Host Defence Peptides BMAP-27 and BMAP-28 Down-Regulate Proliferation of Porcine T Cells Through Induction of T Cell Anergy

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By

Tova Dybvig

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ABSTRACT

Host Defense Peptides (HDPs) are small, cationic and amphipathic molecules with inherent antimicrobial and immunomodular function. However their effects on blood-derived T cells is unknown and is the focus of this investigation. In this thesis, porcine peripheral blood mononuclear cells (PBMCs) were stimulated with bovine myeloid antimicrobial peptide (BMAP)-27, BMAP-28, Indolicidin (Indol), or HH2 in the presence and absence of Concanavalin A (ConA). It was observed that BMAP-27, BMAP-28, and Indol inhibited ConA-stimulated porcine PBMC proliferation. To ensure that the observed effect on cell proliferation was not simply due to a physical interaction between the peptide and ConA, addition of peptide and ConA was staggered. Porcine $CD4^+/CD8^+$ T cells were isolated from blood using magnetic activating cell sorting (MACS) and it was determined that BMAP-27 and BMAP-28 inhibited ConA-stimulated T cell proliferation. They did not promote T cell necrosis, but approximately 40 % of the activated T cells undergoes apoptosis in the presence of BMAP-27 and BMAP-28. The remaining 60 % of the T cells consumed very little ATP and showed an increase in expression of cytotoxic T lymphocyte antigen-4 (CTLA-4), indicating the induction of T cell anergy. The addition of exogenous IL-2 decreased the surface expression of CTLA-4 in ConA-activated $CD4^+$ T cells and induced renewed $CD4^+/CD8^+$ T cell proliferation, an indicator that these cells underwent activation-induced anergy. Thus, we submit that BMAP-27 and BMAP-28 may play a role in returning the activated T cell population to a homeostatic state through induction of peripheral tolerance mechanisms.

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LIST OF ABBREVIATIONS

ACAD	Activation autonomous death
ACID	Activation induced cell death
APAF-1	Apoptotic-protease-activating-factor-1
ATP	Adenosine triphosphate
AP	Activator protein
APC	Antigen presenting cell
Bac2a	Bactenecin 2a
Bcl	B cell lymphoma
BMAP	Bovine myeloid antimicrobial peptide
BSA	Bovine serum albumin
BTLA	B and T lymphocyte activation
Ca	Calcium
cAMP	Cyclic adenosine monophosphate
CD	Cluster of differentiation
CFSE	Carboxyfluorescein succinmidyl ester
CLTA-4	Cytotoxic T-lymphocyte antigen 4
ConA	Concanavalin A
CRAMP	Cathelin related antimicrobial peptide
CTL	Cytotoxic T lymphocytes
DC	Dendritic cell
DGK	Diacylglycerol kinase
DR	Death receptor
E. coli	*Escherichia coli*
EDTA	Ethylenediamine tetraacetic acid
ERK	Extracellular regulated kinase
FADD	Fas associated death domain
FOXp3	Fork head protein family
FPR	Formyl peptide receptor
GITR	Glucocorticoid-induced tumor necrosis factor receptor

GM-CSF	Granulocyte-macrophage colony-stimulating factor
hBD	Human beta defensin
HCEC	Human colonic epithelial cells
HDP	Host defense peptides
HNP-1	Defensin human neutrophil peptide-1
IBD	Irritable bowel disorder
ICOS	Inducible co-stimulatory molecule
iDC	Immature dendritic cell
IDR	Innate defense regulator
Ig	Immunoglobulin
IκB	Inhibitor of kappa B
IL	Interleukin
Indol	Indolicidin
IFN	Interferon
IP_3	Inositol triphosphate
JNK	C-Jun N-terminal kinase
LAT	Linker of activated T cells
Lck	Leukocyte
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
MAC	Membrane attack complex
MAP	Mitogen associated protein
MCP-1	Monocyte chemotactic protein-1
MHC	Major histocompatibility complex
MPTP	Mitochondrial permeability transition pore
NO	Nitric oxide
NFAT	Nuclear factor associated T cells
NF-κB	Nuclear factor kappa B
NK	Natural killer
NPY	Neuropeptide Y
nTreg	Natural T regulatory cell

PAMP	Pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PD	Programmed death
PI	Propidium iodide
PKC	Protein kinase C
PLC	Phospholipase C
PLCγ1	Phopholipase C gamma 1
RAP	Ras proximase
RIP1	Receptor interacting protein 1
RPMI	Roswell park memorial institute
SLAM	Signaling lymphocyte activation molecule
TCR	T cell receptor
TGF	Tumor growth factor
Th	T helper
THP-1	Human acute monocytic leukemia cell line
TLR	Toll like receptor
TNF	Tumor necrosis factor
TRADD	TNF receptor death domain
TRAIL	Tumor necrosis factor apoptosis related ligand
Treg	T regulatory
VIP	Vasoactive intestinal peptide
ZAP70	Zeta chain-associated protein 70

1.0 LITERATURE REVIEW

1.1 Interplay Between Innate and Adaptive Immunity

The immune system is a complex system responsible for protecting the body against pathogenic organisms while simultaneously discriminating self from non-self. The two main divisions of the immune system are the innate immune system, which is activated rapidly and is non-specific, and the adaptive immune system, which takes days to become fully activated, but is antigen-specific (Janeway Jr 1998; Medzhitov 2003). There is increasing evidence that the innate immune system instructs the adaptive immune system and this interplay is essential to protecting the host against infection (Janeway Jr 1989; Iwasaki 2010).

1.2 Innate Immunity

The innate immune system consists of multiple anatomical, mechanical, and chemical barriers which kill and/or prevent pathogen entry into the body. For instance, mucus and cilia in nasal passages prevent pathogen attachment and sweep away pathogens, respectively. Peristaltic action of coughing expels pathogens from the upper respiratory tract. Gastric acid in the stomach kills a majority of microbes. Enzymes, such as lysozyme and phospholipases and biochemical factors such as fatty acids, HDPs, carbohydrates, and various small molecules are present in bodily secretions such as tears, saliva, sweat, urine, breast milk, and nasal and vaginal secretions (Medzhitov 2003; Lee 2007). Thrombin found in blood plasma is released which cleaves fibrinogen, which can aggregate to form a blood clot and act as a chemoattractant for phagocytic cells. (Fearon 1996; Medzhitov 2003; Kaisho 2009). Normal flora of the skin, respiratory, vaginal, and gastrointestinal tract compete with pathogenic bacteria for nutrients and/or attachment to cell surfaces (Frank 1991; David 2003).

Major cellular components of the innate immune system are neutrophils, dendritic cells (DCs), macrophages, natural killer (NK) cells, and eosinophils. Resident neutrophils, which are situated throughout the tissues, are often the first cells to respond to infection. Upon infection, pathogen associated molecular pattern (PAMP), such as bacterial lipopolysaccharide (LPS), flagellin, and lipoteichoic acid, are recognized by toll like receptors (TLR), which activate neutrophil degranulation and initiate cellular recruitment. Neutrophils, DCs, and macrophages are recruited to the site of infection where they are activated by PAMPs or danger associated

molecular patterns (DAMPs) to release cytokines and to phagocytose invading organisms and kill them intracellularly via induction of oxidative molecules, such as superoxide, myeloperoxidase, and nitric oxide (NO) (Tufet 2007). DCs migrate to draining lymph nodes to promote T and B cell activation (Kaisho 2009).

NK cells are important in the clearance of pathogens during acute inflammation. Upon engulfment of pathogens or their components, these cells have the ability to kill invading pathogens via cytotoxic activity (Strowig 2008; Girardin 2009) and can differentiate self from non-self. NK cells are activated by signals, such as cytokines, released from macrophages including interferon (IFN)-α, and IFN-β (Schoenborn 2007; Stuart 2008). The cells express the Fc receptor on their surface, which can bind to the Fc portion of antibodies. Antibody bound NK cells release cytokines, such as IFN-γ and cytotoxic granules containing perforin and granzymes that enter the targeted cell and promote cell death (Brown 2001).

The three main pathways of the complement system include classical, alternative, and mannose-binding lectin pathways, all of which culminate in the activation and release of complement system's C3b protein. C3b binds to the cell walls of pathogens promoting their uptake by phagocytes, which is opsonization, a mechanism by which bound antibodies promote phagocytosis of pathogens (Kemper 2007).

Complement protein C3a promotes vasodilation through the stimulation of histamine release from mast cells, which promotes cellular infiltration to the site of infection. Complement protein C5a acts as a chemoattractant for neutrophils to the site of infection. The complement system triggers the activation of the membrane attack complex (MAC) to lyse infected cells (Morgan 2005; Chou 2008). The innate immune system consists of cells and mechanisms that defend the host from infection by other pathogens. Their first response to an infection also promotes activation of the adaptive immune system through various mechanisms.

1.3 Adaptive Immunity

The adaptive immune response depends on the two main cellular components, B cells and T cells, both of which have antigen-specific receptors on their surfaces (Iwasaki 2010). Antigen presenting cells (APCs), such as DCs, present antigen on the surface of their cell in the context of major histocompatability complex (MHC). DCs travel from the site of infection to the draining lymph node, where they interact with and activate naïve T cells, which recognize the cognate

antigen (Lee 2007; Hammad 2008). Activated T lymphocytes differentiate into short-lived effector cells to combat the current infection, as well as memory cells that can become re-activated if challenged by the same pathogen in the future (Van Leeuwen 2009). APCs express MHC I and MHC II on their surface, which are key molecules to help T cells and B cells decipher whether an antigen is self or non-self. MHC I molecules are cell surface glycoproteins that present antigen fragments synthesized within the cell. Cluster of Differentiation (CD)8^+ T cells, which recognize antigens presented on MHC I molecules, kill cells that are invaded by intracellular pathogens (Mescher 2007). MHC II molecules display digested antigen to $CD4^+$ T cells. Activated $CD4^+$ T cells are involved in regulating the cellular and humoral immune response by their sub-classes of T helper (Th) cells including Th1 (cell-mediated), Th2 (humoral), and Th17 (regulatory) cells (Williams 1991). The details of T cell development, activation, proliferation and differentiation will be discussed below.

1.3.1 T Cell Development

In the thymus, T cells undergo differentiation into distinct subpopulations including CD4 and CD8 subsets. T cells originally do not express CD4 or CD8 surface markers ($CD4^-CD8^-$ T cells) but they do express the adhesion molecule CD44 and the α chain of the interleukin (IL)-2 receptor (CD25). Productive rearrangement of the β chain of the IL-2 receptor triggers the T cell receptor (TCR) to complex with CD3 on the surface of the T cell. Thymic cortical epithelial cells express either MHC class I or II, which bind to T cells that promote differentiation into $CD4^+CD8^-$ if they are selected by MHC class II and $CD4^-CD8^+$ if they are selected by MHC class I molecules (von Boehmer 2008). A T cell becomes a $CD4^+$ cell by down regulating expression of its CD8 cell surface molecules. If the cell does not lose its signal through the immunoreceptor tyrosine-based activation motifs (ITAM) pathway, it will continue down regulating CD8 and become a $CD4^+$, single positive cell. If the signal drops, the cell stops down regulating CD8 and switches over to down regulating CD4 molecules instead, eventually becoming a $CD8^+$, single positive cell. Any T cell with high affinity for self peptides undergo apoptosis to prevent autoimmunity (David 2003). The percent of each specific subset population in human blood is approximately 60 % $CD4^+CD8^-$ (CD4) and 40 % $CD4^-CD8^+$ (CD8) (Ethan 2006).

Unlike murine and human T cells, porcine T cells have unique subpopulations identified as $CD4^+CD8^{lo}$ that make up 60 % of the total T lymphocytes of adult porcine peripheral blood.

The majority of these are memory $CD4^+CD8^{lo}$ T cells (Werner 1990; Zuckermann 1996). In humans, $CD4^+CD8^{lo}$ T cells are found in patients suffering from rheumatoid arthritis, neoplasia and infectious mononucleousis (Andrea De 1987; Gianello 1994; Saalmüller 1999; Charerntantanakul 2006).

1.3.2 T Cell Activation

Upon T cell activation, the α and β chains of the TCR interact with a CD3γε heterodimer, a CD3δε heterodimer, and a CD3ζ homodimer on the T cell surface (Lenschow 1996; Nel 2002; David 2003; Smith-Garvin 2009). This TCR/CD3 complex interacts with the antigen-MHC complex expressed on APCs, which constitutes the first signal for T cell activation, as illustrated in figure 1.1. The second activation signal is the interaction of co-stimulatory molecules such as CD28, CD27, B and T lymphocyte attenuator (BTLA), inducible co-stimulatory molecule (ICOS), CD134, CD30, CD137, signaling lymphocyte activation molecule (SLAM), cytotoxic T-lymphocyte antigen 4 (CTLA-4), and program death (PD)-1, which interact with corresponding signaling molecules on the APC (Viola 1996; Shaw 1997; Kroczek 2005). Co-stimulatory molecules interaction form the immunologic synapse which is the molecular arrangement on the T cell surface responsible for concentrating the TCR and its co-stimulatory molecules into one central zone. Co-stimulatory T cell surface molecule CD28 interacts with CD80/86 on the surface of APCs triggering CD3 chain tyrosine protein phosphorylation by the Src kinases, leukocyte (Lck) and Fyn (Weiss 1994; Chu 1998; Van Leeuwen 1999; Nel 2002). This change in phosphorylation status triggers activation of downstream proteins, such as phospholipase C (PLC), Ras and Rac, protein kinase C (PKC) and mitogen associated protein (MAP), which leads to the production of interleukin (IL)-2. Concurrently, interaction with the TCR and co-stimulatory molecules promotes cytokine release from the APCs, which acts as the third signal for T cell activation. IL-1 family cytokines are released from APCs and participate in inducing T cell activation (Zhu 2010). For further differentiation of $CD4^+$ T cells into Th1 and Th2, IL-4 or IFN-γ are secreted from $CD4^+$ T cells, respectively. The third, most recent T-effector cell subset is Th17. IL-1β, IL-23, IL-6, tumor growth factor (TGF)β and IL-21 are secreted for the differentiation of Th17 T cells (Katia 2008). IL-12 is secreted for $CD8^+$ T cell differentiation (Curtsinger 1999; Curtsinger 2010). These cytokines can act directly on the $CD4^+$ or $CD8^+$ T cells or enhance the APCs by promoting increased expression of co-stimulatory molecules on

their surface such as CD40 ligand and CD80/86. This would thereby enhance signal two (Grewal 1995; Grewal 1996; McLellan 1996). Ultimately, activated T cells express genes whose corresponding proteins promote cellular proliferation and differentiation (Trowbridge 1993). Without co-stimulation, T cells enter into a state of non-responsiveness, known as anergy (Guerder 2001).

