The Immunomodulation of Porcine Immune Cells by Innate and Synthetic Host Defense Peptides

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ABSTRACT

Dendritic cells (DCs) are potent antigen presenting cells (APCs) that link the innate and adaptive immune system by their unique ability to induce and direct immune responses towards various T helper (Th)-type of immune responses such as Th1-, Th2-, Th9-, Th17-, Th22- or T regulatory (TR). The type of Th response generated very much depends on the nature of the antigen encountered and allows for an effective and proficient immune response. For example, Th1 responses are used to clear intracellular pathogens while Th2 responses are needed to clear extracellular pathogens The ability to specifically modulate Th-responses is an area of intense research, as it allows for the development of more effective vaccines and immunotherapeutics. Immunomodulation of DCs is one strategy by which specific Th-type immune responses may be tailored. Current research is focused on identifying agents that have the capacity to immunomodulate DCs such as host defense peptides (HDPs). Apart from their anti-microbial activities, HDPs have a number of immune functions including recruitment and subsequent activation of DCs.

The goal of this study was to examine the immunomodulatory effects of HDPs on porcine DC functions. This research was part of a larger multinational research project to develop a novel adjuvant platform for single-immunization vaccines against pertussis in neonates. The pig model was used for this research because of its physiological similarities to humans and the recently developed pertussis infection model in young piglets. A series of experiments was conducted to characterize and describe porcine DC functions. Two subsets of DCs were successfully characterized and tested for their response to stimulation with HDPs. Initial results demonstrated a minimal effect of HDPs on DC functions, therefore we expanded the number of HDPs used to include both synthetic derivatives of HDPs known as innate defense regulators (IDRs) and naturally- occurring HDPs. We examined these effects on peripheral blood mononuclear cells (PBMC) *in vitro* and found that HDPs induce expression of the chemokine interleukin (IL)-8, which resulted in PBMC recruitment *in vitro.* We then proceeded to evaluate the HDPs *in vivo* by intradermally administering them into the flank of pigs. Surprisingly, treatment with the HDPs did not result in recruitment of neutrophils *in vivo.* We also examined the effects of formulating IDR-1002 as an adjuvant with the academic antigen Keyhole

Limpet Hemocyanin (KLH) on the development of KLH-specific immune responses in vaccinated pigs. While there was no difference in antibody titers between vaccinated and control animals, we found that co-formulation with IDR-1002 decreased both antigen-specific and mitogen-induced proliferation in KLH/IDR-1002 vaccinated animals as long as four weeks post-treatment. These results demonstrate that specific IDRs can suppress certain aspects of the pro-inflammatory immune response making them potentially highly versatile tools to modulate and tailor the immune response in disease states characterized by a pro-inflammatory component.

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β-galactosidase	β-gal

Chapter 1 LITERATURE REVIEW

1.1 Dendritic cells

DCs were initially observed and characterized in murine peripheral lymphoid organs by Dr. Ralph Steinman in 1973 [1, 2]. Since then, DCs have been identified as an important link between the innate and adaptive immune system [3, 4]. As potent APCs, DCs are located at sites of pathogen entry in the periphery and in primary and secondary lymphoid tissues, where they are specialized in antigen uptake and processing. Subsequently, antigen is presented via the molecules of the major histocompatibility complex (MHC)I or MHCII for the engagement and activation of T lymphocytes (T cells), important members of the adaptive immune system. The functions attributed to DCs include playing a role in preventing or limiting infectious diseases, allergy, autoimmunity and graft rejection [5]. As such, DCs are being used in the design of novel vaccines and immunotherapeutic agents for cancer and autoimmune diseases. Accordingly, by modulating DC activity, we hypothesize that it is possible to modulate adaptive immune functioning. For this reason we chose to examine DC behavior in response to stimulation by various HDPs in pigs.

DCs can be categorized as plasmacytoid DCs (pDCs) or conventional DCs (cDCs), which can be further sub-classified as migratory, lymphoid tissue-resident, monocyte-derived (Mo) and inflammatory DCs. pDCs differ from cDCs both phenotypically and functionally [6-8]. In the following section, some of these differences will be discussed in both human and murine models. Finally, porcine DC subsets, their morphology and behavior will be discussed.

1.1.1 Plasmacytoid dendritic cells

Historically, pDCs were known as interferon-producing cells (IPCs) and had been

characterized based on their robust type 1 interferon (IFN) production [9], as compared to other accessory cells present in human PBMC [10]. Further characterization of IPCs established them as being DCs [11, 12]. At steady state, IPC resemble plasma cells in appearance until they are activated, subsequent to which they develop the classical DC morphology of cytoplasmic extrusions and an irregularly shaped nucleus [13]. In the following paragraphs, DCs in general will be described with regards to their phenotype, migration, receptor expression, cytokine production, antigen uptake and T cell stimulatory capacity.

