillus anthracis* Sterne strain, which harbors plasmid pXO1 encoding the toxin, but lacks plasmid pXO2 and consequently lacks the capsule. WT C57BL/6 mice are resistant to the Sterne strain; however, complement C5 depletion renders C57BL/6 mice sensitive to the i.t. challenge of *Bacillus anthracis* Sterne strain [(146) and unpublished data from our lab]. Protective antigen (PA) has been used to vaccinate human beings and generate antibody protection (147). In the proposed experiments, WT and *Camp* -/- mice will be immunized on day 0 and day 21 with PA absorbed in aluminum hydroxide by i.p. injection. 14 days after secondary immunization, serum anti-PA antibody will be measured by ELISA and same amount of serum from WT or *Camp* -/- mice will be adoptively transferred into *C5* -/- mice. 24 hours later, these *C5* -/- mice will be i.t. challenged with lethal dose of *Bacillus anthracis* Sterne strain and survival rate will be monitored daily for 2 weeks. By performing this experiment, we expect to reveal whether enhanced antibody production help to control infections and diseases in *Camp* -/- mice.

Although several cell surface and intracellular receptors have been suggested for

LL-37, only mFPR2 has been identified as mCRAMP receptor. mCRAMP can induce migration of monocytes, neutrophils, macrophages, and peripheral blood leukocytes, initiate calcium flux and activate MAPKs pathway in monocytes (95). Our RT-PCR result showed that all B and T cell subset express mFPR2 (Fig. 10A), which leads us to hypothesize that mCRAMP act through mFPR2 to regulate B cell IgG1 production. But further studies demonstrated that mCRAMP does not induce B cell calcium mobilization (Fig. 10B) or cell migration (Fig. 10C) within the concentration we tested, leaving the question as on which receptor or signaling pathway mCRAMP acts to regulate IgG1 production? Since mCRAMP activates MAPK pathway through mFPR2 in macrophages (95, 116), the first experiment we propose is to investigate the phosphorylation status of signaling molecules in this pathway, including p38, ERK and JNK, by western blot. Recently Chen *et al* had generated the mFPR2 knockout mice (148), which provide a good model to study the effect of mFPR2 pathway on antibody production. We plan to obtain mFPR2 knockout mice and measure splenic B cell antibody production after in vitro activation in the presence or absence of mCRAMP. We will also analyze mice immunized with T-independent and T-dependent antigens.

The study with mFPR2-/- mice also showed that mFPR2-deficiency causes reduced severity in OVA-induced allergic airway inflammation. These mice showed diminished recruitment of dendritic cells in to the airway and reduced Th2 cytokines production. Since mCRAMP is one of the mFPR2 ligands that promote cell migration and regulate cytokine expression, we have began conducting pilot experiments to study the effect