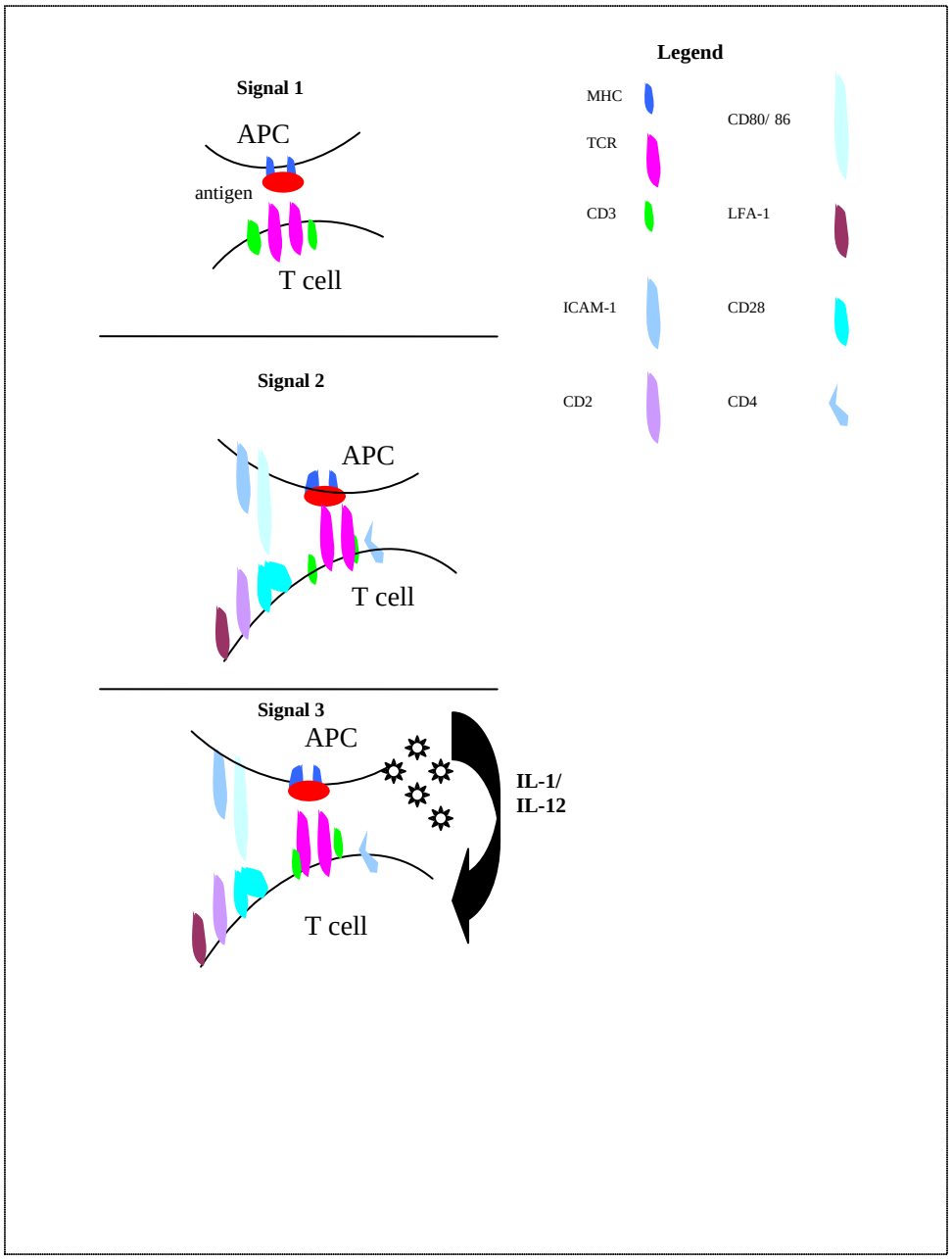


Figure 1.1 Three Signaling Events Must Occur to Trigger T Cell Proliferation and Differentiation. The 1st signal is TCR/CD3 complex interaction with the antigen-MHC complex expressed on APCs. The 2nd signal involves the interaction of co-stimulatory molecules, which trigger downstream signaling and tyrosine phosphorylation in the T cell. The 3rd signal is the release of cytokines from APCs, such as IL-12 for the phenotypic differentiation of $CD8^+$ T cells and IL-1 in the case of $CD4^+$ T cell differentiation.

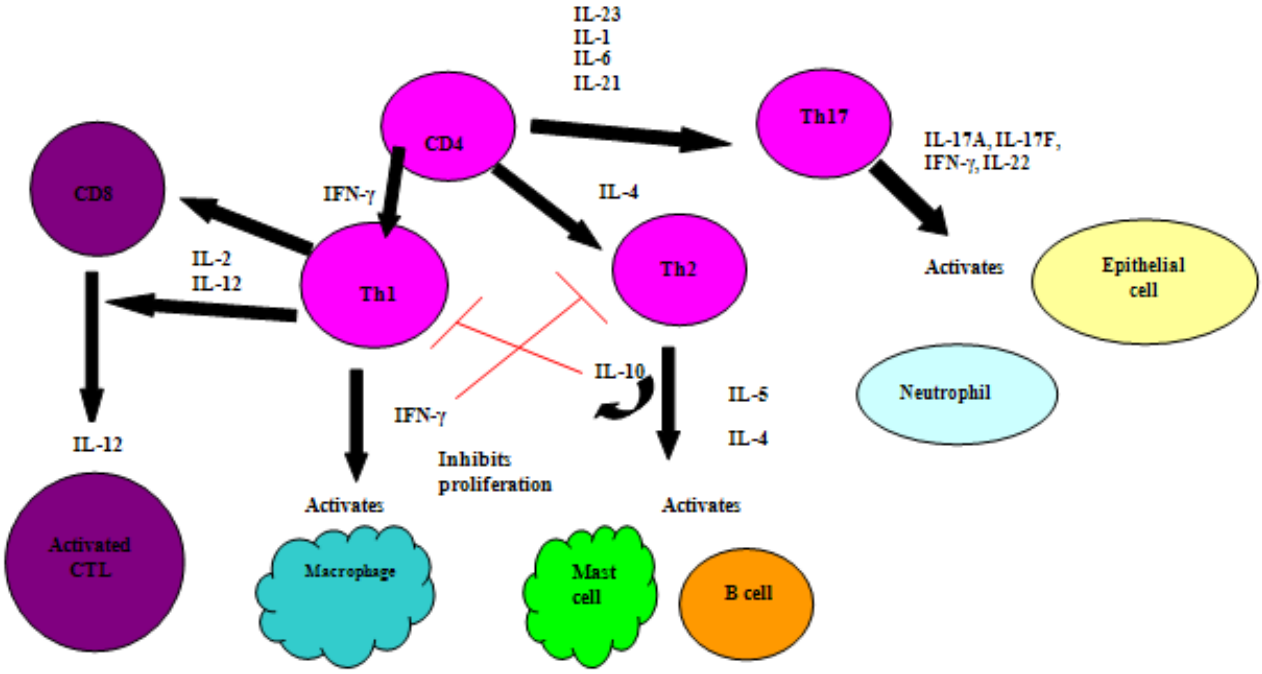


Figure 1.2 CD4 T cells differentiate into Th1, Th2 or Th17 cells. IFN-γ and IL-4 are secreted from CD4 T cells for the differentiation of Th1 and Th2 cell, respectively. Th2 cells activate B cells and mast cells with the secretion of IL-5 and IL-4, while Th1 cells activate macrophages and help in the activation of CTLs with the release of IL-12 and IL-2. Th1 differentiation inhibits Th2 cells by secreting IFN-γ, while Th2 differentiation inhibits Th1 cells by secreting IL-10. IL-23, IL-6, IL-21, and IL-1, IL-6, IL-21 and IL-23 secretion differentiate CD4 T cells into Th17 cells. Th17 cells further activate epithelial cells to release antimicrobial peptides such as beta defensin-2. Th17 cells also activate and recruit neutrophils.

1.3.3 Th Cell Characterization and Function

$CD4^+$ T cells can differentiate into Th1, Th2, or Th17 cells, which can further promote a cell-mediated immune response, humoral immune response, or Th17-mediated autoimmunity, respectively as seen in figure 1.2. Briefly, Th1 cells are characterized by a cytokine profile including IL-12, IL-2, and IFN-γ. Th2 cytokine profile includes IL-4, IL-5, IL-9, and IL-13 (Mosmann 1996; Thomas MJ 1998; Abbas 2004). Th17 cytokine profile includes IL-17A, IL-17F, IL-22, IFN-γ, IL-26, IL-6 and Tumor Necrosis Factor (TNF)-α (Katia 2008).

1.3.3.1 Th2 and Humoral Immunity

While B cells can be activated in a T cell independent manner, the majority of B cells require interaction with cognate T cells in the lymph node to become activated when an APC presents an antigen to a Th2 cell, a specific cytokine profile (IL-4, IL-5, IL-13) is secreted from the T cell. These cytokines bind to their receptors on B cells which in turn stimulate B cell growth and differentiation into plasma cells. Plasma cells secrete immunoglobulin (Ig)A, IgD, IgE, IgG, and IgM, the main effector molecules of the humoral immune response (Janeway Jr 1989; Pier 2004; Carroll 2008). Each immunoglobulin class differs in function. Th2 cytokines promote antibody production for the removal of extracellular pathogens, such as helminths and nematodes, by activating B cells to produce more immunoglobulin as well as directly activating mast cells and eosinophils by binding to their receptors (Finkelman 1991; Sher 1992).

1.3.3.2 Th1 and Cell-Mediated Immunity

Th1 cells promote cell-mediated immunity through three main mechanisms: 1) The release of IFN-γ, which activates phagocytosis and killing of intracellular pathogens by macrophages (Steinman 2007), 2) activating macrophages to produce TNF-α, NO, and reactive oxygen intermediates, which kill target cells by induction of apoptosis. 3) Th1 cells secrete IL-2 and IFN-γ, which signal cytotoxic T lymphocytes (CTLs), antigen and MHC-restricted $CD8^+$ T cells, to release enzymes such as perforin that promote death of the target infected cell. 4) Th1 cells secrete IFN-γ and IL-2, that will in turn activate NK cells, which will then recognize foreign proteins on the surface of infected cell or tumor cell (Douglas MS 1997; Wan 2009). These pro-inflammatory properties of Th1 cells are important in clearing intracellular infections, such as *Leishmana major*, a protozoal parasite (Sadick 1987), *Mycobacterium avium* (Kobayashi 1997),

Salmonella typhimurium (Mastroeni 1999), and *Listeria monocytogenes* (Buchmeier NA 1985), or intracellular fungi, such as *Cryptococcus neoformans* (Zhang 1997). Viruses, such as Herpes Simplex Virus (Fujioka 1999), Influenza A virus (Sareneva 1998), and Vaccinia virus (Tanaka-Kataoka 1999) require a Th1 response for host protection. Th1 cells are also involved in the removal of tumor cells (Micallef 1997).

1.3.3.3 Th17-Mediated Autoimmunity and Normal Immune Defense Mechanisms

Th17 cells have a cellular function that is more established in their role in autoimmunity compared to their role in normal immune defense mechanisms. Th17 cells secrete IL-12, IFN-γ, IL-23, and IL-17 cytokines, which activate inflammation in the colonic *lamina propria* by stimulating macrophages and recruiting more cells to the site of infection (Jovanovic 1998). There is strong evidence that Th17 cells are linked to Irritable Bowel Disorder (IBD) and addition of neutralizing antibodies to IL-12 and IL-17 cytokines in patients, reduced symptoms of inflammation (Fujino 2003).

Th17 cells act at epithelial/mucosal barriers to promote epithelial cell release of HDPs including β-defensin to clear microbes, such as *Candida* and *Staphylococcus* (H. Riechelmann 2005). They also activate and recruit neutrophils by the expression of chemokine receptors such as CCR6, CXCR3, CXCR6, and CCR4 (Liang 2007) and the enhanced expression of CXCL1, CXCL5, and CXCL2 in response to IL-17A, which is secreted from Th17 cells (Ferretti 2003). However, an overproduction of IL-17 and Th17 cells can lead to neutrophilia, a condition recognized by an excessive amount of neutrophils in a patient's blood (Ferretti 2003).

1.4 Resolution of T Cell Activation

Upon clearance of the foreign pathogen or antigen, immune responses are dampened to allow the immune system to return to a homeostatic state and rest before a new challenge (Parijs 1998). The proper resolution of cellular activation is just as important as the propagation of a response. A homeostatic state of the immune system is a sustainable environment, promoted by an array of mechanisms in peripheral tolerance. These mechanisms will be further discussed below and include adaptive tolerance, clonal anergy, activation induced anergy, apoptosis, and induction of T regulatory (Treg) cells.

1.4.1 Tolerance

Tolerance is defined as the acquired specific non-reactivity of immunological mechanisms to respond to a given antigen. Immune tolerance is known to have a "two tiered system" starting in the thymus where cells develop central tolerance to eliminate the immature T cell population of thymocytes which may recognize self proteins. The second tier of tolerance is peripheral tolerance, which culminates in induction of anergy, which directs activated T cells to undergo a temporary state of unresponsiveness post activation, deletion of activated T cells by apoptosis, and suppression of immune responses through up-regulation of T regulatory cell function (Walker 2002; Rathmell 2003; Starr 2003; Kronenberg 2005). Tolerant T cell signaling differs to proliferating T cell signaling. Unlike non-self reactive T cells, tolerant T cells show poor phosphorylation of the TCR-ζ and TCR-ε chains and are unable to activate the lymphocyte specific protein tyrosine kinases, zeta chain associated protein (ZAP)-70, Ras, c-Jun N terminal kinases (JNK), and extra signal regulated kinase (ERK) (Kang 1992; Cho 1993; Gajewski 1994; Boussiotis 1996; Fields 1996; Mondino 1996; Slaughter 2002). The TCR is unable to activate activator protein 1 (AP-1) and nuclear factor of activated T cells (NFAT) binding sites in the IL-2 promoter, suggesting that T cell tolerance involves novel signaling events.

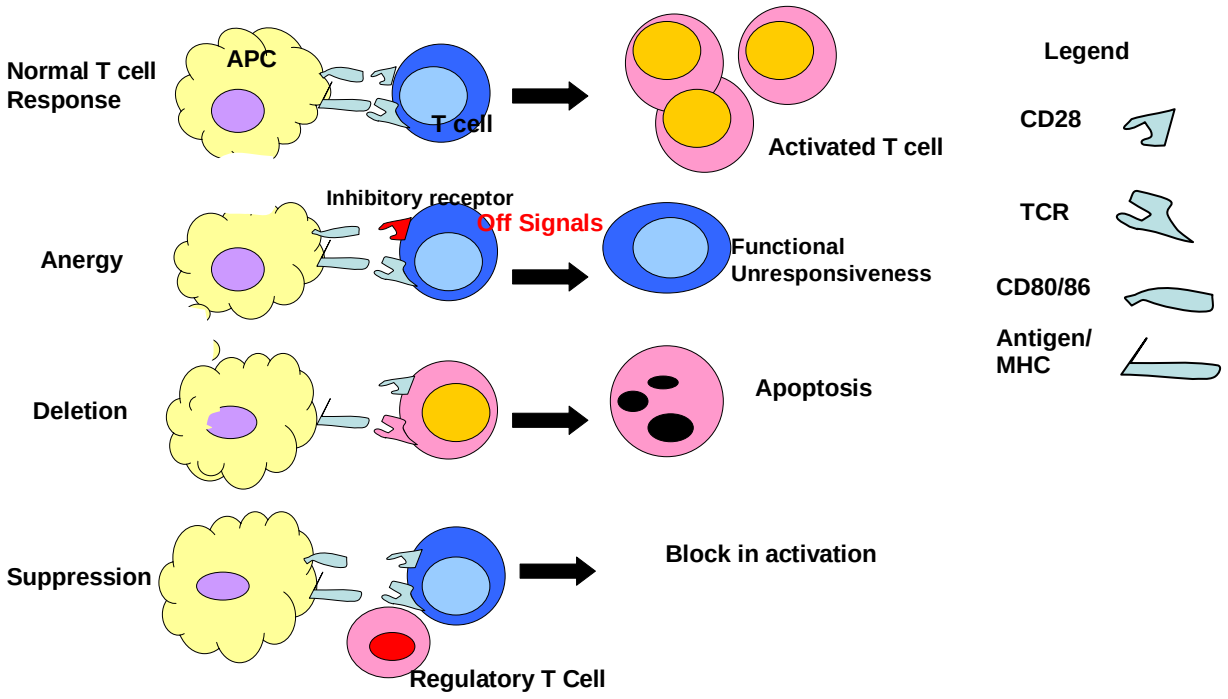


Figure 1.3 T Cell Anergy, Deletion, and Suppression: Three Forms of Peripheral Tolerance
An anergic T cell has increased surface expression of inhibitory receptors, which outcompetes with the CD28 molecule for CD80/86 on the APC. This leads to functional unresponsiveness. T cells which interact with the APC's Ag and MHC, but not the co-stimulatory molecules undergo deletion (apoptosis), which is programmed cell death. Suppression is the blocking of activation and is controlled by regulatory T cells, which control the signaling between T cells and APCs.