1.1.1.1 pDC cell surface marker expression

Phenotypically (i.e. in regard to cell surface marker expression) there are several commonalities between human and murine pDCs. Neither express various T cell related antigens (CD3, CD8β or the T-cell receptor), B cell related antigens (CD19, CD20 or surface antibodies) or myeloid related antigens (CD14 and CD11b) [12, 14-16]. Both however express co-stimulatory molecules MHCI and MHCII, CD40, CD54, CD80 and CD86 [16]. In contrast human pDCs are positive for CD4, CD123 (IL-3R), blood dendritic cell antigen (BDCA)2 (also known as CD303), BDCA4 and immunoglobulin-like transcript 7 (ILT7) [12, 15, 17, 18]. Murine pDCs, unlike those of humans, are positive for sialic-acid-binding immunoglobulin-like lectin H (Siglec-H) (endocytic marker), CD45R (B220), Ly6C and $CD11c^{low}$. Human blood pDCs express chemokine receptors (CCRs)-2, -5 and -7 and C-X-C chemokine receptor type (CXCR)-3 and -4 [19] and mouse pDCs express CCR1, CCR5, CCR7, CXCR3 and CXCR4 [20]. The expression of these chemokine receptors is crucial for DC migration.

1.1.1.2 Migration of pDCs

The migration of pDCs from the periphery into lymph nodes (LNs) remains unclear. LNs can be accessed either via afferent lymphatic vessels or via high endothelial venules (HEVs), but the route by which pDCs access LNs remains controversial. In humans, pDCs have been shown to roll and adhere to HEVs in a L-selectin dependent manner and

migrate into T cell areas of the LN [15, 21]. Cella et al. (1999) observed pDCs in the HEV lumen and in inflamed LNs [15] corroborating the hypothesis that human pDCs enter LNs via HEVs. Further, studies in sheep and pigs determined that pDCs were detectable in afferent lymph [22]. In contrast, rat pDCs treated with Toll-like receptor (TLR) 9 agonists were not observed in intestinal or hepatic afferent lymph [23] suggesting that pDCs do not travel via lymphatics. In mice, however, pDCs do not migrate via afferent lymphatic vessels but instead enter inflamed lymph nodes via HEVs [7]. Further, studies have shown that during steady state, murine pDCs do not transmigrate into the LNs [21], however, in the case of an inflammatory stimulus such as intranasal administration of influenza virus in mice, pDCs can be observed in LNs, as suggested by a decrease in pDCs in the blood and increase in the number of pDCs present in mediastinal LNs [24]. Within the LNs, pDCs are located in the T cell area and red pulp with limited numbers present in the marginal zone. Six hours following injection into the retro-orbital vein with TLR9 agonists, CpG oligodeoxynucleotides (ODNs), murine spleen pDCs formed clusters in marginal zones in the T cell area [25]. A similar finding was made following infection with murine cytomegalovirus (MCMV) [25] and confirmed that pDCs can migrate into LNs. In summary, pDC migration to LNs in humans and mice occurs via HEVs and is dependent on the expression of selectins and chemokine receptors.

1.1.1.3 pDC expression of pattern recognition receptors

pDCs express a multitude of surface receptors, which allow them to respond to a wide variety of invading organisms. Examples of these receptors include BDCA2, a C-type lectin, ILT7, NKp44 (an Ig-like receptor), dendritic cell immunoreceptor (DCIR) and FcεRIα (high-affinity Fc receptor for IgE) and TLRs [14]. In contrast to mice, human pDCs do not express TLR2, TLR3, TLR4 or TLR5 and therefore human pDCS cannot respond to agonists for these receptors such as peptidoglycans, LPS, flagellin, or double-stranded ribonucleic acid (RNA), respectively [26]. Both human and mouse pDCs express TLR7 and TLR9 which detect single stranded (ss) RNA and deoxyribonucleic acid (DNA) from viruses and bacteria, respectively. Thus human and mice pDCs can

detect bacteria, viruses and synthetic agonists [27]. TLR engagement triggers a signaling cascade that involves various adapter proteins, including myeloid differentiation primary-response gene 88 (MyD88), TIR-domain containing adaptor protein (TIRAP) and TIRAP inducing IFN-β (TRIF) and TRIF-related adaptor molecule (TRAM). Mobilization of these adapter proteins activates nuclear factor (NF)-κB, mitogen-activated protein kinases (MAPKs) and IFN regulatory factors (IRFs) that subsequently induce production of cytokines, chemokines and up-regulation of co-stimulatory molecules [27, 28]. For example, in response to TLR9 stimulation by CpG class A and C ODNs, pDCs produce large amounts of IFN-α and subsequently locate to lymphoid tissues to stimulate T cell responses [29]. The rapid and extensive production of IFN-α is thought to play a protective role during infection by bacteria or viruses.