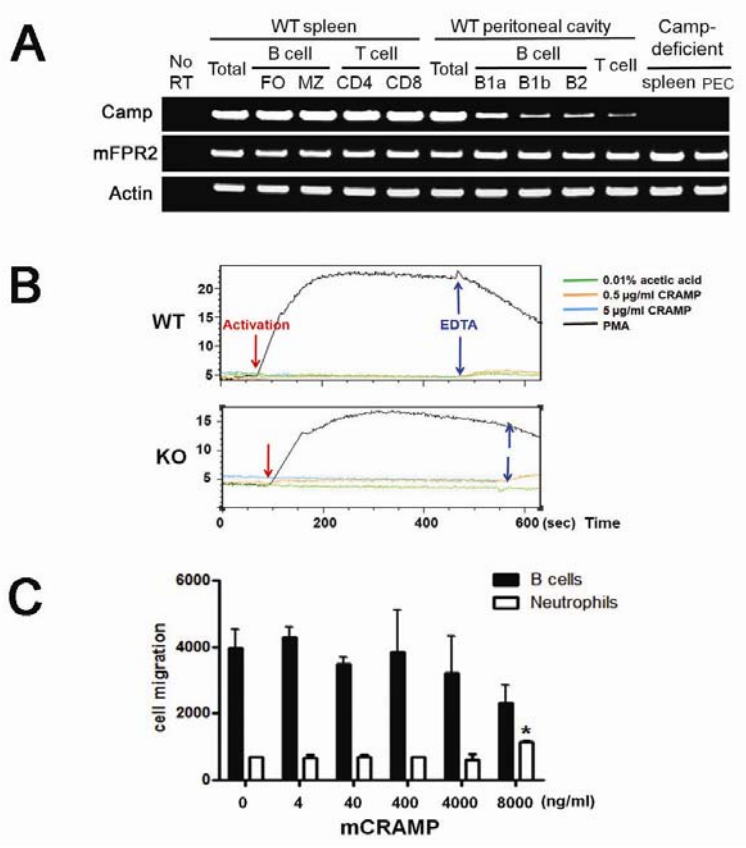

Figure 10**:** *B and T cell subsets express mFPR2.* Marginal zone (MZ), follicular (FO) B cells, $CD4^+$, and $CD8^+$ T cells were sort-purified from C57BL/6 spleens while B1a, B1b, B2, and T cells were sort-purified from peritoneal cavity washes. Total RNA was isolated and analyzed using (A) RT-PCR analysis for the mRNA level of mFPR2. (B) Sort-purified $CD43^-$ B cells were activated by mCRAMP for fluo-4 calcium flux assay; PMA was used as positive control and 0.01% acetic acid solution was used as negative control. (C) Chemoattractant activity of mCRAMP was analyzed using a transwell system. Cells that migrated from transwell inserts to the outside compartments were enumerated under microscope and the percentages of B cell and neutrophil were determined by FACS staining.

of endogenous mCRAMP on OVA-induced asthma model. Our preliminary data showed that similar amount of CD4+, CD8+ T cell, B cells and eosinophils are flushed out in the BAL fluid from WT and *Camp* -/- mice (Fig. 11A). However, the number of alveolar macrophages is significantly lower in the BAL liquid from *Camp* -/- mice when compared to those from WT mice. Neutrophils were a trend to decrease in *Camp* -/- mice, but the difference is not statistically significant. The alveolar macrophage is the predominant immune effector cell resident in the alveolar spaces and conducting airways, and it is responsible for activating inflammatory responses sufficient to kill invaded pathogens (149). Depletion of resident alveolar macrophages with clodronate results in enhanced airway hyperresponsiveness in association with enhanced eosinophilic inflammation and increased lavage levels of IL-4 and IL-5 and decreased levels of IFN-γ (150). IL-1β expression is regulated by LL-37 (93) and its expression in human BAL cells is located exclusively within alveolar macrophages (151). We performed RT-PCR to detect IL-1β, IL-4, IL-5, IL-6, IL9, IL-10, TNF, TGF and IFN-γ expression in total cells from BAL fluid. Less IL-1β expression in total BAL cells was measured from *Camp* -/- mice, which is consistent with decreased number of alveolar macrophages (Fig. 11B). However, the expression levels of other cytokines various dramatically within group of WT and Camp -/- mice (data not shown) and needs further investigation. We plan to use BD cytometric bead array to measure cytokine proteins in BAL fluid. Lung from WT and Camp -/- mice will be frozed and sectioned followed by H&E or immunohistological staining. Furthermore, instead of OVA-induced asthma, we have established a more physiological