1.4.1.1 Adaptive Tolerance

Adaptive tolerance is known as *in vivo* anergy, which refers to the persistent antigen-specific stimulation of T cells in the absence of co-stimulation (Schwartz 2003). Adaptive tolerance has four characteristics that differ from clonal anergy. Firstly, in adaptive tolerance, there is a down-regulation of multiple effector cytokines, such as IL-4 and IFN-γ. Secondly there is a dependence of persistent antigen and, thirdly, exogenous IL-2 cannot reverse the state of anergy (Chiodetti 2006). Fourthly, increased expression of CTLA-4 competes with CD28 for interaction with the CD80/86 site on APCs, as seen in figure 1.1. The addition of CTLA-4 inhibitors circumvents adaptive tolerance and proliferation then continues. Experiments have shown that mice deficient in CTLA-4 expression have excessive proliferation of T lymphocytes and die due to this overload of an immune response (Tivol 2005). T cell signaling in adaptive tolerant cells is initiated when Zap-70 phosphorylation of Linker of activated T cells (LAT), is prevented. LAT, which is part of the TCR complex, requires phosphorylation so phospholipase C-γ1 (PLCγ1) and Grb2/mSOS can dock at the cell surface, which culminates in IL-2 production and T cell activation. Mice deficient in CTLA-4 or its receptor succumb to massive multifocal inflammation or lymphoproliferative disease with progressive accumulation of lymphocytes in peripheral organs. Without adaptive tolerance helping to monitor the homeostasis of the periphery, unwanted disease occurs (Gatzka 2007).

1.4.1.2 Clonal Anergy

Clonal anergy, known as growth arrest, occurs when there is incomplete activation of T cells (Schwartz 2003). Clonally anergic T cells experience an ineffective MAP kinases activation and mobilization of nuclear factor kappa B (NF-κB) to the nucleus. Specifically, there is a down-regulation of phosphorylation of proteins, such as ERK, JNK, p38, and PKC/IkBα (Choi 2007). In addition, Ras is found to reside in the inactive guanosine 5-triphosphate (GTP)-bound state. This in turn increases the levels of diacylglycerol kinase (DGK)-α, which impedes T cell signaling (Zha 2006). Clonal anergy can be reversed with the addition of IL-2, DGK inhibitor, or anti-OX40 signaling (Alegre 2001; Schwartz 2003) and it is not influenced by CTLA-4 surface expression. There is increased CTLA-4 expression in $CD4^+$ T cell clones, however the protein is mostly expressed intracellularly. Blocking antibodies or genetic CTLA-4 deficiency did not

influence the induction of anergy *in vitro* or allow clonally anergic cells to respond to antigen stimulation.

1.4.1.3 Activation-Induced Anergy

Activation-induced anergy differs from other anergic events in that it occurs after TCR engagement and co-stimulation and it is reversed if exogenous IL-2 is added (De Mattia 1999; Tham 2002). Addition of anti-CTLA-4 mAb neither prevents nor reverses activation-induced anergy (Tham 2002). Interestingly, after addition of exogenous IL-2, activation-induced anergic cells can revert back to functioning T cells, which produce their own IL-2 and are able to sustain their effective response to antigen (Dubey 2007). After reversal, anergic T cells do not need co-stimulatory cells for full activation, only the engagement of the TCR (Dubey 2007; Mescher 2007). This type of anergy is known to occur as a regulatory checkpoint, where for example, an autonomous CD8 response is converted to one requiring IL-2 from $CD4^+$ T cells for continued activation. $CD4^+$ T cells that undergo activation-induced anergy are highly susceptible to cell death (Tham 2002).

1.4.1.4 Apoptosis

As a measure to control immunity, activated T cells eventually undergo apoptosis, which is characterized by cellular morphological changes, including increasing cell density, chromatin condensation, internucleosomal degradation of DNA, and nuclear collapse (Lakhani 2006; Rüdiger 2006; Gatzka 2007). Unlike necrotic cells, T cells undergoing apoptosis do not induce an inflammatory response. T cells undergo apoptosis by extracellular and intracellular events. There are two major apoptotic pathways; the intrinsic pathway and the extrinsic pathway (Zhang 2005).

The intrinsic apoptotic pathway, also referred to as activated T cell autonomous death (ACAD), is thought to be initiated by cytokine withdrawal including IL-2 and IL-6 (Zhang 2005; Rüdiger 2006). ACAD is determined by the ratio of anti-apoptotic to pro-apoptotic B cell lymphoma (Bcl)-family members (Brenner 2008). Bcl-family control cell death signals at the mitochondria outer membrane, which results in a loss of membrane integrity. Anti-apoptotic subfamily members including Bcl-2, Bcl-x, Mcl-1, and A1/Bfl-1 inhibit pro-apoptotic Bcl-2 family members by acting as a downstream effector of NF-κB signaling (Zhang 2005).

The pro-apoptotic family is composed of subfamily BH3-only, which include Bad, Bid, Bik, Bim, Noxa, and Puma and subfamily BH1-3, which include Bax, Bok, and Bak proteins. Subfamily BH3 only promotes pro-apoptotic BH1-3 subfamily activity or inhibits the anti-apoptotic subfamily members. Activated Bax and Bok are the main executioners of the intrinsic apoptosis pathway which act by forming holes in the outer mitochondrial membrane. Mitochondria contents, such as cytochrome C, are then released into the cytoplasm where they interact with apoptotic-protease-activating-factor-1 (Apaf-1). An apoptosome complex is formed in the cytoplasm where Caspase-9 is activated, which in turn, activates Caspase-3, 6, and 7. Caspase activation leads to cleavage of hundreds of cellular components, which results in irreversible cell death (Krammer 2007).

The extrinsic pathway, also referred to as activation induced cell death (ACID), is induced when pre-activated and expanded T lymphocytes are re-stimulated and cell death receptors, such as TNF receptor 1, Fas, Death Receptor (DR) 3, tumor necrosis factor apoptosis related ligand (TRAIL) receptor 1, TRAIL-R2, and DR6 are stimulated (Hildeman 2002; Douglas 2003; Peter 2003). Once ligands bind to the death receptors, one of two major adaptor protein signaling pathways are initiated, Fas associated death domain (FADD)-dependent and TNF receptor death domain (TRADD)-dependent (Majno 1995).

Activation of FADD signaling, leads to activation of Caspase 8 and 10, which in turn activates Caspase 3, resulting in apoptosis. Activation of TRADD signaling triggers TNFR1 binding and the subsequent formation of a signaling complex with Receptor Interacting Protein 1 (RIP1), which in turn inhibits NFκB activation (Cohen 1992; Hetz 2002; Zhang 2005; Rüdiger 2006; Girkontaite 2007).

1.4.1.5 Regulatory T Cells

T regulatory cells function to maintain homeostasis and self tolerance, failure of which results in autoimmune disorders, such as systemic lymphoproliferative autoimmune syndrome (Shevach 2000; Sakaguchi 2005). Naturally occurring regulatory T cells (nTregs) and induced Tregs (iTregs) are two types of $CD4^+$ Tregs, which are primarily defined by where they develop (Workman 2009). nTregs develop in the thymus and constitutively express CD25, CTLA-4, and glucocorticoid-induced tumor necrosis factor receptor (GITR), while iTregs develop in the periphery from conventional $CD4^+$ T cells following antigenic stimulation and can be induced to

become X-linked transcription factor of the Fork head protein family (Foxp3)$^-$ Type 1 regulatory T cell (Tr1) cells via IL-10 or Foxp3$^+$ Th3 cells via TGFβ (Groux 1997; Howard 2001; Skapenko 2005). IL-4 and IL-13 also induce iTreg independently of TGFβ and IL-10 (Skapenko 2005). Both nTregs and iTregs share cell surface markers characteristic of an activated T cell, such as CD25, CTLA-4, GITR, CD62L, and CD45RB^{lo} (Zheng 2008).

nTregs develop in response to self-antigen, whereas iTregs develop in response to weaker, suboptimal TCR stimulation and exogenous antigens in the periphery (Apostolou and von Boehmer 2004; Kretschmer 2005). To generate nTregs, CD28 co-stimulation is required. In contrast, iTregs do not require CD28 co-stimulation, but instead require presence of anti-inflammatory cytokines (Apostolou 2002); (Taylor 2002). Other Treg cell populations have been discovered, which are unique from nTreg and iTreg cells. A marker common to all Tregs has yet to be found (Fontenot 2003; Vieira 2004; Riley 2009).

1.5 Host Defense Peptides

1.5.1 Structure and Function of Host Defense Peptides

HDPs are peptides with inherent antimicrobial and immunomodulatory functions. They are naturally present in many forms of life including plants (DeGray 2001), insects (Bulet 1999), and animals (Boman 2003). HDPs generally range in size from 12 to 50 amino acids (Bowdish 2005). They have a positive charge provided by arginine and lysine residues and their amphipathic structure enables them to interact with bacterial membranes. Their secondary structures can range from, α-helical, β-sheet, β-hairpin, and to an extended conformation (Bowdish 2005). They mediate their antimicrobial activity by binding to and disrupting the integrity of the negatively-charged microbial membranes. These peptides were first classified simply as “antimicrobial peptides”, but also have since been proven to have immunomodulatory activities (McPhee 2005; McPhee JB 2005).

Cationic HDPs are sub-divided into two extensively studied mammalian families, defensins and cathelicidins. Defensins are classified based on their secondary structure including α-defensins, β-defensins, and θ-defensins. There are over 100 different defensins identified in mammals. Defensins are stored in granules of Paneth cells or neutrophils and secreted by monocytes, macrophages, mast cells, NK cells, keratinocytes, and epithelial cells. Cathelicidins,

the other extensively studied cationic family, will be the focus of this project and, therefore, I will provide a more in-depth description of this family.

1.5.2 Structure and Function of Cathelicidins

Cathelicidin family members have been identified in human, pig, mouse, sheep, and bovine (Zanetti 1995; Boman 1998; Ganz 1999; Lehrer 1999; Bals 2000; Ramanathan 2002; Yang 2004). They are characterized by a 99-114 amino acid long pro-region, known as the cathelin domain (Ritonja 1989), which is highly conserved. The C-terminal domain of cathelicidins is very heterogenous in its amino acid sequence and it is this region that regulates the antimicrobial effect and/or immunomodulatory functions (Zanetti 1995; Yang 2004).

Cathelicidins have broad range antimicrobial activity against Gram-positive (Friedrich 2000) and Gram-negative (Matsuzaki 1997) bacteria, fungi (Lemaitre 1997), protozoa (Rivas 2009), enveloped viruses (Robinson 1998), and tumor cells (Lichtenstein 1986). Their immunomodulatory functions include induction of chemotaxis of neutrophils, T cells, and monocytes (Wieprecht 1997; Dathe 1999; Giangaspero 2001; Yang 2004) and these functions will be elaborated on below.

Previously in our laboratory, a microarray analysis was performed to establish whether distinct bovine cathelicidin family members influenced gene expression patterns of porcine leukocytes. The microarray results (unpublished data) uncovered BMAP-27, BMAP-28, and Indol influenced the gene expression patterns of genes, whose corresponding proteins play a role in T cell proliferation, despite the lack of homology in both amino acid sequence and tertiary structures between these peptides. Some genes included were part of the MAP kinase signaling pathway, TCR signaling pathway, and IL-2 signaling; NFkB, MAPK, CD3ε, GRB, AKT, IL-2R, JAK, and ICAM. My project was designed to validate at a functional level whether these distinct cathelicidins influenced T cell signaling. HH2, a derivative of a bovine peptide Bac2a, was incorporated to compliment BMAP-27, BMAP-28, and Indol.

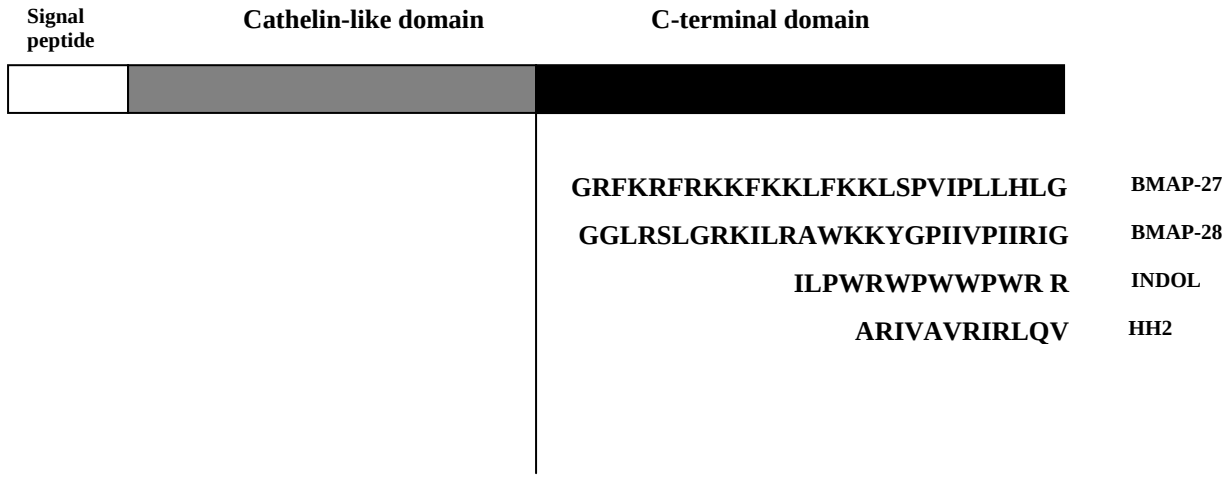


Figure 1.4 Primary Sequence and Functional Domains of BMAP-27, BMAP-28, Indol, and HH2. The amino acid sequence of the C-terminal domain is provided for BMAP-27, BMAP-28, Indol, and HH2. BMAP-27 is 27 amino acids rich in lysine, while BMAP-28 is 28 amino acids in length, with isoleucine being the most prominent amino acid. Indol is rich in proline and tryptophan. HH2 is 12 amino acids in length and rich in arginine and valine.

1.5.2.1 Effect of Cathelicidins on Pro-Inflammatory and Anti-Inflammatory Responses

The role of cathelicidins with respect to regulating a pro- or anti-inflammatory response varies greatly depending on the type of HDP, the species, and the cell type under investigation. Generally, cathelicidins influence epithelial cell proliferation, promote wound healing and angiogenesis, stimulate chemokine production, inhibit pro-inflammatory cytokine production, direct chemotaxis of leukocytes, mast cell degranulation, and modulation of host cell gene expression (Bals 2000; Travis 2001; Zasloff 2002; Cunliffe 2003; Oppenheim 2003). Evidence for pro- or anti-inflammatory responses will be outlined below to clarify contradictory information in the literature.

1.5.2.1.1 Pro-inflammatory Role

Indolicidin, LL-37, PR-39, and Cathelin Related Antimicrobial Peptide (CRAMP) have been shown to promote a pro-inflammatory response by stimulating the expression of chemokines, such as IL-8 and monocyte chemotactic protein (MCP)-1 from epithelial cells, as well as induction of G-protein-coupled, seven transmembrane–domain receptors on B and T lymphocytes (Fearon 1996; Medzhitov 1997; Travis 2001; Bals and Wilson 2003; Aarbiou 2004). Inhibiting $G_i\alpha$ receptors including CXCR1, CXCR2, CCR1, CCR2, CCR4, CCR5, CCR6, and formyl peptide receptor (FPR) (Yang 2001) with pertussis toxin inhibited LL-37 chemotactic function. Recent efforts have been put forth to generate peptides with less pro-inflammatory activity, such as innate defense regulator (IDR)-1, a synthetic peptide derivative of LL-37 (Biragyn 2008; Easton 2009). The above examples illustrate how cathelicidins affect the pro-inflammatory response, however they also have direct consequences on the anti-inflammatory response (Biragyn 2002; Aarbiou 2004; Easton 2009).

1.5.2.1.2 Anti-Inflammatory Role

Cathelicidins have been shown to inhibit pro-inflammatory responses through neutralization of endotoxin in endothelial cells. For example, after LL-37 administration, mice and rats were able to overcome LPS-mediated lethality by binding to LPS, thereby neutralizing its toxic effects (Davidson 2004; Braff 2005). LL-37 has been shown to inhibit the expression of pro-inflammatory cytokines, such as TNF-α and IL-6 through inhibition of NFκB nuclear translocation in human and bovine monocytes (Mookherjee 2006).

1.5.2.2 Structure and Function of BMAP-27 and BMAP-28

BMAP-27 and BMAP-28 are bovine derived α-helical cathelicidins that are stored in the cell as pro-peptides in cytoplasmic azurophil granules of neutrophils. They are activated by elastase, an enzyme that cleaves off the precursor of the peptide (Gennaro 1998). They are cytotoxic to Gram-negative and Gram-positive bacteria and fungi at a concentration of 3 mg/ml (Benincasa 2003); and they are anti-parasitic at 39 mg/ml (Brogden 2007).