1.1.1.4 Cytokine production in pDCs

Type I and type II IFN are the main types of IFN that are produced by pDCs, with type I IFN comprising of IFN-α, -β, -ω and -τ and type II IFN consisting of IFN-γ [30]. Type I IFNs are produced by most cells with pDCs being the main producers, whereas IFN-γ is produced by natural killer (NK) cells, $CD4^+$ Th1 cells and $CD8^+$ cytotoxic T cells [30]. The functions of IFNs range from inhibiting viral proliferation to modulating B [31] and T [32] cell responses and activation of NK cells [33].

1.1.1.5 Antigen uptake in pDCs

With regards to antigen uptake and presentation, antigen can be taken up either in a receptor-mediated manner or via endocytosis. Antigen then depending on whether it is endogenously or exogenously derived is presented on either MHCI or MHCII. Endogenous and exogenous antigen can be presented via MHCI while exogenous antigen is presented via MHCII [34]. pDCs in both humans and mice have been demonstrated to take up and present endogenous and exogenous antigen. In mice it has been demonstrated that pDCs preferentially take up and present endogenous antigens (soluble proteins) as opposed to extracellular or exogenous antigens [8]. A reason for this is due to the manner

in which MHCII is regulated [35]. In pDCs MHCII is continuously turned over even following activation. This means that stable complexes of exogenous antigen for presentation are not available. In contrast to exogenous peptide, endogenous peptides are continuously presented. In mice it was shown that despite the high turnover of MHCII, pDCs continuously present endogenous peptide derived from viruses [35]. In humans, pDCs take up exogenous antigen mostly via receptor-mediated endocytosis [36]. This has made human pDCs potential candidates for the targeted delivery of particulate vaccines *in vivo* via receptor-mediated endocytosis [34].

1.1.1.6 T cell activation by pDCs

Mature pDCs prime naive T cells [8] more strongly than freshly isolated pDCs [37, 38]. In contrast to naïve T cells, pDCs regardless of their maturation state are very capable of inducing the expansion of memory T cells [39]. When irradiated, splenic pDCs were co-cultured with human naïve $CD4^+$ T cells, proliferation was lower as compared to pDCs co-cultured with antigen-experienced T cells [40]. Such functional differences may be related to the lower expression of co-stimulatory molecules, such as CD80/86, by pDCs [41]. In mice, it was demonstrated by Sapoznikov et al. (2007) that pDCs can directly stimulate T cells [38]. This group used CD11c-diptheria toxin (DTR) transgenic mice in which the DTR receptor was under the control of the *Itgax* (CD11c) gene promoter. Endocytosis of diphtheria toxin (DTx) and its subsequent binding to the DTR terminated protein synthesis and resulted in the ablation of CD11c cells. In the absence of CD11c cells, pDCs were able to prime $CD4^+$ T cells but not $CD8^+$ T cells in the lymph node [38]. This study demonstrated that mouse pDCs can prime naïve $CD4^+$ T cells. In summary, both human and mouse pDCs have been demonstrated to prime naïve T cells, just not to the same extent as cDCs.

Recent evidence suggests that pDCs also play a key role in suppressing immune responses. Using a murine model of hapten-specific skin delayed type hypersensitivity (DTH), Goubier et al. (2008) demonstrated that oral tolerance relied on liver pDCs to

suppress DTH responses. Depletion of these hepatic pDCs abrogated suppressive responses in an antigen-specific manner [42]. In addition, pDCs in the thymus have been demonstrated to be responsive to thymic stromal lymphopoietin (TSLP) and subsequently increase the generation of Forkhead Box $P3^+$ ($FOXP3^+$) TR [43]. The ability of pDCs to detect self nucleic acid as well as the chronic production of IFN-α in the absence of viral infection was shown in the context of human autoimmune diseases such as Systemic Lupus Erythematosus (SLE) [14, 27]. Therefore, in both humans and mice pDCs play a role in suppressing immune responses.

1.1.2 Conventional dendritic cells

Conventional (c)DCs are derived from a common myeloid progenitor that gives rise to pre-cDCs, which can be divided into migratory and lymphoid tissue-resident DCs. Migratory DCs encompass DCs present at dermal, pulmonary and mucosal surfaces that act as sentinels and carry antigen from the periphery to LNs. In contrast, lymphoid tissue-resident DCs do not travel via lymphatics but remain in a fixed location such as the thymus or spleen [44]. For a more detailed review of migration of DCs please refer to Appendix B for a book chapter that we published on this topic [45].