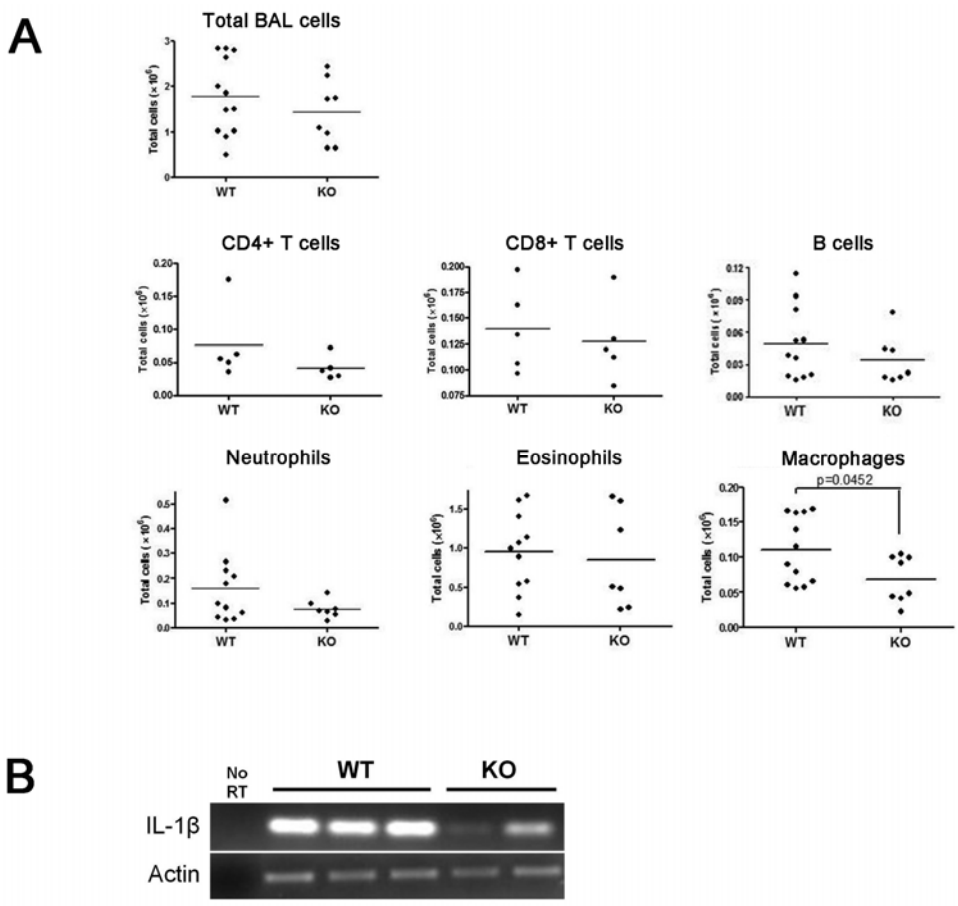

Figure 11: *BAL fluid from Camp-deficient mice contains less alveolar macrophages in OVA-induced asthma model.* WT and *CAMP* -/- (KO) mice were sensitized with TNP-OVA absorbed in Alum on day 1 and day 21. On day 28, 29, 30, mice were given 50μg TNP-OVA (in 50 μl PBS) through i.t. challenge every day. 24 hours after last i.t. challenge, mice were sacrificed and BAL was performed. (A) Total BAL cells were enumerated under microscope and the percentages of each type of cells were determined by FACS staining. (B) Half BAL cells were pelleted and stored in TRIzol reagents. Total RNA was isolated and analyzed using RT-PCR analysis for the mRNA level of IL-1β. Each line represents one mouse.

relevant model of asthma. We give mice low dose of *Aspergillus fumigatus* by i.t. challenge twice a week for 8 weeks. These mice demonstrate a large influx of neutrophils, eosinophils, macrophages, B cells and T cells into the lung, an antigen-specific antibody response as well as altered cytokine production (data not shown). Using this asthma model, we plan to measure the effect of mCRAMP on cell migration, cytokine production and inflammatory response. We will also measure airway hyperreactivity using a BUXCO nebulizer control, BUXCO Max II Strain-Gage Preamplifier and BUXCO Bias Flow Regulators kindly provided by Dr. T. Prescott Atkinson from UAB. Using fluorescent-labeled aspergillus, we will tract down the location of the bacteria and their association with specific types of alveolar cells.