BMAP-27 and BMAP-28 share 28.5 % identity in their C-terminus (Brogden 2007). Since their sequences are quite diverse, the differences in their function are not surprising. For example, BMAP-27 is much less cytotoxic than BMAP-28 in human epithelial cells. BMAP-27 and BMAP-28 are also cytotoxic to human neutrophils at an elevated concentration, determined using propidium iodide method (Skerlavaj 1996; Shai 2002). There are no known receptors for BMAP-27 or BMAP-28. Their cationic nature allows them to associate with the negatively charged membranes in human epithelial cells, transformed cells, and activated lymphocyte cellular membrane proteins and lipids. For example, removing cell surface sialyl lipoprotein with neuramidase resulted in the cells being less susceptible to permeabilization by BMAP-27 and BMAP-28 at a concentration of 3 mg/l (Risso 1998). It is believed that the cellular membrane of activated lymphocytes undergo changes in the biochemical composition as well as in polarization compared to the cellular membrane of resting lymphocytes, which may effect the interaction of peptide with the cellular membrane (Risso 1998). For example, U937 cells, a human macrophage transformed cell line, incubated with 1 mg/l of BMAP-27 or BMAP-28 increased the intracellular Ca^{2+} within minutes from 136 to 551nm suggesting that these peptides disrupted the membranes of the U937 cells a human macrophage transformed cell line.

Concentration of 3 μmg/μl, BMAP-27 and BMAP-28 do not induce necrosis as confirmed by lactate dehydrogenase (LDH) release and do induce apoptosis as confirmed by propidium iodide (PI) uptake, which interacts with DNA from membrane damaged cells, in transformed cells and human lymphocytes. However, this apoptosis occurred without induced expression of Bcl proteins, which are part of the intrinsic apoptotic pathway, nor did they depend upon Caspase 1 to induce apoptosis as evident by the little effect of Caspase 1 inhibitor (Risso 1998). Other studies have shown that BMAP-27 and BMAP-28 promote apoptosis by depolarizing the mitochondria through the mitochondrial permeability transition pore (MPTP) and increasing permeabilization to calcein, releasing cytochrome C. (Risso 2002). A recent finding suggested

that two small stretches of leucine and isoleucine zipper sequences at the C and N terminal of BMAP-28 maintain the cytotoxic effect on human red blood cells and transformed cells (Ahmad 2009). By substituting one amino acid at these sites to an alanine, BMAP-28 showed decreased membrane permeabilization. Decreased permeability occurred with increased alanine substitutions in the zipper sequences (Figure 1.4). This further proves the functional role of the C terminal region of the cathelicidin (Ahmad 2009). A similar substitution in the N and C termini of BMAP-27 decreased its ability to interact with the cellular membrane of human red blood cells (Ahmad 2009).

There are no studies involving BMAP-27 and BMAP-28 and T cell modulation.

1.5.2.3 Structure and Function of Indolicidin

Indol is a 13 amino acid cathelicidin that lacks a coherent secondary structure and is naturally present in cytoplasmic granules of bovine neutrophils. It resides in the granules in a mature form unlike BMAP-27 and BMAP-28, which stay in their pro-form until activated by elastases (Selsted 1992). Indol has antimicrobial activity against various types of microorganisms including Gram-positive (Friedrich 2001) and Gram-negative (Falla 1996) bacteria, fungi (Lee 2003), and viruses (Bhargava 2007).

Indol is cytotoxic towards T lymphocytes and this activity is dependent on its random coiled structure as deletion of tryptophan residues at 4 and 11 positions at the C termini abrogated this function (Figure 1.4) (Brogden 2007). In contrast, Indol is hemolytic to human erythrocytes, but this activity was maintained even when these tryptophan residues were deleted as evident in circular dichroism spectra experiments (Setsuko 1998). Addition of leucine at these positions increased the hemoloytic activity (Setsuko 1998).

There is evidence that Indol binds acidic phospholipids and inserts beneath the lipid head groups of the membrane to induce local disorder (Oreopoulos and Yip 2009). After entering the cell, 20 μM of Indol was shown to interact with 40 μM of DNA to promote cell death. (Marchand 2006; Nan 2009). In LPS stimulated mouse macrophage cell line, Indol inhibited iNOS expression and NO production, however it is not known whether this inhibition occurred by inhibiting iNOS mRNA stability or if it mediated this effect via an upstream signal in the cytosol (Yong Hai 2009). However, an Indol analog which was less hydrophobic than Indol, was

unable to inhibit iNOS expression and NO production, which suggest that this hydrophobic region in Indol is critical to maintain its anti-inflammatory functions.

Indol has been shown to bind to LPS and block TLR-4 signaling in a macrophage-like cell line (Nan 2009). In human bronchial epithelial cell lines a minimal concentration of 10 µg/ml of Indol may promote cellular recruitment induces IL-8 production. The induction of chemokine expression by Indol is an example of a how cathelicidin can modulate innate and adaptive immune responses by acting as a link between them. There is no direct evidence that Indol plays a role in T cell modulation.

1.5.2.4 Structure and Function of HH2

HH2 is an arginine-rich peptide derived from Bac2a, which is a cathelicidin found in bovine neutrophils. Bac2a is a derivative of bactenecin, which is antimicrobial towards Gram-negative bacteria at concentrations ranging from 2 to 32 g/ml, and Gram-positive bacteria at a concentration of 0.25 to 16 g/ml. At a concentration of 20 µg/ml, Bac2a has been shown to disrupt *S. aureus* and *E. coli* cellular membranes, as assessed by membrane positive-sensitive fluorescent dye (Hilpert 2006). HH2's antimicrobial effect is unknown. Analogs of Bac2a which did not disrupt the bacterial cell membranes to the same extent as the parent compound were shown to have less microbicidal activity suggesting that these activities are linked (Hilpert 2006).

There is evidence that HH2 does play a role in modulating the immune response. Bowdish et al showed that HH2 was more strongly chemotactic for macrophages then were BMAP-27, BMAP-28, and Indol (Bowdish 2005). However, Indol significantly induced the expression of IL-8 in an epithelial cell line, whereas Bac2a did not (Bowdish 2005; Jenssen 2006; Håvard 2007). HH2 is yet to be investigated for T cell modulation.

1.5.3 Effect of Cathelicidins on the Innate and Adaptive Immune System

Cathelicidins released by neutrophils and cells of the mucosal surface at the site of infection play a role in linking the innate and adaptive immune response by inducing macrophages and T cells to release chemokines, which recruit leukocytes to the site of infection (Territo 1989; Chertov 1996; Yang 1999; Jenssen 2006). Cathelicidins recruit phagocytic cells, neutrophils, and monocytes to sites of infection, and LL-37, a human cathelicidin, has been shown to recruit mast cells, DCs, and memory T cells. This increase in cell density at the site of infection promotes leukocyte activation, cytokine release, and DC maturation. For instance when taken up by the monocyte, LL-37 localizes to the nucleus, promotes increased surface expression HLA-DR and the co-stimulatory molecule, CD86 (Bandholtz 2006). DCs that have obtained an antigen at the site of infection migrate from the periphery to regional lymph nodes where they present antigens to naïve T cells (Jenssen 2006). Thus, cathelicidin promotes DC maturation, an innate immune response, which indirectly results in the activation of adaptive immunity. There is direct evidence that LL-37 can promote CD4 effector T cell cytokine expression which leads to phagocyte activation and LL-37 has been shown to promote CD8 effector T cell activation and killing of intracellular pathogens (Goya 1992; Yang 2000; Yang 2001; Yang 2001; Oppenheim 2003). However, the ability to modulate the adaptive immune system varies depending on the host and cathelicidin under investigation.

1.5.4 Effect of Cathelicidins on Resolution of Infection

One role for cathelicidins is the promotion of resolution of infection and restoration of homeostasis after lymphocyte activation. This action is carried out by inducing neutrophils and macrophages to clear the debris caused by pathogens and tissue damage (Martin 2005). Angiogenesis and induction of cell growth are important for promoting a homeostatic environment and these activities have been shown to be induced by LL-37 (Constance 2009; Easton 2009). LL-37 can promote fibroblast cell proliferation (Tomasinsig 2008), keratinocyte migration (Carretero 2007), and to promote wound healing by induction of epithelial cell regeneration (Carretero 2007).

Little is known of how cathelicidins function in anergic cells, however there is evidence HDPs are present during a tolerant state. LL-37 gene expression was up-regulated 4 hours after human colonic epithelial cells (HCEC), which were previously tolerized with flagellin, were re-

challenged with *P. aeruginosa*. Pro-inflammatory cytokine, TNF-α and IL-8, however were not induced (Kumar 2007). This illustrates that HDPs can be triggered independently of cytokine activation and may play a role in tolerization of cells.

Neuropeptides are neuroendocrine mediators expressed by the nervous system which share important characteristics with cathelicidins including size, (<10 kDa), high positive charge, and amphipathic α-helix structures upon interaction with cellular membranes. The positive charge of the neuropeptides interacts with the negative charged leaflet of the cellular outer membrane, thereby inserting itself into the cell (Allaker 2006). Neuropeptide activity is largely anti-inflammatory. They have been shown to decrease macrophage phagocytic activity, free radical production, cellular adherence and migration, and they have been shown to reduce the production of inflammatory cytokines such as TNF-α, IL-12, IL-6, and IL-1β (Chorny and Delgado 2008; Delgado and Ganea 2008). The peptides are released at the peripheral peptidergic endings of sensory and efferent nerves located in close proximity to lymphocytes promoting anti-inflammatory functions. They are also found in immune cells such as neutrophils, macrophages, and mast cells. Neuropeptides such as vasoactive intestinal peptide (VIP), neuropeptide Y (NPY) and npT1 are neuroendocrine mediators are currently being used as therapeutics to treat autoimmune diseases through induction of Treg cell activation and function. Evidence suggests that incubation with neuropeptides with self-reactive effector T cells generates a Treg cell that is dependent on CTLA-4 production as well as IL-10 or TGF-β (Gonzalez-Rey and Delgado 2007; Delgado and Ganea 2008). This was evident in type-1 diabetic mouse model where neuropeptide administration led to increased pancreatic FoxP3 and TGF-β expression (Amelia 2006). Cathelicidins have yet to be studied for inducing anergy and therefore neuropeptides, which share similar structure and function to HDPs, may act as a model in understanding HDPs role in anergy.

2.0 HYPOTHESIS AND OBJECTIVES

Prior to the beginning of this project, microarray analyses were performed on blood-derived PBMCs from adult pigs to identify whether BMAP-27, BMAP-28, and Indol significantly alter the expression profiles of a number of genes whose corresponding proteins play a role in immune regulation. It was observed that one or more of these peptides significantly inhibited the expression of a number of genes involved in T cell activation.

Hypothesis: Bovine and synthetic host defense peptides modulate the activation of porcine PBMCs and T cells by inducing activation-induced anergy.

Objectives: To elucidate whether HDPs have a functional role in regulating T cell activation

Objective 1: Ascertain whether BMAP-27, BMAP-28, Indol, or HH2 influence cell proliferation

Objective 2: Ascertain whether BMAP-27, BMAP-28, Indol, or HH2 promote T cell necrosis or apoptosis

Objective 3: Ascertain whether BMAP-27, BMAP-28, Indol, or HH2 promote T cell anergy

3.0 MATERIALS AND METHODS

3.1 Reagents and Chemicals

All Chemicals reagents required to carry out experiments throughout this thesis are listed in **Table 3.1**.

Table 3.1 List of Selected Chemicals, Reagents and their Suppliers

Chemicals	Supplier and Address
Bromophenol Blue	Sigma-Aldrich Canada Ltd., (Oakville, ON, CAN)
ConA	Sigma-Aldrich Canada Ltd., (Oakville, ON, CAN)
EDTA	Sigma-Aldrich Canada Ltd., (Oakville, ON, CAN)
FACola	General Maintenance Protocols (GMP) (Saskatoon, SK, CAN)
FBS	Invitrogen (Burlington, ON, CAN)
Ficoll	GE Healthcare Life Sciences (Piscataway, NJ, USA)
Formaldehyde	VWR International (Edmonton, AB, CAN)
PBS	Sigma-Aldrich Canada Ltd., (Oakville, ON, CAN)
NaCl	
Na_2HPO_4	
KH_2PO_4	
RPMI	Invitrogen (Burlington, ON, CAN)
Sodium azide	VWR International (Edmonton, AB, CAN)
Trypan Blue	Invitrogen (Burlington, ON, CAN)

Antibodies	**Supplier and Address**
CD3	Becton Dickinson Biosciences (Mississauga, ON, CAN)
CD4	Becton Dickinson Biosciences (Mississauga, ON, CAN)
CD8	Becton Dickinson Biosciences (Mississauga, ON, CAN)
CD152 (CTLA-4)	Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA)
Goat anti-mouse Ig FITC	Becton Dickinson Biosciences (Mississauga, ON, CAN)
Mouse IgG1	Invitrogen (Burlington, ON, CAN)
Mouse IgG2a	Invitrogen (Burlington, ON, CAN)
Mouse IgG2b	Invitrogen (Burlington, ON, CAN)

Antibiotics	**Supplier and Address**
Ampicillin	Sigma-Aldrich Canada Ltd., (Oakville, ON, CAN)

Assay Kits	**Supplier and Address**
Caspase Glo3/7 ™ Assay	Promega Biosciences (San Luis Obispo, CA, USA)
QuantiChrom™ Lactate Dehydrogenase Assay	BioAssay Systems (Hayward, CA, USA)
MTT solution	
NAD solution	
PMS buffer	
Substrate buffer	
CellTitre-Glo ™ Luminescent Cell Viability Assay	Promega Biosciences (San Luis Obispo, CA, USA)

3.2 Peptide Selection Process

Cathelicidins are the most abundant family of HDPs (Zanetti 1995; Boman 1998; Ganz 1999; Lehrer 1999; Ramanathan 2002; Yang 2004). Previously in our laboratory, a microarray analysis was performed to establish whether distinct bovine cathelicidin family members influenced gene expression patterns of porcine leukocytes. The microarray results uncovered that, despite the lack of amino acid sequence homology in the functional domain, BMAP-27, BMAP-28, and Indol inhibited the expression of genes whose corresponding proteins play a role in T cell proliferation**.** Some genes included were part of the MAP kinase signaling pathway, TCR signaling pathway, and IL-2 signaling; NFkB, MAPK, CD3ε, GRB, AKT, IL-2R, JAK, and ICAM. My project was designed to validate the microarray analysis at a functional level to establish whether BMAP-27, BMAP-28 and Indol influence T cell signaling. HH2, a derivative of a bovine peptide Bac2a, was incorporated to compliment BMAP-27, BMAP-28, and Indol.

3.3 Cell Isolation

3.3.1 Peripheral Blood Mononuclear Cell Isolation

PBMC isolation was performed as indicated in (Mena 2003). Briefly, porcine blood was collected from 6-8 week old pigs by venipuncture using an 18 gauge needle attached to 60 ml syringe (Becton Dickinson Biosciences, Mississauga, ON, CAN) from the jugular vein in a final concentration of 0.2 % EDTA (Sigma-Aldrich Canada Ltd., Oakville, ON, CAN). Blood was centrifuged at 1400 g for 20 minutes and the white buffy cell layer was collected and re-suspended in phosphate-buffered saline; PBS (Sigma-Aldrich Canada Ltd.) (0.137 M NaCl, 8mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.3 /0.1 % EDTA). The cells were layered on a 20 % Ficoll Gradient (GE Healthcare Lifesciences, Piscataway, NJ, USA) and centrifuged for 40 minutes at 400 g at 20 ºC. PBMCs were then collected at the interface between Ficoll and PBS and washed two more times in PBS at 300 g for 5 minutes at 4 ºC. A 0.1 mm hemocytometer (Reichert, Buffalo NY, USA) was used to count cells on an Olympus CX41 light microscope (Olympus Canada, Markham, ON, CAN) at 20X magnification. PBMCs were re-suspended in either RPMI (Invitrogen, Burlington, ON, CAN), supplemented with 2 % fetal bovine serum (FBS) (Invitrogen) or 100 µl MACs buffer (Miltenyi Biotec GmbH, Auburn, CA, USA) to a final concentration of $1x10^7$ cells/ml. The percent of T cells in porcine peripheral blood is

approximately 53 %. The percent of B cells and non B or T cells in the porcine peripheral blood is approximately 32 % and 15 %, respectively (Yang and Parkhouse 1996).