In the skin, the epidermis and dermis contain different populations of DCs namely Langerhans cells (LCs) and dermal DCs. LCs are present in the epidermis where they represent less than 5% of the population of nucleated cells [46] and traverse from the dermis to LNs. LCs are phenotypically described as being $Langerin^+$ (CD207, a C-type lectin pattern recognition receptor (PRR) involved in carbohydrate moiety recognition), $EpCAM^-$, and $CD103^-$ [46]. Also, unique to LCs are the presence of intracytoplasmic granules known as Birbeck granules. Similarly, both LCs and dermal DCs are $Langerin^+$, $MHCII^+$, $CD86^+$, $CD11c^+$ but only dermal DCs are $Langerin^+$, $CD103^+$ and $EpCAM^-$ [47]. LCs require transforming growth factor (TGF)-β but not FMS-related tyrosine kinase 3 ligand (Flt3L) for differentiation, dermal DCs require Flt3L but no TGF-β [48, 49] demonstrating that these two DC subsets are distinct.

LCs are suggested to play a role in humoral responses and are considered T cell stimulatory cells. For example, Th1-dependent IgG2a and IgG2c synthesis was increased in LC-deficient skin in mice [49]. Because LCs migrate into T cell areas in draining LNs, they are thought to play a tolerogenic role. DCs migrate during homeostasis and are proposed to constantly present self-antigen to T cells thereby preventing induction of auto-reactivity [50]. During inflammation, LCs take up antigen, upregulate expression of MHCII and CCR7 and migrate to draining lymph nodes. For example, following infection with *Leishmania major* parasite, mice lacking LCs had higher concentrations of IFN-γ and a smaller lesion size compared to control mice suggesting that LCs can play an immunosuppressive role [30]. In contrast, dermal DCs are more prominent in directly driving $CD8^+$ T cell responses. For example, following epidermal inoculation with Herpes simplex virus (HSV)-1, $CD8^+$ DCs, which are involved in class I–restricted presentation of HSV antigen, were isolated from the draining brachial LNs [51]. This observation raised the question as to whether dermal DCs interacted directly with $CD8^+$ T cells or if antigen was received by LN resident $CD8^+$ DCs obtained from migratory DCs. LangDTR mice, which express the DTx receptor (DTR) under the control of the Langerin promoter, treated with DTx, resulted in ablation of $Langerin^+$ DCs. Using LangDTR mice Stoecklinger et al. (2011), demonstrated that following gene gun vaccination dermal DCs directly activated $CD8^+$ T cells and also induced more IFN-γ secreting cells [16]. Similarly, the priming of $CD8^+$ T cells was also demonstrated in a model of *L. major* infection [52].

Another type of cDCs are lymphoid tissue-resident DCs, which include spleen and LN resident DCs. These DCs originate from cDC precursors in the bone marrow, which then migrate to the spleen and LNs where they fully differentiate into cDCs. These DCs survey blood and lymphoid tissue for foreign antigens and are known to take up soluble antigen from lymphatics until DCs from the periphery arrive in the LN [53]. In contrast to migratory DCs, that arrive in LNs in a mature state, cDCs in the lymph node and spleen are immature [54].

Inflammatory DCs are another type of cDC that can stem from monocytes. Monocytes are leukocytes that originate in the bone marrow in a cytokine- and hematopoietic-growth factor receptor (Csf-1R; also known as c-fms) dependent manner and that exist in various subsets [55]. Monocytes originate in the bone marrow and are recruited into tissues both during inflammation and at steady state [56]. This recruitment occurs into the dermis, LN, spleen, and mucosal surfaces [57]. Recruited monocytes can subsequently differentiate into DCs *in vivo* [58] during inflammatory conditions and potentially at steady state as well [59]. Inflammatory DCs produce inflammatory factors including tumor necrosis factor (TNF-α), nitric oxide and reactive oxygen species. DCs derived from monocytes originate from a myeloid progenitor whereas pDCs and cDCs derive from a common DC progenitor, which stems from a myeloid progenitor [59].

Given the ease of isolating monocytes, MoDCs have been studied extensively *in vitro* by culturing monocytes in the presence of IL-4 and granulocyte-macrophage colony-stimulating factor (GM-CSF). In humans, cultured MoDCs express high levels of MHCI and II, CD1, FcγRII, CD40, CD80/86, CD44, and intracellular cell adhesion molecule (ICAM)-1, but they lack CD14. When human MoDCs were cultured with TNF-α or CD40L the expression of MHCI and II, CD80/86, and ICAM-1 was increased [60]. Similar to human MoDCs, cultured murine MoDCs display MHCI and II, CD80 and CD86, CD40 and ICAM [61]. Cytokines produced by MoDCs following stimulation with LPS include TNF-α, IL-6 and IL-12 which, can indirectly increase T cell production of IFN-γ and IL-4 [62]. MoDCs are also described as being highly stimulatory in a mixed lymphocyte reaction (MLR) [60]. *In vivo* examination of MoDCs demonstrated that MoDCs contributed to $CD4^+$ T cell activation, $CD8^+$ T cell cross priming and bacterial killing by TNF-α/iNOS production [57]. TNF-α/iNOS dependent killing was carried out by a subset of inflammatory DCs known as TNF-α and iNOS producing DCs (Tip-DCs). These DCs are involved in bacterial clearance as was demonstrated using an infection model of *Listeria monocytogenes* [63].