In our study we focused mainly on IL-4-induced IgG1 production. Since IL-4 also induces IgE isotype switch (152), we did some pilot experiments to measure IgE production in purified B cells upon in vitro activation. Our preliminary data indicated that IgE has similar trend as IgG1; mCRAMP enhances IL-4-induced IgE production in vitro. But the effect of mCRAMP on in vivo antigen-specific IgE production needs further investigation. In asthma patients, allergen-specific IgE antibodies are produced after exposure and released into the bloodstream. These IgE antibodies may bind to the receptors on inflammatory cells such as mast cells or remain free floating in the bloodstream. When an individual is re-exposed to the same allergen, mast cells may undergo receptor-bound IgE cross-linking, leading to the release of chemical mediators such as histamine, prostaglandins and leukotrienes (153). These chemical mediators can cause in-

flammatory responses in the body that have been linked to asthma signs and symptoms such as bronchial constriction, coughing and wheezing We will measure antigen-specific antibody production in the low-dose *Aspergillus fumigatus*-induced asthma model as well as histamine, prostaglandins and leukotrienes production from mast cells. Although mFPR2-deficiency causes reduced severity in OVA-induced allergic airway inflammation, things may be complicated in *Camp* -/- mice by involvement of mCRAMP in the regulation of antibody production. We predict the decreased severity caused by mCRAMP-deficiency may be attenuated by increased IgE production.

Recently studies show that mCRAMP is a potent and important immunomodulatory molecule. We show that B and T cells express and respond to mCRAMP. Purified B cells clearly respond to mCRAMP and produce more IgG1 antibody in vitro while in contrast; in vivo more IgG1 antibody is produced in the *Camp*-deficient mice. The future studies described above will reveal detailed molecular mechanism through which receptor and signaling pathway mCRAMP regulates IgG1 production. Furthermore, the proposed experiments with asthma model will expand our knowledge about the role of mCRAMP in the adaptive immune response and correlate it with allergic disease, suggesting that clinical application of AMPs in infectious disease needs more considerations. The more we know about AMPs, the better we can use them.

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APPENDIX

IACUC APPROVAL

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

NOTICE OF APPROVAL WITH STIPULATIONS

DATE: September 15, 2009

TO: Kearney, John F.
SHEL-410 2182
934-6557

FROM: Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee

SUBJECT: Title: Regulation of B Cell Clonal Diversity
Sponsor: NIH
Animal Project Number: 090907732

On September 15, 2009, the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) approved above referenced application for use of the following species and numbers of animals:

Species	Use Category	Number in Category
Mice	C	272
Mice	A	2800

Approval is granted with the following stipulation(s):

Animal procurement and initiation of studies may not commence until the Animal Use Safety Information Sheet is authorized by OH&S. Once the AUSI is authorized, you will be contacted by Earle Durboraw (934-3538) to discuss specific safety precautions which may be necessary for the ARP care staff. Animal procurement and use of potentially hazardous agents in live animals may not occur until Mr. Durboraw has informed the IACUC Office that a satisfactory discussion has occurred.

This protocol must be submitted to the IACUC for renewal no later than September 2010. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.
Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 090907732 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at 934-7692.

Institutional Animal Care and Use Committee
B10 Volker Hall
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205.934.7692
FAX 205.934.1188

Mailing Address:
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1530 3RD AVE S
BIRMINGHAM AL 35294-0019

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

NOTICE OF APPROVAL

DATE: April 9, 2010

TO: Kearney, John F.
SHEL-410 2182
934-6557

FROM: Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee

SUBJECT: Title: Immunobiology of Bacillus Anthracis Spore-Host Interactions
Sponsor: NIH
Animal Project Number: 100408755

On April 9, 2010, the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) reviewed the animal use proposed in the above referenced application. It approved the use of the following species and numbers of animals:

Species	Use Category	Number in Category
Mice	B	586

Animal use is scheduled for review one year from April 2010. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 100408755 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at 934-7692.

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