3.3.2 Isolation of $CD4^+$, $CD8^+$, and $CD4^+/CD8^+$ T cells

Magnetic labeling was performed according to the protocol provided by the manufacturer (Miltenyi Biotec GmbH, Auburn, CA, USA). PBMCs ($1x10^7$ cells) were incubated with 0.5 mg/ml of anti-CD4 monoclonal antibody (Becton Dickinson Biosciences) and/or 0.5 mg/ml anti-CD8 monoclonal antibody (Becton Dickinson Biosciences) alone or in conjunction for 15 minutes at 4 ºC. Cells were washed in MACs buffer at 300 g for 10 minutes at 4 ºC. A 0.5 mg/ml of goat anti-mouse IgG (Becton Dickinson Biosciences) was added to 100 µl of MACs buffer per $1x10^7$ cells and incubated at 4 ºC for 15 minutes. The cell suspension was twice passed through an equilibrated LS Midi MACS™ Column (Miltenyi Biotech GmbH) and the adherent cell fraction was collected and identified as the $CD4^+$, $CD8^+$, or the $CD4^+/CD8^+$ population, respectively, using flow cytometric analysis as described in section 3.3.3. The cell populations were re-suspended in $1x10^8$ cells/ml of PBS and 0.2 % of Sodium azide (VWR International, Edmonton, AB, CAN) (PBSA).

3.3.3 Flow Cytometry Analysis of Cell Phenotype and Cell Surface Markers

$CD4^+$, $CD8^+$, and, $CD4^+/CD8^+$ cell fractions were incubated with PBSA containing 0.5 mg/ml CD4 mAb (Becton Dickinson Biosciences), CD8 (Becton Dickinson Biosciences) 0.5 mg/ml, CD3 (0.5 mg/ml) (Becton Dickinson Biosciences), and/or CD152 (0.5 mg/ml) (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) monoclonal antibody to identify $CD4^+$, $CD8^+$, or $CD4^+/CD8^+$ cells and/or CD152 surface expression, respectively. Cells were washed at 300 g for 5 minutes at 4 ºC in PBSA and then cells were incubated for 15 minutes at 4 °C with Fluorescein isothiocyanate-(FITC) conjugated isotype-specific goat anti-mouse immunoglobulin (Becton Dickinson Biosciences). Cells were re-suspended in PBSA to a final concentration of $2x10^5$ cells/well and fixed in a 2 % PBS buffered formaldehyde (VWR International, Mississauga, ON, Canada) solution. The purity of $CD4^+$, $CD8^+$, or $CD4^+/CD8^+$ cell fractions were assessed using the Cell Quest™ Program for data analysis and acquisition with a FACSCALIBUR Flow Cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA).

3.3.4 Primary Cell Culture Conditions and Stimulation of Cells

MACS® purified $CD4^+$, $CD8^+$, or $CD4^+/CD8^+$ T cells were incubated overnight in 100 µl of RPMI cell media at a concentration of $2x10^5$ cells per well in 96-well round bottom plates (NALGENE NUNC Thermo Scientific, Rochester, NY, USA). Unless otherwise indicated, cells were incubated at 37 ºC with 20 µg/ml of BMAP-27, BMAP-28, Indol, or HH2 for 4 hrs before 1 µg/ml of ConA was added. Activated lymphocytes undergo changes in the biochemical composition as well as in polarization compared to the cellular membrane of resting lymphocytes, which may effect the interaction of peptide with the cellular membrane (Risso 1998). A concentration of 20 µg/ml peptide is equivalent to physiological concentration of peptides during an inflammatory response (Huang 2006). This concentration is similar to the concentration used by Risso 1998 where in BMAP-27 and BMAP-28 did not induce necrosis on activated lymphocytes. The stimulants were not introduced at the same time to avoid complex formation prior to cellular stimulation. Cells were stimulated for several time points (2 day, 4 day, and 6 days) as indicated in each relevant figure after which cells were washed in FACola (0.1 M PBS, 0.20/0 gelatin, 0.030/0 NaH_3) (GMP, Saskatoon, SK, CAN) and collected at 300 g for 5 minutes. For Figure 4.9 and 4.10, 100 U/ml of IL-2 was added to the cell culture after 3 days of initial stimulation and the cells continued to incubate for 2 more days.

3.3.5 Cellular Proliferation Assays using CFSE-based Flow Cytometry

Following PBMC and MACS®-purified T cells isolation, cells were re-suspended at a concentration of $1.5x10^7$ cells in PBS and then incubated with 10 µM CFSE dye (Invitrogen). The cell/ CFSE dye mixture was incubated for 5 minutes at 20 °C prior to stimulation with ConA and HDPs for 2 days, 4 days, or 6 days as indicated in section 3.3.4. At the end of the culture period, cells were washed at 300 g for 5 minutes at 4 °C with FACola and fixed in 3.7% formaldehyde VWR International (Edmonton, AB, CAN) (37% formalin, 0.01 M PBSA) for 15 minutes. Cells were analyzed using CellQuest™ on the FACSCALIBUR™ Flow cytometer (Becton Dickinson Immunocytometry Systems).

3.4 Cell Death Assays

3.4.1 Trypan Blue Assay

A Trypan Blue Assay was used to determine whether cellular stimulation induced cellular cyotoxicity. For these experiments, porcine PBMCs and MACS®-purified $CD4^+$/$CD8^+$ T cells were stimulated as previously described in section 3.3.4 and incubated at 37 ºC for 4 days before incubation with 0.025 % Trypan Blue (Invitrogen, Burlington, ON, CAN) using a hemotocrit and light microscopy 20x (Olympus Canada). The number of dead and live cells was tabulated.

3.4.2 Caspase-Glo3/7 ™ Assay

These experiments were performed using the protocol provided by Promega Biosciences (San Luis Obispo, CA, USA). Equal portions of Caspase-Glo™ Buffer and Caspase-Glo™ Substrate (Promega Biosciences) were combined immediately prior to experimentation. Caspase-Glo™ solution was added to the samples in a 1:1 ratio of reagent to culture medium. Cells were plated at $2x10^5$ cells/well in 96 well plates (NALGENE NUNC Thermo Scientific). Cells were incubated for 1 hr with limited exposure to light. Cells were analyzed on the TD21 Luminometer (Turner Designs, Sunnyvale, CA, USA) which measured luminescence at 420 nm.

3.4.3 Lactate Dehydrogenase Assay

QuantiChrom™ Lactate Dehydrogenase Kit was performed according to the manufacturer's instructions (BioAssay Systems, Hayward, CA, USA). The Lactate Dehydrogenase Assay was performed to determine whether cellular stimulation with HDP and ConA-induced cell lysis. MACS® isolated T cells and PBMCs were stimulated as previously described in section 3.3.4 for 4 days. Cells were incubated with LDH buffer at a final cell concentration of $1x10^5$ cell/well on day 4. The cell mixture was incubated for 5 minutes at 20 ºC on day 4 and subjected to spectrophotometric analysis at 565 nm.

3.5 Anergy Assays

3.5.1 CellTiter-Glo™ Luminescent ATP Consumption Assay

CellTiter-Glo™ Luminescent ATP Consumption Assay was performed according to the manufacturer's instructions (Promega Biosciences (San Luis Obispo, CA, USA). MACS® isolated T cells were stimulated for 4 days, as previously described in section 3.3.4. On day 4

CellTitre-Glo™ buffered substrate was added to an equal volume of $2x10^5$ cells/well. The mixture was incubated for 10 minutes with limited exposure to light at 20°C. The luminescent signal was detected using the TD21 Luminometer (Turner Designs) at 420 nm.

3.6 Statistical Analysis

All statistical analyses and graphing were performed using GraphPad Prism™ 5 software (GraphPad Software, San Diego, CA). Outcome variables were found to be distributed normally. Differences among groups were examined by using a 1-way ANOVA where means were compared using Tukey's Multiple Comparison Test and Bartletts Test ($p>0.05$). Differences were considered significant if $p<0.05$.

4.0 RESULTS

4.1 Effects of HDPs on Cellular Proliferation

4.1.1 CFSE Cell Proliferation Assays

To determine whether HDPs influence lymphocyte proliferation, PBMCs were isolated from porcine blood and stimulated with four distinct host defense peptides: BMAP-27, BMAP-28, Indol, and HH2. The mitogen ConA was introduced 4 hrs later to activate the lymphocytes. It was not added at the same time as the peptides to ensure that HDPs and ConA did not form a complex. All peptide stimulations used 20 µg/ml of peptide for $2x10^5$ cells per well. CFSE staining was introduced to monitor changes in cell proliferation over time. The cellular proliferation was arrested on day 6 of peptide stimulation. PBMCs stimulated with BMAP-27 ($p<0.01$), BMAP-28 ($p<0.01$), or Indol ($p<0.05$) in the presence of ConA showed a significant decrease in cell proliferation compared to PBMCs stimulated with ConA alone (Figure 4.1). HH2 did not inhibit ConA-stimulated lymphocyte proliferation.

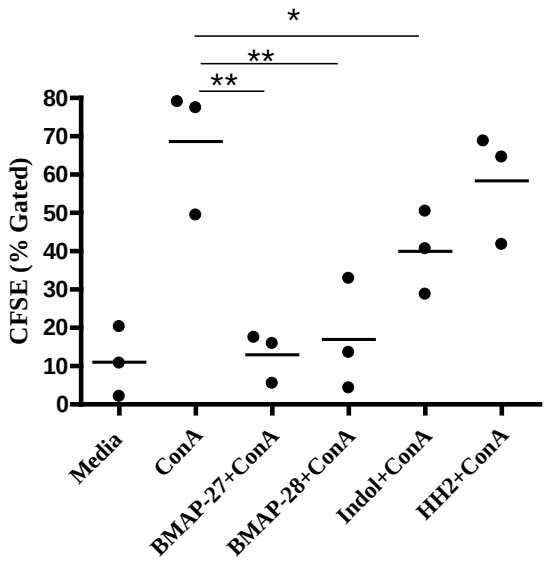
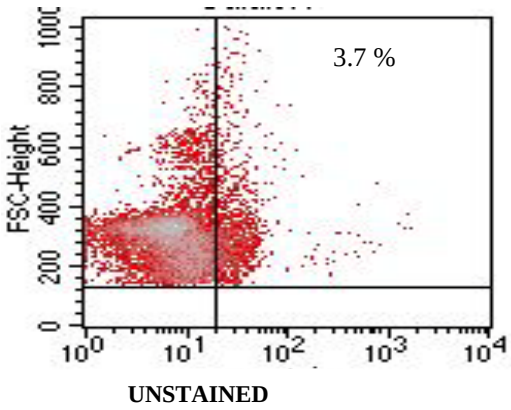


Figure 4.1: ConA-Stimulated Porcine PBMC Proliferation is Inhibited by BMAP-27, BMAP-28, and Indol. Porcine PBMCs (n=3) were cultured in $2x10^5$ cells/well with media alone, ConA, or ConA plus BMAP-27, BMAP-28, Indol or HH2. Cells were labeled with CFSE and incubated with and without stimulants for 6 days prior to flow cytometric analysis. The percent gated refers to the percent of total viable cells gated within the cell population. The horizontal bar represents the mean values for 3 biological replicates of PBMCs. A 1-way ANOVA and Tukey's Multiple Comparison Test indicated that there were statistically significant decreases in proliferating cells stimulated with BMAP-27, BMAP-28, and Indol compared to PBMCs stimulated with ConA alone (*$p<0.05$) (**$p<0.01$).

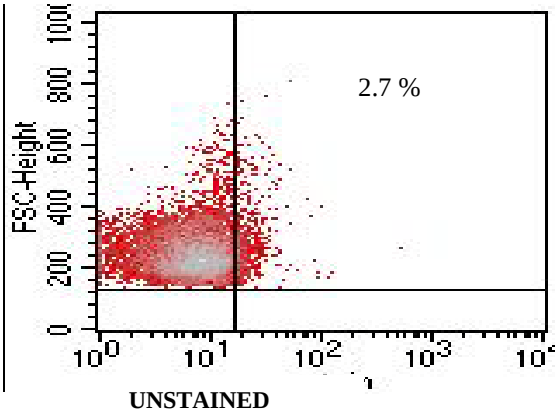
4.1.2 FACs Analyses of Isolated Porcine Lymphocytes

T cells were isolated by MACS purification technique using CD4 and CD8 microbeads that bind to T cells expressing $CD4^+$ and $CD8^+$ surface proteins, respectively. Purified T cells were subjected to FACs analysis to ensure that contaminating cell types from the PBMC population were not present (Figure 4.2). The purity of the $CD4^+$ and $CD8^+$ T cell population was 95.7% and 96.5%, respectively. Because a subpopulation of APCs can also express CD4, the T cell population was counterstained with CD3 and it was observed that 98.1 % of the cells were CD3 positive. The viability of the cells was 94.7% based on Trypan blue cell death analysis.

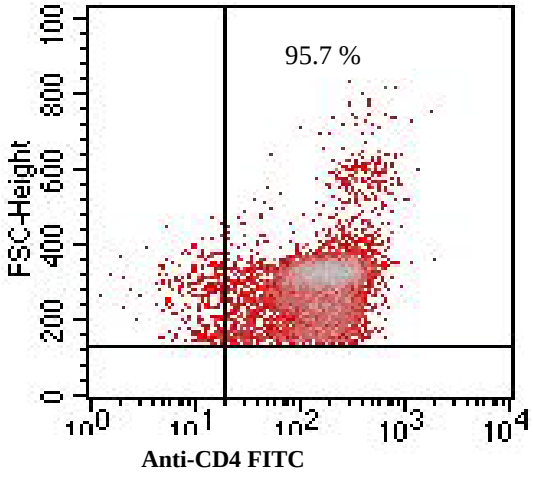
A) CD4 T cells unstained



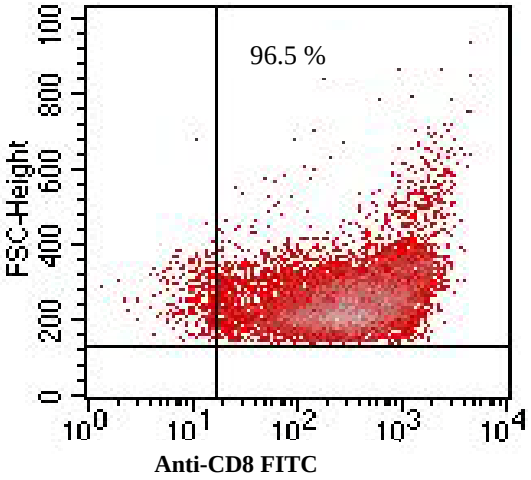
B) CD8 T cells unstained



C) CD4 T cells fluorescently labeled with Anti-CD4 FITC



D) CD8 T Cells fluorescently labeled with Anti-CD8 FITC



E) CD4/CD8 T Cells fluorescently labeled with Anti-CD3 FITC

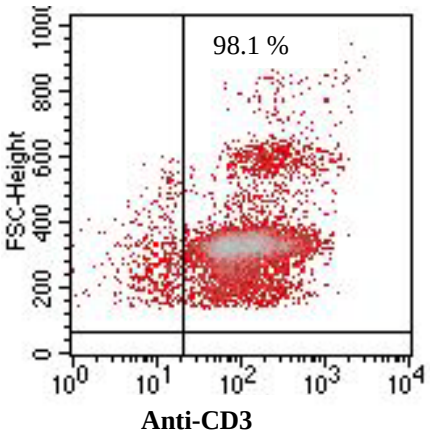
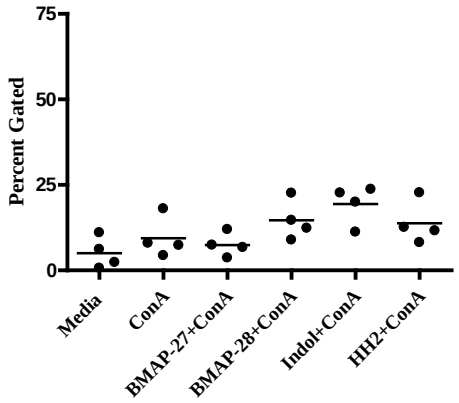
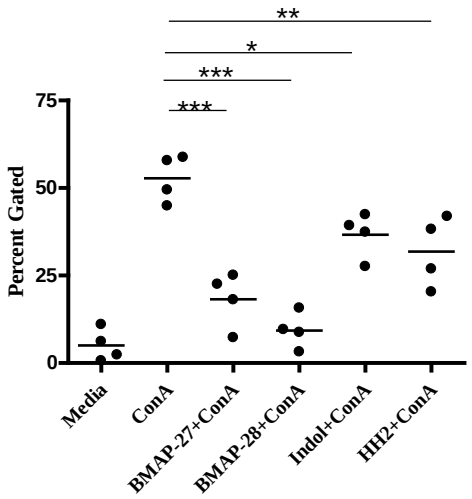


Figure 4.2 Representative FACs Analysis of Purified CD4 and CD8 T cells. A) $CD4^+$ (unstained) of B) $CD8^+$ (unstained) C) $CD4^+$ of (Anti-CD4 FITC) of D) $CD8^+$ (Anti-CD8 FITC) and of E) $CD4^+/CD8^+$ (Anti-CD3 FITC) porcine lymphocytes. Isolated T cells were stained with anti-CD4 FITC or anti-CD8 FITC and analyzed by flow cytometry. C) The sample purity of CD4 T cells was 95.7 %. D) The sample purity of CD8 T cells was 96.5 %. E) The sample purity of CD3 T cells was 98.1 %.