1.1.3 Porcine dendritic cells

The pig model is an important experimental model given the similarity between human and pigs with respect to their physiology and immune biology [64]. Due to growing toolbox of pig reagents becoming available, the field of porcine DC immunobiology has significantly expanded. Many porcine DC subsets have been phenotypically described and work is underway to describe their function in various contexts. The following section provides an overview of various aspects of porcine DCs including subtypes, phenotype and functionality.

Porcine DC are comprised of various DC subsets including blood (B) DCs [65], bone marrow-derived (BM) DCs [66], Langerhans-type cells [67], MoDCs [66-71], thymus DCs [72], LN and spleen DCs [73], gut DCs [74] and lung DCs [75].

Similar to humans and mice, porcine BDC subsets are comprised of pDCs and cDCs, which constitute approximately 0·1–0·3% of all PBMC [65] and they are present in a ratio of 1:0.8 of cDC to pDC [76]. Characterization of an antibody for the swine workshop cluster number 3 (SWC3) [77], also known as CD172a, led to BDCs being phenotypically described as $CD172a^{+}$, $MHCII^{+}$, $CD80/86^{+}$, $CD1^{+/-}$ and $CD14^{-}$ with pDCs being $CD4^{+}$ and cDCs being $CD4^{-}$ [65]. pDC isolation and characterization has proven to be challenging in the pig given the lack a specific cell surface marker to select for this cell type. Initially, producers of porcine IFN-α in PBMC *in vitro* in response to infection with transmissible gastroenteritis virus (TGEV) were described as non-T, non-B, $MHCII^{-}$ and $CD4^{-}$ [78]. Most studies examining porcine pDCs in PBMC have been characterized using CD172a which include pDCs, cDCs and monocytes [79-81]. In our studies, we characterized BDCs and MoDCs as $CD172a^{+}$ expressing cells [82].

1.1.3.1 Porcine pDCs

Functionally, pDC characterization has been carried out both *in vitro* and *in vivo* using viruses including classical swine fever virus (CSFV) [76], foot-and-mouth disease virus

(FMDV) [83, 84], TGEV [85, 86] pseudorabies virus (PrV) [86], swine influenza virus (SIV) [86] and porcine circovirus type 2 (PCV2) [87, 88]. These works have contributed much regarding pDC behavior as it relates to IFN production. For example, studies have shown that in pigs, IFN regulatory factor 3 (IRF3) and IRF7 regulate IFN-α/β production. CSFV induces proteasomal degradation of IRF3 [89] and modulates IRF7 turnover thereby potentially limiting Type I interferon production at initial sites of replication in cDCs, macrophages and epithelial cells [90]. However, during CSFV infection pDCs are found in a mature state (increased expression of CD80/86 and decreased expression of CD1) in the blood, tonsils and spleens of infected pigs where they produce TNF-α and IFN-α [76]. Infection models using FMDV have demonstrated that natural IFN producing cells (NIPC) produce high levels of IFN-α when FMDV was complexed with antibody thereby demonstrating FcγRII-dependent activation of NIPCs [83]. Stimulation of pDCs with CSFV, FMDV, SIV but not PCV resulted in increased IFN-α production *in vitro* and to a lesser extent *in vivo* [91]. pDCs are also able to respond to CpG ODN. Stimulation with class A CpG ODN resulted in the production of IFN-α [91] and TNF-α by $CD172a^{+}$ cell fractions [81]. Data from our laboratory demonstrated that while BDCs derived from adult pigs do not produce IFN-α in response to CpG ODN stimulation [92], neonatal BDCs produce IFN-α after poly I:C, imiquimod, class A or class C CpG stimulation (Gael et al, accepted PLOS ONE). Whether BDCs respond differently than pDCs alone in response to stimulation with CpG ODN remains to be determined. In summary, with the availability of reagents and tools it has become possible to demonstrate that porcine pDCs are producers of IFN-α following their stimulation by viruses and CpG ODN.

Porcine blood cDCs can be phenotyped as $CD4^{-}$, $CD14^{-}$, $CD11R2^{+}$, $CD1^{+/-}$, $CD16^{+/-}$, $CD3^{-}$, $CD5^{-}$, $CD8^{-}$, $CD21^{-}$ and represent 0·2–0·6% of PBMC [65]. Blood cDCs in culture up-regulate MHC class II and CD80/86, have endocytic activity and they can promote T cell proliferation [65].