DAY 2



DAY 4



DAY 6

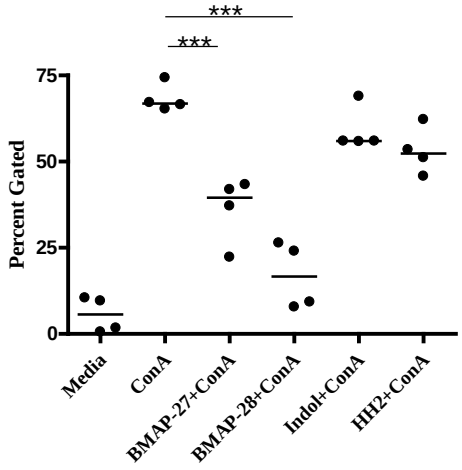


Figure 4.3: ConA-Stimulated Porcine T Cells Incubated With BMAP-27 and BMAP-28 Show Decreased Cellular Proliferation MACs-purified $CD4^+/CD8^+$ T cells were cultured in $2x10^5$ cells/well with media alone, ConA, or ConA plus BMAP-27, BMAP-28, Indol or HH2. Cells were labeled with CFSE and incubated with and without stimulants for 2 days (Fig 4.3A), 4 days (Fig 4.3.B) and 6 days (Fig 4.3.C) prior to flow cytometric analysis. Percent gated refers to the percent of total viable cells gated within the cell population. The horizontal bar represents the mean values for 4 individual animals (PBMCs). (*$p<0.05$) (**$p<0.01$) (***$p<0.001$)

Results from Figure 4.1 indicated that HDPs inhibited proliferation within a mixed-cell population. To ascertain whether this population was specific to T lymphocytes, porcine $CD4^+/CD8^+$ T cells were purified from the PBMC population and stained with CFSE in the presence or absence of ConA and HDPs (Figure 4.3). T cells were incubated with HDPs and activated by ConA 4 hrs later. After 2 days (Figure 4.3A), little difference was observed between ConA-stimulated T cell proliferation in the presence or absence of HDPs. BMAP-27, BMAP-28, Indol, and HH2 significantly inhibited ConA-stimulated T cell proliferation after 4 days, (***$p<0.001$; **$p<0.01$; *$p<0.05$) as indicated in Figure 4.3B. After 6 days, only ConA-stimulated T cells incubated with BMAP-27 or BMAP-28 (***$p<0.001$) showed significant inhibition of T cell proliferation relative to T cells stimulated with ConA alone (Figure 4.3C).

4.2 Effect of HDPs on Cell Death

BMAP-27 and BMAP-28 have potent antimicrobial activities, which they partially mediate through disruption of the bacterial cell membrane and thus it was predicted they may also be cytotoxic to the host cells (Brogden 2007). To ensure that the observed inhibition of activated T cell proliferation was not an artifact of increased cell death, three distinct cell death assays were performed.

4.2.1 Lactate Dehydrogenase Assay

An LDH assay was performed to determine if ConA-stimulated T cells co-incubated with HDPs were undergoing necrosis. LDH assay is a non radioactive colorimetric assay that measures the amount of LDH in the supernatant after the cells have been lysed. MACS-purified $CD4^+/CD8^+$ T cells were stimulated with 20 μg/ml of BMAP-27, BMAP-28, Indol, or HH2 followed 4 hrs later with 1 μg/ml of ConA. Four days post-stimulation, no significant difference in LDH activity was observed between ConA-stimulated $CD4^+/CD8^+$ T cells co-cultured alone or in the presence of HDPs (Figure 4.4). Therefore, any HDP-specific decrease in cellular proliferation observed in Figure 4.3 cannot be attributed to induction of cellular necrosis.

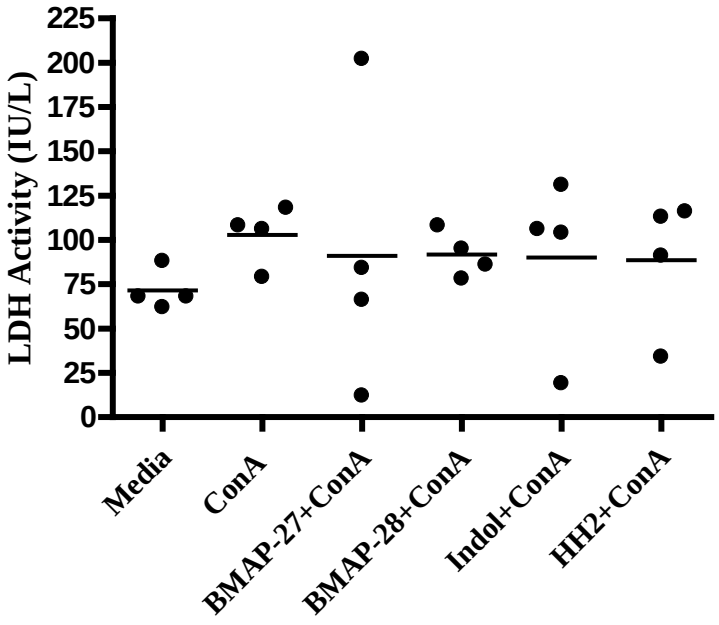


Figure 4.4: ConA-Stimulated Porcine T Cells Incubated With BMAP-27, BMAP-28, Indol, or HH2 Do Not Undergo Necrosis. LDH assays were used to measure the reduction of tetrazolium salt (MTT) to a colored state at 565 nm. Ten µl of supernatants generated from T cells (stimulated with 20 µg/ml of HDP and 1 µg/ml of ConA 4 hrs later) was added to the colourmetric reagent and measured at 565 nm. The assay was performed 4 days after stimulation. Each data point represents an individual animal. A 1-way ANOVA and Tukey's Multiple Comparison Test indicated that there was no statistically significant difference in levels of LDH in the supernatants between the groups. The bars represent the mean difference between 4 biological replicates in each group.

4.2.2 Trypan Blue Assay

Living cells are very selective in what they transport across their plasma membrane. Trypan blue stains cells undergoing apoptosis and is excluded from live cells (Strober 2001). Thus, use of a Trypan Blue Assay allows for a simple and effective method to identify apoptotic, blue cells from live, uncoloured cells. To determine if HDPs induced T cell apoptosis, $CD4^+/CD8^+$ T cells were stimulated with HDPs (20 µg/ml) for 4 hrs and then stimulated with 1 µg/ml of ConA. Cells were incubated at 37ºC for 4 days then subjected to Trypan blue analysis. It was observed that T cells stimulated with ConA and BMAP-28 ($p<0.01$) and ConA and BMAP-27 ($p<0.05$) showed a significant number of cells positive for Trypan blue compared to the other stimulated cells. However, there was no significant increase in cells staining positive for Trypan blue for Indol and HH2 ConA-stimulated cells, suggesting that these peptides do not promote T cell apoptosis **(**Figure 4.6**)**. Despite the fact that almost 40% of the cells stimulated in the presence of BMAP-27 or BMAP-28 underwent cell apoptosis, the remaining 60% of cells remained alive and may still influence T cell proliferation (Figure 4.6).

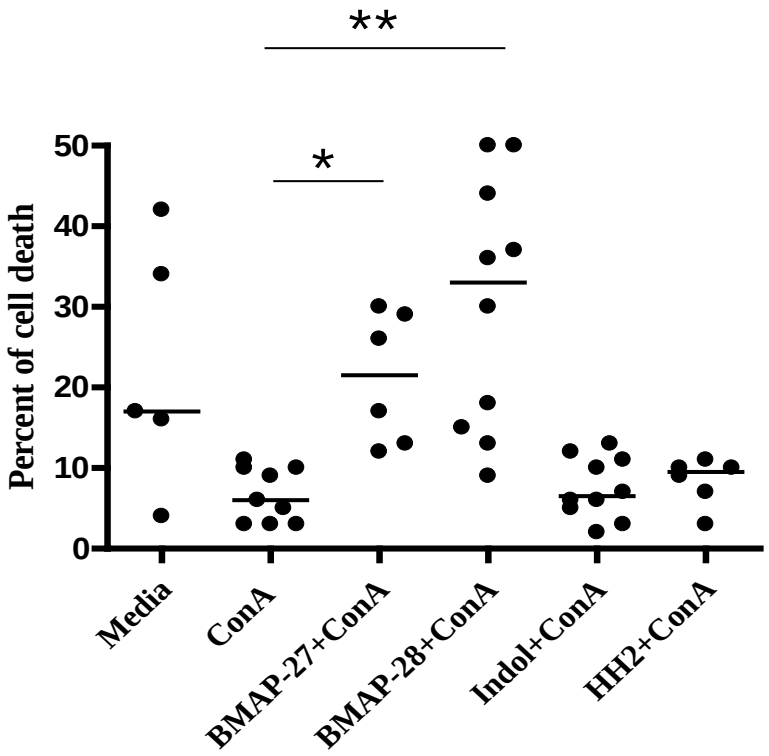


Figure 4.5: BMAP-27 and BMAP-28 induce ConA-stimulated T cells to undergo apoptosis. T cells were stimulated with 20 µg/ml of HH2, BMAP-27, BMAP-28, or Indol for 4 hrs before being stimulated with 1 µg/ml ConA. The cells were subjected to Trypan Blue Assay after 6 days. The percent of cell death was determined by the absolute number of cells originally plated ($2x10^5$ cells per well) divided by the number of cells after day 4. Each data point represents an individual animal, and mean values are indicated by horizontal bars. A 1-way ANOVA and Tukey's Multiple Comparison Test indicated that there was statistically significant differences in apoptosis (*$p<0.05$) (**$p<0.01$).

4.2.3 Caspase Glow Assay

Caspase Glow 3/7 is an assay that measures the enzyme activity of Caspase 3 and 7, which are proteins induced in apoptotic cells (Hetz 2002). Porcine $CD4^+/CD8^+$ T cells were stimulated with 20 µg/ml of each peptide as indicated, followed by stimulating with 1 µg/ml of ConA, 4 hrs later. The caspase enzyme activity was measured on day 4. Briefly, a luminogenic substrate was added to the cells in a 1:1 ratio and incubated for 2 hrs. The unstimulated cells (Media) showed very little Caspase 3/7 activity yet there was significant induction of Caspase 3/7 activity in ConA-stimulated cells regardless of the presence or absence of HDPs (Figure 4.5A). If this assay is used to measure apoptosis and yet ConA-stimulated T cells are highly proliferative, then these results appear to be contradictory. A more detailed search of the literature revealed that Caspase 3 and 7 may in fact be induced in proliferating T cells (Lakhani 2006).

Figure 4.5 indicates that approximately 40 % of cells treated with BMAP-27 and BMAP-28 were not viable. Further, we would predict that cells treated with ConA alone would undergo proliferation and thus their cell numbers would increase over time. If we re-examine the Caspase 3/7 activity taking into account projected changes in cell number across treatment groups, the ConA-stimulated T cells treated with BMAP-27 and BMAP-28 showed modestly higher Capase 3/7 activity compared to ConA-stimulated T cells.

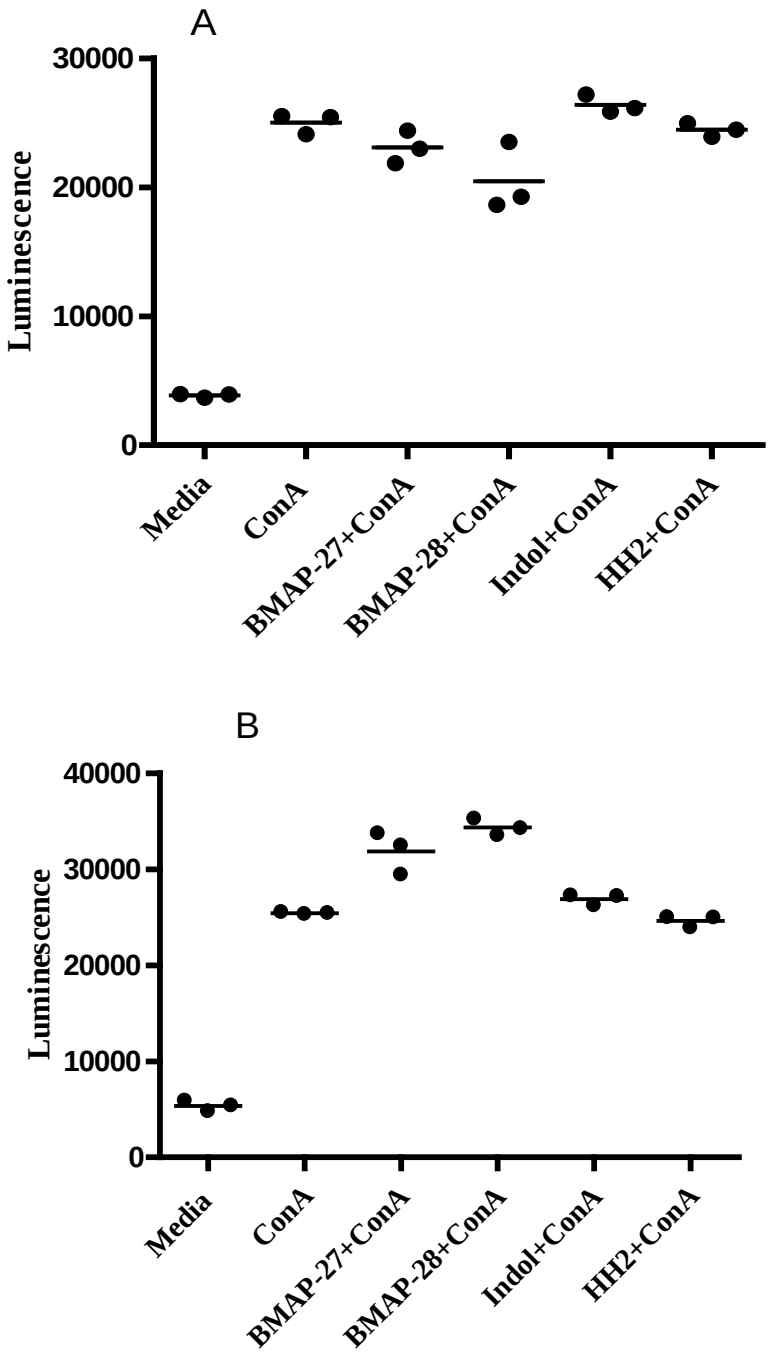


Figure 4.6: ConA-Stimulated Porcine T Cells Incubated With BMAP-27, BMAP-28, Indol, or HH2 Show Caspase 3/7 Activity. Caspase-Glo 3/7 Assay analysis was used to measure the activity of caspase-3 and caspase-7 using a luminogenic substrate. A 1:1 ratio of reagent to medium culture was added to unstimulated T cells, T cells stimulated with ConA alone or cells stimulated with the indicated HDP before the addition of ConA. The wells were measured in a

luminometer after 4 days. Each data point represents an individual animal. A 1-way ANOVA and Tukey's Multiple Comparison Test indicated that there were no statistically significant differences in Caspase-Glo 3/7 levels. The bar represents the mean difference between 3 biological replicates in each group. B) ConA-Stimulated Porcine T Cells Incubated With BMAP-27, BMAP-28, Indol, or HH2 Show Caspase 3/7 Activity with corrected cell count. Cells stimulated with BMAP-27 and BMAP-28 experienced approximately 25% and 35% cell death (Figure 4.6). Cell stimulated with ConA, Indol, HH2 or media underwent approximately 5%, 5%, 10% and 20% cell death, respectively. When the cell counts were normalized and the results from the Caspase Glo assay was reevaluated using these numbers, we observed that BMAP-27 and BMAP-28 stimulated with ConA-activated T cells underwent a modest induction of Capase activity.