1.1.3.2 Porcine monocyte-derived DCs

MoDCs can be phenotyped as $CD1^+$, $CD14^{+/-}$, $CD16^+$, $CD80/86^+$, $CD172a^+$, $MHCI^+$, $MHCII^+$, $CD4^-$, $CD3^-$ and $CD8^-$ [66, 67]. Porcine MoDCs, similar to mice and humans, could be generated by differentiating monocytes using growth factors IL-4 and GM-CSF. Porcine MoDCs express TLR4, 5, 7 and 9 [93, 94] and respond to a variety of stimuli including PRRSV [95] and various TLR ligands including LPS [66]. Stimulation with LPS and TNF-α increased the maturation of MoDCs as seen by an up-regulation of MHC class II and a down-regulation of CD1 expression [67]. MoDCs were able to sample antigen via both receptor- and non-receptor-mediated endocytosis as demonstrated by their uptake of dextran (DX)-fluorescein isothiocyanate (FITC), BSA-FITC and β-galactosidase (β-gal) immune complexes (ICs) [67]. MoDCs pulsed with the superantigen staphylococcal enterotoxin B (SEB) and matured with either TNF-α or LPS were able to stimulate T cell proliferation as well as increase the production of the cytokines IFN-γ and IL-4 [66]. T cell proliferation was also seen by MoDCs in MLRs [67].

1.1.3.3 Porcine BM-derived DCs

BM cells cultured with GM-CSF and TNF-α or Flt3 can generate both pDCs and cDCs [96]. Stimulation of BMDCs with TNF-α and LPS gave rise to an increase in the expression CD80/86 respectively, with TNF-α also increasing MHCI and MHCII expression [66]. Furthermore, in MLRs, BMDCs were potent stimulators of allogeneic T lymphocytes [66].

1.1.3.4 Porcine DCs in tonsils, mesenteric LN and spleen

Populations of cDCs ($CD11R1^+$ and $CD172a^+$) and pDCs ($CD4^+$ and $CD172a^+$) were present in large numbers in the spleen. Furthermore, in spleen, LNs and tonsils low levels of IFN-α were expressed mostly by pDCs with cDCs being the major producers of TNF-α in the tonsils [73].

1.1.3.5 Porcine mucosal DCs

The first description of mucosal porcine DCs was by Makala et al. (1998) whereby a $MHCII^+$ population of cells that lack B and T cell markers was identified in Peyer's patch cells [97]. Subsequently, four populations of porcine DCs were identified as follows: lamina propria (LP) DCs characterized as $CD11b^+$ and $CD172a^+$, Peyer's patch DCs characterized as $CD11b^-$ and $CD172a^+$ in subepithelial dome regions, DCs in interfollicular regions characterized as $CD11b^-$ and $CD172a^-$, whereas MLN DC were characterized as $CD11b^+$ and $CD172a^-$ [98]. It was also demonstrated that a population of intestinal DCs ($MHCII^+$, $CD16^+$ and $CD11b^+$) extended cytoplasmic processes between epithelial cells and also that small intestinal DCs were present adjacent to microvilli (M) cells [99]. Interestingly, of the four populations identified, only $CD11b^+$ and $CD172a^+$ and $CD11b^+$ and $CD172a^-$ were present in afferent lymph thereby suggesting that DCs migrate from the LP to MLN [98].

1.1.3.6 Porcine pulmonary DCs

Porcine lung DCs were phenotypically described as $CR4^+$(CD11c), $MHCI^+$, $CD80/86^+$, $CD172a^+$, $CD1^+$ and 50% were $CD16^+$ and MHCII $^+$. Functionally, lung DCs were capable of dextran and ovalbumin uptake as well as driving T cell proliferation in MLRs [75].

1.1.3.7 Porcine dermal DCs

Characterization of porcine Langerin/CD207 led to the observation that 50–70% of skin DCs express Langerin and are therefore LCs. Swine epidermal LCs are $CD163^-$ $CD172a^+$, $CD207^+$ and $Langerin^+$ [100, 101]. Three dermal DC subsets have been characterized, namely: (1) $CD163^-$ and $CD172a^-$ (2) $CD163^+$ and $CD172a^+$ and (3) the $CD163^{lo}$ and $CD172a^+$ DCs [101]. Porcine skin DCs have been phenotypically described as $CD172a^+$, $CD1^+$, $MHCII^+$, $CD80/86^+$, $S100\beta^+$, $CD80/86^+$, $CD3^-$, $CD4^-$ and $CD8^-$ [102]. Functionally, they were able to take up fluorescent-labeled OVA, induce T cell proliferation in MLRs and also in antigen specific T cells assays [102].