4.3 Effects of HDPs on T Cell Anergy

4.3.1 CellTiter-Glo Luminescent ATP Consumption Assay

To determine if BMAP-27 or BMAP-28 influenced the cellular activity of surviving T cells, ATP consumption was measured. ConA-stimulated T cells incubated with or without HDPs for 4 days were than incubated for 5 hrs with a reagent that becomes luminescent if ATP is consumed. In an active cell ATP is present and will react with luciferin to produce light at 420 nm**.** In a relatively inactive cell, little ATP is present and less light is produced, thus ATP in a cell can be quantified. Results from this assay indicate that $CD4^+/CD8^+$ T cells stimulated with ConA and BMAP-27 ($p<0.01$) or BMAP-28 ($p<0.001$) for 4 days consumed less ATP per cell compared to cells stimulated with ConA alone (Figure 4.7). Therefore, we hypothesize that ConA-stimulated T cells show decreased proliferation in the presence of BMAP-27 and BMAP-28 because they have entered into a quiescent or anergic state. ConA-stimulated $CD4^+/CD8^+$ T cells incubated with HH2 and Indol showed comparable consumption of ATP with T cells stimulated with ConA alone.

4.3.2 Induced Surface CTLA-4 Levels are Markers for Anergy

Increased surface expression of CTLA-4 is a marker for T cells which have entered into a state of anergy. This protein competes with CD28 to bind CD80/86 on APCs and thus prevents T cell activation. MACS-purification was employed to isolate $CD4^+/CD8^+$ T cells from porcine blood, as well as $CD4^+$ T cell and $CD8^+$ T cell subpopulations. Each population was stimulated with 20 μg/ml of BMAP-27, BMAP-28, Indol, or HH2 for 4 hrs prior to the addition of 1 μg/ml of ConA.

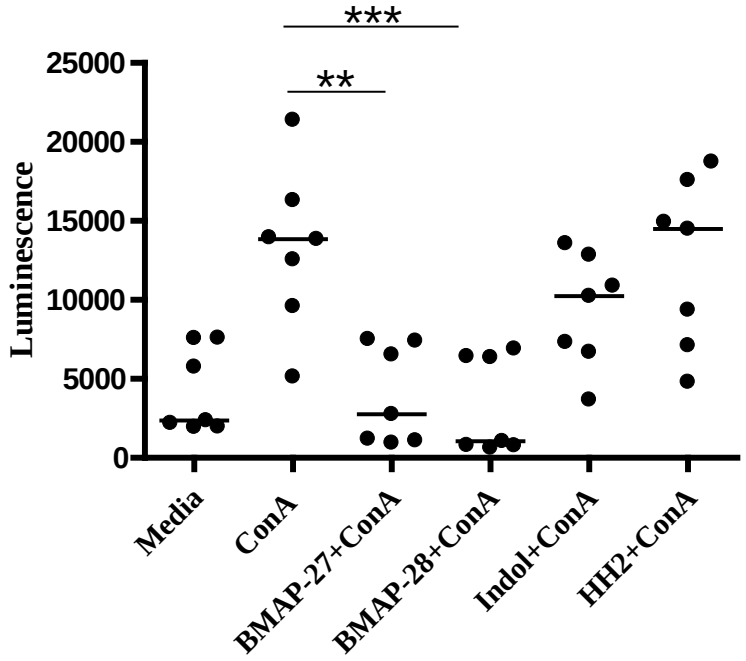


Figure 4.7: ConA-Stimulated Porcine T Cells Incubated With BMAP-27 and BMAP-28 Consume Relatively Little ATP compared to Con alone stimulated T cells. ATP consumption was determined by CellTiter-Glo Luminescent Cell Viability Assay for 7 biological replicates. Cells were stimulated for either 4 days with 1 μg/ml of ConA and 20 μg/ml of BMAP-27, BMAP-28, Indol, or HH2. Each data point represents an individual animal, and mean values are indicated by horizontal bars. A 1-way ANOVA and Tukey's Multiple Comparison Test indicated that there was statistically significant differences in ATP consumption (**$p < 0.01$) (***$p<0.001$).

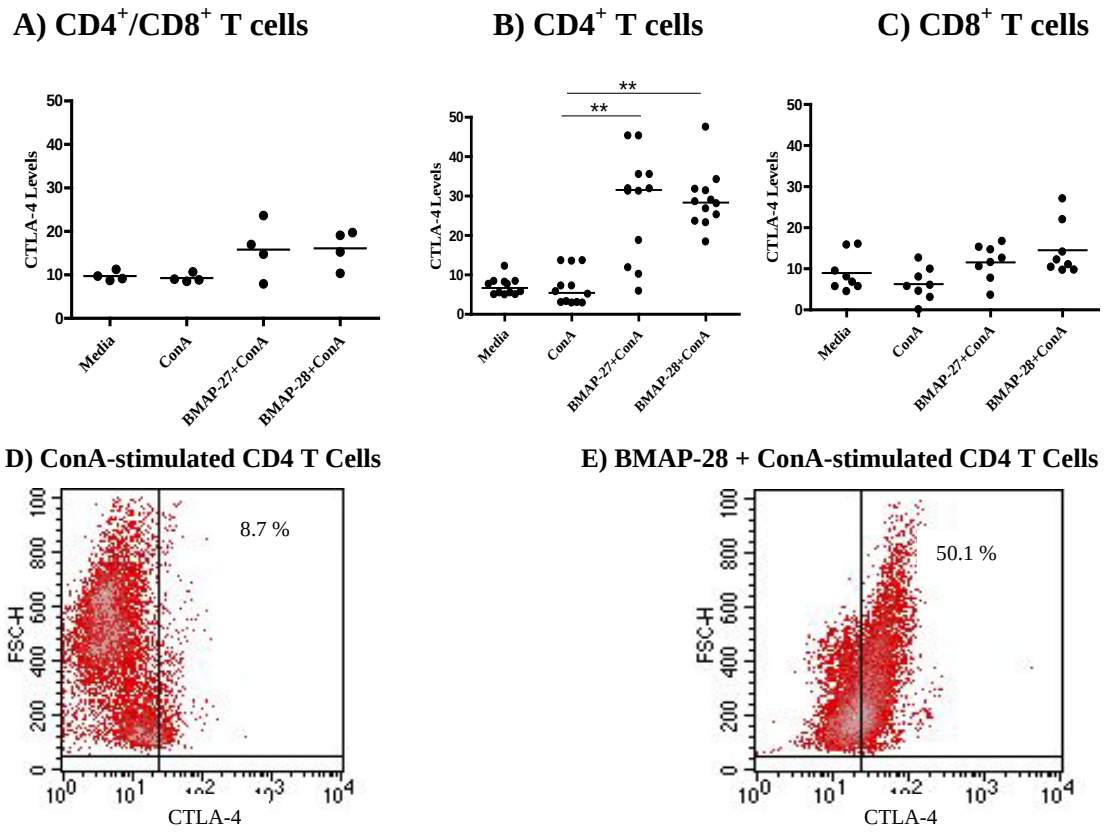


Figure 4.8: ConA-Stimulated Porcine T Cells Incubated With BMAP-27 and BMAP-28 Show Induced Surface Expression of CTLA-4. T cells were stimulated with 20 μg/ml of either, BMAP-27, or BMAP-28 followed by 1 μg/ml of ConA 4 hrs later. The stimulations were arrested on Day 4 and 5 μg/ml of CD152 monoclonal antibody was added to the wells. Cells were fixed in 3.7% formaldehyde. Graph A represents porcine $CD4^+/CD8^+$ MACs isolated T cells. Graph B represents $CD4^+$ MACs isolated T cells. Graph C represents $CD8^+$ MACs isolated T cells. D) $CD4^+$ T cells stimulated with ConA and stained for CTLA-4 expression illustrates that 8.7 % of the $CD4^+$ T cells isolated express CTLA-4. E) $CD4^+$ T cells stimulated with BMAP-28 and ConA 4 hrs later express 50.1 % CTLA-4 after 4 days. Each bar represents the mean and each data point represents an individual animal. A 1-way ANOVA and Tukey's Multiple Comparison Test indicated that there are statistically significant differences in $CD4^+$ T cell CTLA-4 levels. (**$p < 0.01$).

In Figure 4.8A, we observed no significant increase in CTLA-4 expression in ConA-stimulated $CD4^+/CD8^+$ T cells among treatment groups. However, as indicated in Figure 4.8B, ConA-stimulated $CD4^+$ T cells incubated with BMAP-27 or BMAP-28 showed significant increase ($p<0.01$) in CTLA-4 expression after 4 days compared to T cells stimulated with ConA alone, which suggested that these $CD4^+$ T cells have entered into a state of quiescence. No significant increase in CTLA-4 expression was evident in $CD8^+$ T cell populations regardless of the stimulants under investigation. Please note, HH2 and Indol were not included in these experiments since previous proliferation experiments indicated that they did not promote T cell proliferation (Figure 4.3) nor did they augment T cell senescence (Figure 4.7).

4.3.3 Effect of IL-2 on HDP-Induced T Cell Anergy

Because these T cells are mitogen-activated and then underwent decreased cellular proliferation in the presence of BMAP-27 and BMAP-28, it was predicted that the surviving T cells are undergoing activation-induced anergy. Because this form of anergy can be reversed in the presence of IL-2 (Beverly 1992), the stimulations were repeated as indicated in Figure 4.3.2, with the exception that 5 ng/ml of recombinant porcine IL-2 was added at day 3 of culture. As expected, ConA-stimulated T cells cultured with 20 µg/ml of BMAP-27 or BMAP-28 underwent significantly less cellular proliferation compared to T cells stimulated with ConA alone. However, upon addition of IL-2, a significant portion of $CD4^+/CD8^+$ T cells stimulated with ConA+BMAP-28 ($p<0.03$) showed a significant increase in cellular proliferation (Figure 4.9A). When evaluating the effect of IL-2 on $CD4^+$ or $CD8^+$ T cell proliferation, it was observed that ConA-stimulated $CD4^+$ or $CD8^+$ T cells incubated with BMAP-28 showed no statistical significance. The presence of IL-2 on BMAP-27 and ConA-stimulated $CD4^+$ or $CD8^+$ T cells did not appear to result in increased cellular proliferation (Figure 4.9B and 4.9C).

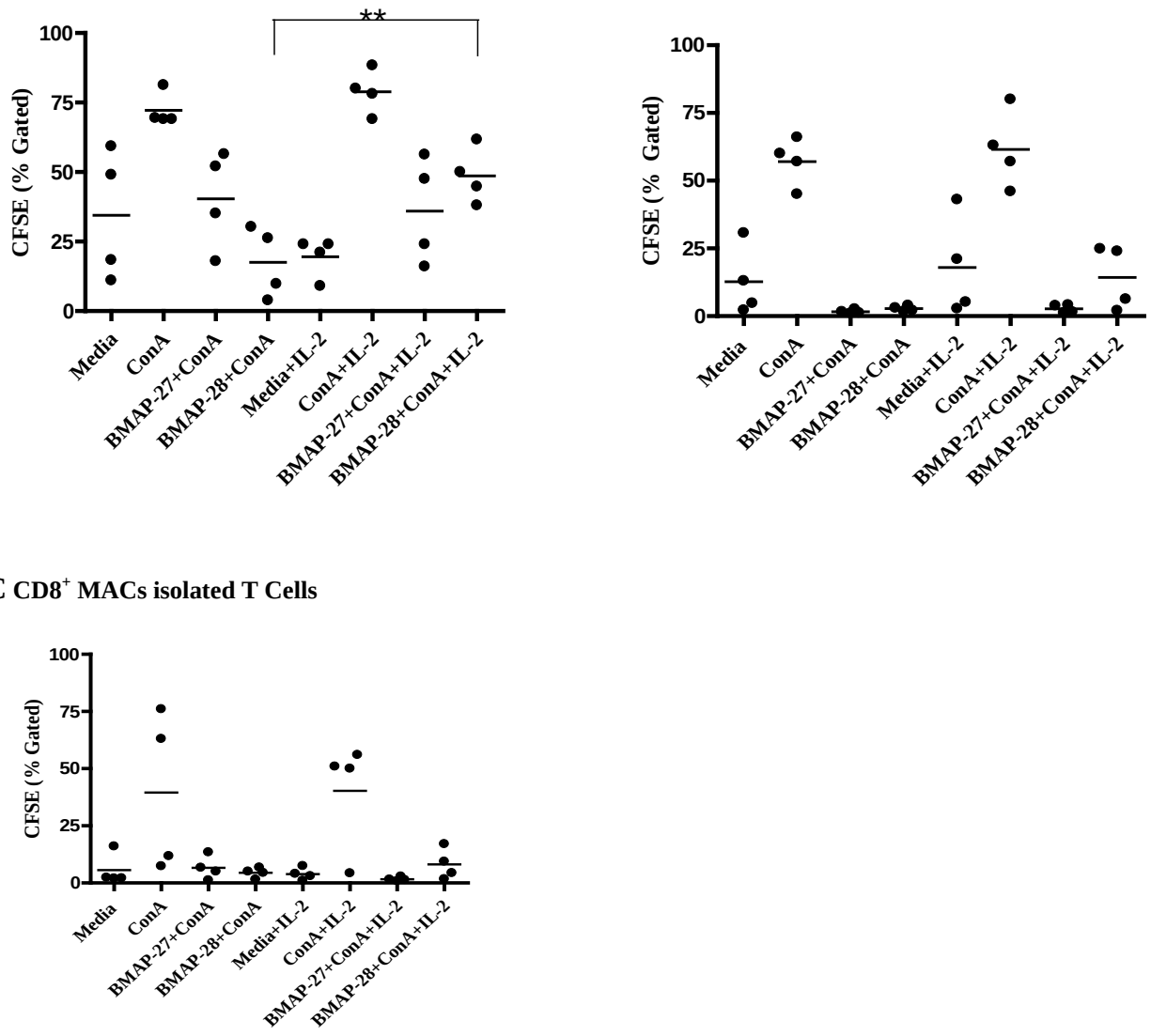


Figure 4.9: ConA-Stimulated Porcine T Cells Incubated With BMAP-27 and BMAP-28 Show Renewed Cellular Proliferation After the Addition of IL-2. $CD4^+CD8^+$ T cells (A) or $CD4^+$ T cells (B), or $CD8^+$ T cells (C) were stimulated with 20 μg/ml of BMAP-27, or BMAP-28 followed by 1 μg/ml of ConA 4 hrs later. The stimulations were stopped on Day 3 and washed. The cells were then incubated with IL-2 for another 3 days. The percent gated refers to the percent of total living viable cells gated in a population. The horizontal bars represent the groups' mean values that are being compared. BMAP-28+ConA before and after addition of IL-2. Each data point represents an individual animal. A 1-way ANOVA and Tukey's Multiple Comparison Test indicated that there are statistically significant differences in CFSE % Gated $CD4^+CD8^+$ T cells. (**P<0.01)

If addition of exogenous IL-2 reverses activation-induced anergy, it was predicted that there would be a decrease in CTLA-4 expression on the surface of T cells. Using the same conditions as above, $CD4^+$ and $CD8^+$ T cells were stimulated with 20 μg/ml of BMAP-27 or BMAP-28 and 4 hrs later 1 μg/ml of ConA was added as illustrated in Figure 4.9A. FACS analysis indicated that $CD4^+$ T cells co-stimulated with ConA and BMAP-27 or BMAP-28 showed a significant decrease in CTLA-4 surface expression upon addition of IL-2 in Figure 4.10. In Figure 4.8C, it was observed that CTLA-4 surface expression was not induced in $CD8^+$ T cells in the presence of BMAP-27 or BMAP-28. Re-stimulation of these cells with IL-2 did not, therefore, show any difference in CTLA-4 surface expression within the $CD8^+$ T cell population.