To summarize, our knowledge of porcine DCs and their phenotype, function and behavior is constantly increasing as recognition of the pig as a model is growing and better and more specific reagents are becoming available.

1.2 Host defense peptides

1.2.1 Cathelicidins and defensins

Hans Boman was one of the first to study HDPs in insects. Driven to find an answer to the question how insects survive infection without antibodies and T cells in hemolymph [103], Boman began in the 1970s to examine the antimicrobial system of insects. He isolated various antibacterial peptides including cecropins and lysozyme in the early 1980's [104]. Since then over 700 antimicrobial peptides have been described in mammals with their activity ranging from antimicrobial to immunomodulatory [105]. HDPs include peptides found in plants, insects, fish, amphibia, bacteria, fungi and mammals. They differ in conformation and length, ranging from 6-59 amino acids [106, 107]. HDPs can be categorized structurally as shown in Table 1-1 [107]. However, recent studies have focused predominantly on two main families of HDPs comprised of cathelicidins and defensins**.**

Table 1-1 Structural characterization of HDPs

Structural classes of HDPs	Examples
1. Anionic	Dermcidin released from human sweat glands
2. Linear cationic α-helical peptides	LL-37- the human cathelicidin
3. Cationic peptides enriched for specific amino acids	Bactenecins (Bac) from cattle
4. Anionic and cationic peptides that contain cysteine residues and form disulphide bonds	Protegrin from pigs and defensins from numerous species
5. Anionic and cationic peptide fragments of larger proteins	Antimicrobial domains from lysozyme and ovalbumin (OVA)

Cathelicidins are characterized by a conserved N-terminal pro-sequence and a variable C-terminal region [108] (Figure 1-1). The release of the mature peptide is regulated by enzymes, which cleave the pro-peptide into its mature form. For example, neutrophil-

derived elastase cleaves Bac7 into its mature form [109], whilst the cleavage of LL-37 from HCAP18 occurs upon exocytosis and consequent cleavage via proteinase 3 [110].

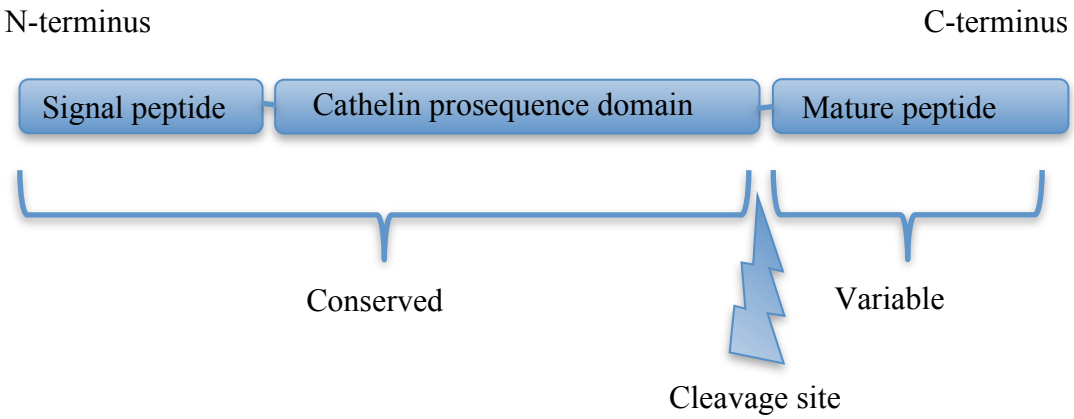

Figure 1-1 Cathelicidin structure.

(Adapted from Nizet et al. (2003) [111]). Cathelicidins are made up of a conserved N-terminal region and a variable C-terminal region. The conserved region consists of the signal peptide and a cathelin domain. The variable region gives each distinct cathelicidin its properties due to variations in amino-acid length and composition. Proteolytic cleavage of the cathelicidin holoprotein at the cleavage site releases the active form of the peptide [108].

Cathelicidins can be grouped into three major peptide groups namely, α-helical, β-sheets, and extended peptide structures which are rich in certain amino acids [112, 113] (Table 1-2). While humans express only one cathelicidin gene which gives rise to the peptide LL-37, other species such as pigs express 11 cathelicidin genes [113, 114]. Pigs express the most diverse repertoire of cathelicidins [113].