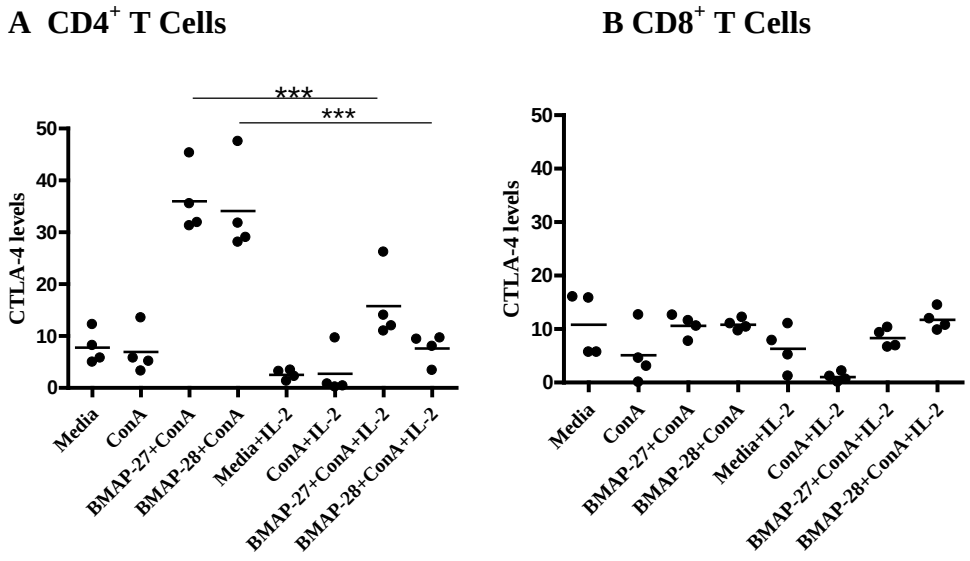


Figure 4.10: ConA-Stimulated CD4$^+$ T Cells Incubated With BMAP-27 and BMAP-28 Show Decreased CTLA-4 Expression After the Addition of IL-2. CD4$^+$ T cells and CD8$^+$ T cells were stimulated with 20 μg/ml of either, BMAP-27, or BMAP-28 followed by 1 μg/ml of ConA 4 hrs later. The stimulations were stopped on Day 3 and washed. The cells were then incubated with IL-2 for another 3 days. The stimulations were stopped on Day 6 and 5 μg/ml of CD152 (CTLA-4) monoclonal antibody was added to the wells. Cells were fixed in 3.7% formaldehyde. The percent gated refers to the percent of total living viable cells gated in a population. Graph A represents CD4$^+$ MACs isolated T cells. Graph B represents CD8$^+$ MACs isolated T cells. Each data point represents an individual animal, and mean values (4 biological replicates per treatment group) are indicated by horizontal bars. A 1-way ANOVA and Tukey's Multiple Comparison Test indicated that there are statistically significant differences in CD4$^+$ T cell CTLA-4 levels. (***$p < 0.001$)

5.0 DISCUSSION

5.1 The Structure and Charge of HDPs May Contribute to Their Diversity of Function

To our knowledge, there is no evidence that BMAP-27, BMAP-28, Indol, and HH2 interact with cell membrane receptors, and yet their ability to modulate eukaryotic cell function is evident (Risso 2002). Thus, peptides may intercalate into eukaryotic cells, not through receptor-mediated endocytosis, but through a local disruption of the cellular membrane. Structure and charge of HDPs influence cellular association and permeabilization. BMAP-27 and BMAP-28 have an α-helical secondary structure and these peptides inhibited ConA-stimulated T cell proliferation. Indol and HH2 are relatively short peptides with no secondary structure and they did not inhibit ConA-stimulated T cell proliferation (Sitaram and Nagaraj 1999; Risso 2002). Thus, an α-helical secondary structure may be required for HDP-specific T cell inhibition of proliferation. This prediction is somewhat supported by studies by Oreopoulos and Yip who showed that if Indol was altered to adopt an α-helical structure, its binding affinity for cellular membranes increased, which resulted in increased cellular polarization and entry of Indol into the cell (Oreopoulos and Yip 2009).

Although Indol failed to inhibit ConA-stimulated T cell proliferation, it did inhibit PBMC proliferation and thus, may be acting on B lymphocytes or myeloid cells. There is evidence that Indol interacts with macrophages to inhibit iNOS expression, thereby contributing to down regulation of the pro-inflammatory response (Yong Hai 2009). Indol inhibits TLR-4 signaling to NFκB, which is central in coordinating the expression of inflammatory cytokines (Mookherjee 2006). Therefore, HDPs secondary structure may be indicative of cell type interaction and function.

5.2 Effect of BMAP-27 and BMAP-28 on Peripheral Tolerance

Peripheral tolerance is coordinated by regulation of apoptosis, activation-induced anergy, adaptive tolerance, and the activation of Treg cells. Induction of peripheral tolerance is a critical mechanism whereby the immune system returns to a homeostatic state. The complexity of regulation of peripheral tolerance is highlighted when one considers that one or more activity may be occurring simultaneously. The results presented in this thesis demonstrate that ConA-activated T cells stimulated with BMAP-27 and BMAP-28 triggered induction of T cell apoptosis in a portion of the cells and induction of activation-induced anergy in the remainder of the cells.

The dual induction of both apoptosis and activation-induced anergy is not uncommon. For instance, after murine $CD8^+$ T cells were incubated with H-Y-soluble peptide for 24 hours, it was determined that 40 % of the cells underwent apoptosis, while the remaining 60 % entered into a state of anergy which was reversed upon the addition of IL-2 (Chai 1998). We speculate that BMAP-27 and BMAP-28 play a role in returning activated T cells to homeostasis through the coordinated regulation of apoptosis and activation-induced anergy based on several lines of evidence.

Firstly, ConA-stimulated porcine $CD4^+/CD8^+$ T cells incubated with BMAP-27 or BMAP-28 showed significantly decreased cell proliferation compared to T cells stimulated with ConA alone or in the presence of Indol or HH2. By measuring LDH levels in the supernatant, it was clarified that reduced T cell proliferation was not due to induction of cellular necrosis.

Secondly, results from Trypan Blue Assay analysis indicated that ConA-activated T cells stimulated with BMAP-27 or BMAP-28 underwent cellular apoptosis. These findings are in agreement with Risso et al 1998 who observed that BMAP-27 and BMAP-28 induced activated lymphocytes and epithelial cells to undergo apoptosis, not necrosis (Risso 1998).

The Trypan Blue Assay is advantageous method for determining apoptosis because it is inexpensive and easy to use. The Trypan Blue Assay underestimates the amount of apoptosis since the trypan blue cannot pass the cellular membrane during early stages of apoptosis. This confirms that membrane integrity is maintained throughout the course of apoptosis. The loss of membrane integrity is usually only seen at the late stages of apoptosis. This is advantageous for our work since we measured apoptosis on day 4 of stimulation. Since the sensitivity of the Trypan Blue Assay is less then other apoptosis assays including Annex, TUNEL, Light Scatter, and Fluorescent microscopy, it may be important to verify the percentage of apoptosis determined by the Trypan Blue Assay with another more sensitive apoptosis assay. The Trypan Blue Assay is used as a measurement for necrosis and apoptosis. Because necrosis occurs rapidly while disruption of an apoptotic cell membrane occurs several days after cell stimulation one could argue the Trypan Blue Assay is appropriate for apoptosis identification.

Thirdly, Risso 1998 also suggests that BMAP-27 and BMAP-28 act through a modified intrinsic apoptotic pathway. In the intrinsic apoptotic pathway, Bcl-2 family members interact with and release proteins such as calcein and cytochrome C from the mitochondrial membrane. These proteins interact with Apaf-1 to form an apoptosome complex in the cytoplasm, which

mediates Caspase-9 activation and the subsequent activation of Caspase-3, 6, and 7 (Zhang 2005). At elevated concentrations, BMAP-27 and BMAP-28 are known to permeablize the mitochondrial membrane and trigger the release of calcein and cytochrome C (Skerlavaj 1996; Shai 2002). Interestingly, although BMAP-27 and BMAP-28 interact with the mitochondrial membrane and promote apoptosis, they do so in a Bcl-2 independent manner (Risso 1998). The Caspase assay did not show a significant difference in apoptosis for ConA-stimulated cells plus BMAP-27 or BMAP-28. It is possible that the Caspase assay if reinterpreted to include the difference in cell number between treatment groups may give a significant difference in apoptosis. The Caspase assay is a more sensitive assay than the Trypan Blue Assay.

Fourthly, anergic cells have minimal essential needs which is less than what would be expected to be used by proliferating cells. The ATP consumption assay demonstrated that ConA-activated T cells stimulated with BMAP-27 and BMAP-28 consumed significantly less cellular ATP compared to T cells stimulated with ConA alone.

Fifthly, BMAP-27 did not show renewed proliferation after the addition of IL-2 after 6 days. A time course analysis may reveal that reversal of BMAP-27 induced anergy may be time dependent. However, $CD4^+/CD8^+$ T cells stimulated with BMAP-28 showed renewed proliferation when exogenous IL-2 was added after 3 days, which is a marker for activation-induced anergy (Dubey 2007). This was not true for purified $CD8^+$ T cells or $CD4^+$ T cell populations. Understanding the discrepancy observed within these separate cell populations is perhaps more complicated in the pig than in other animal models. Since there is a large $CD4^+CD8^{lo}$ porcine T cell population, one must consider that a MACS-purified $CD4^+$ T cells consists of the $CD4^+$ single positive cell population and the $CD4^+CD8^{lo}$ population. Further, MACS-purified $CD8^+$ T cells consists of purified $CD8^+$ and $CD4^+CD8^{lo}$ double positive cells. The sorting methods and the purity of our CD4 and CD8 single positive populations are therefore difficult to calculate and it is difficult to ascribe any definitive attribution to a distinct cell population.

Sixthly, ConA-activated $CD4^+$ T cells stimulated with BMAP-27 and BMAP-28 showed induced surface expression of CTLA-4, which is a marker for anergic T cells. Why BMAP-27 and BMAP-28 mitogen-stimulated $CD8^+$ T cells do not show an increase in CTLA-4 expression but an increase is observed in BMAP-27 and BMAP-28 mitogen-stimulated $CD4^+$ T cells will require further investigation. However, we submit that, due to the fact that a large percentage of

the T cells are double positive in the pig, it is difficult to interpret the results for the single positive T cell populations.

5.3 Biological Relevance of HDP-Induced T Cell Peripheral Tolerance

HDPs may play multiple roles during the time course of an infection. HDPs are released at the site of infection where they have direct antimicrobial, antifungal, and antiviral activity towards invading pathogens (Lehrer 2002). Their secondary structure allows them to interact with the negative motifs common to pathogens and, upon binding, HDPs invade and kill pathogens often within minutes of infection (Brogden 2007). Outside of the antimicrobial functions, HDPs modulate the initial host immune responses by inducing cytokine and chemokine secretion from resident cells and through promotion of APC maturation (Bowdish 2004). They specifically promote inflammation by inducing production of pro-inflammatory cytokines such as IFN-γ and TNF-α, which in turn, enhance the function of local cells, such as phagocytes. However, over time HDPs promote resolution of inflammation by inhibiting pro-inflammatory cytokines, NFκB signaling, and NO production in macrophages (Yang 1999). Further, HDPs act on neutrophils and macrophages to clear the debris produced by pathogens and damaged tissue (Martin 2005). HDPs released from epithelial cells induce angiogenesis and migration and proliferation of fibroblasts, both of which play a role in wound healing and the return to homeostasis (Carretero 2007).

Little is known about the role HDPs play in the induction of peripheral tolerance, however recent studies outlined that human colonic epithelial cells, which underwent decreased cellular proliferation in response to secondary antigen challenge, showed up-regulated LL-37 gene expression (Kumar 2007), which one may speculate contributes to induction of tolerance. Therefore, BMAP-27 and BMAP-28 induced cellular apoptosis and T cell anergy may have biological relevance in resolution of an inflammatory response.

5.4 Porcine $CD4^{+}CD8^{lo}$ T Cell Phenotype and Activation-Induced Anergy

Porcine T lymphocyte biology is distinct from that observed in human and mice. Porcine T cells have a $CD4^{+}CD8^{lo}$ T cell population in the peripheral blood, which increases from less than 5 % at 1 week of age to greater than 50 % at 3 years of age (Zuckermann 1996). $CD4^{+}CD8^{lo}$ T lymphocytes are memory Th cells which recognize recall antigens in an MHC II

restricted fashion and respond to antigens by up-regulating expression of the IL-2 receptor, inducing cell proliferation, and producing cytokines, such as IFN-γ, IFN-α, and IL-2 (Werner 1990; Charerntantanakul 2006).

It is known that memory T cell populations are more sensitive to activation-induced anergy than naïve T cells. As illustrated in De Mattia 1999, memory Th cells were un-responsive to secondary antigen stimulation, while a corresponding naive T cell population proliferated vigorously (De Mattia 1999). The observed activation-induced anergy of $CD4^+/CD8^+$ MACs isolated T cells stimulated with BMAP-27 and BMAP-28 may be a consequence of the porcine memory $CD4^+CD8^{lo}$ T cell phenotype. Further investigation with other models would be important to determine if this anergy is species specific.

5.5 Clinical Relevance of Therapeutics Known to Induce T Cell Anergy

Neuropeptides such as VIP, NPY, and npT1 are neuroendocrine mediators currently being used as therapeutics to treat autoimmune diseases through induction of Treg cell activation and function. NPY share important characteristics with BMAP-27 and BMAP-28 including size (<10 kDa), a strong positive charge, and an amphipathic α-helix structure which mediates interaction with cellular membranes. Neuropeptides also share similar anti-inflammatory functions with cathelicidins, including inhibition of macrophage adherence and migration, phagocytic activity, free radical production, and inflammatory cytokine production such as TNF-α, IL-12, IL-6, and IL-1β (Delgado and Ganea 2008). NPY also induce apoptosis in infected macrophages, which is also a functional attribute of BMAP-27 and BMAP-28 (Chorny and Delgado 2008; Delgado and Ganea 2008). Similar to Indol, NPY can down regulate iNOS and NO production by macrophages. Self-reactive effector T cells incubated with NPY showed increased CTLA-4 surface expression and production of IL-10 and TGF-β on Tregs (Amelia 2006; Gonzalez-Rey and Delgado 2007; Delgado and Ganea 2008). Thus, NPY mediates anti-inflammatory effects and promotes induction of peripheral tolerance. Further studies should elucidate whether BMAP-27 and BMAP-28 have therapeutic potential.

5.6 Future Studies

The hypothesis was true that BMAP-27 and BMAP-28 modulate the activation of porcine PBMCs and T cells by inducing activation-induced anergy. There is little known about the promotion of T cell peripheral tolerance by host defense peptides, BMAP-27 and BMAP-28. From our studies, further work could be done to determine why BMAP-27 and BMAP-28 mitogen-stimulated $CD4^+/CD8^+$ T cell population showed a decrease in cellular proliferation compared to $CD4^+$ and $CD8^+$ single positive T cell populations. Analysis of cytokine profiles for Th1, Th2, cytotoxic T cells, and Treg cells could also provide information about the specific T cells that are being affected by peptide stimulation which may help determine how $CD4^+$ and $CD8^+$ T cell populations are affected by ConA and HDPs. Future studies should also include a time course adding ConA 4 hours before the addition of peptide to ensure the results we have seen are not an artefact of the stimulation method.

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