Table 1-2 Structural groupings of Cathelicidins

Structure	Peptide	Origin
α-helical	BMAP-27, -28, -34	Cow
	LL-37/hCAP18	Human
	CRAMP	Mouse
	PAMP-23, -36, -37	Pig
	SMAP-29	Sheep
β-sheets	Protegrin-1, -2, -3, -4, -5	Pig
Extended peptide structures	Indolicidin (tryptophan rich)	Cow
which are rich in certain	Bac-4, -5, -7	Cow
amino acids	Prophenin-1, -2 (phenylalanine rich)	Pig
	PR-39 (proline and arginine rich)	Pig

(Adapted from Lehrer et al. (2002) [114] and Linde et al. (2008) [112]). Cathelicidins can be structurally grouped based on the presence of α-helical or β-sheet conformations or enrichment of certain amino acids.

Defensins contain six cysteine residues that form disulfide bridges and comprise three families: α-defensins, β-defensins and θ-defensins. Mature peptides range from 18-45 amino acids, contain three intra-molecular disulfide bridges and have a positive charge. In contrast, θ-defensins have a cyclized peptide backbone. The expression of defensins differs among species [115, 116] and by cell type. In humans, six α-defensins have been identified [117], whereas none are detected in pigs [113]. More than 30 different β-defensins have been described in humans [118] and 13 in pigs [119]. The expression of θ-defensins has only been observed in Old World monkeys, lesser apes and orangutans [120].

Defensin gene expression is developmentally regulated. For example, in human neonatal lungs, no human β-defensin (hBD)-2 was detected at 18 and 22 weeks gestation as compared with 42 weeks of gestation, seven months and 13 years of age. In contrast LL-37 was expressed in tissues taken from all of the ages sampled [121]. Similarly, Elahi et

al. (2006) demonstrated that neonatal piglets failed to express porcine beta-defensin (pBD)-1 in their lungs compared to >2 months old pigs, and that expression was correlated with protection against infection with *Bordetella pertussis* [122]. Whereas newborn piglets were susceptible to infection, four week old piglets were protected.

1.2.2 Structure and function

HDPs vary in size, sequence, charge, conformation and structure, hydrophobicity and amphipathicity [107]. For example, hBD-3 contains three disulfide bonds, which are not required for antimicrobial activity but are necessary for chemotactic functions [123]. In contrast, in the case of hBD-1, the reduction of these disulfide bridges was crucial for its antimicrobial activity against the fungus *Candida albicans* and the anaerobic and Gram-positive commensals *Bifidobacterium* and *Lactobacillus* species [124]. In these examples, both sequence and structure were essential for biological activity. A hydrophobic structure of some HDPs, including hBD-3, has been attributed with increased interactions with membranes, cytotoxic and hemolytic effects on eukaryotic cells [125]. Based on these observations, novel peptides have been synthesized to possess certain desired characteristics. For example, Omiganan, a molecule based on the HDP Indolicidin, is undergoing clinical trials for its antimicrobial effects in the prevention of catheter-related bloodstream infections and for the treatment of acne [126]. Results demonstrate that Omiganan *in vitro* demonstrates activity against pathogens commonly causing catheter associated infections including Gram-positive pathogens such as staphylococci, enterococci, streptococci, as well as *Enterobacteriaceae* [127]. Similar to Omiganan the design of novel peptides for their antimicrobial activity may aid in the fight against antimicrobial resistance to current antibiotics.

1.2.3 Antimicrobial activity

Several HDPs demonstrate antimicrobial activity against a broad spectrum of bacteria through various mechanisms including, disrupting the cell membrane. Models describing trans-membrane pore formation include the barrel stave model, the torodial pore

(wormhole) model and the (micellisation) carpet model [107]. Some HDPs function by binding components of the cell membrane, nucleic acids and proteins [107, 128]. In the case of *Pseudomonas aeruginosa,* cationic α-helical HDPs bind the outer membrane protein OprI which triggers peptide internalization followed by membrane permeability and subsequent death [129]. HDPs have also been demonstrated to bind surface lipids. For example, hBD-3 displayed antimicrobial activity against *Staphylococcus aureus* by binding lipid II, a cell wall precursor, resulting in disruption of cell wall synthesis and causing cell wall lesions [130]. Other peptides such as Indolicidin bind inner membrane components to mediate the transport of anionic compounds across the membrane [131]. Furthermore, Indolicidin has been shown to bind both single- and double-stranded DNA [132]. HDPs may also act by binding intracellular proteins. An example is the peptide PR39, which was demonstrated to prevent the proteasomal degradation of inhibitor of NF-κB alpha, by binding to the 26S proteasome thereby preventing the expression of NF-κB-dependent genes [133]. Interestingly peptide function may be dependent on their level of processing and/or maturity. For example, the immature form of Bac7 was chemotactic [134] without any antimicrobial activity, whereas in its mature form it proved antimicrobial against *Klebsiella pneumoniae* and *Salmonella typhimurium* [109].

1.2.4 Immunomodulatory activity

In addition to their antimicrobial activity, HDPs can recruit immune cells to sites of pathogen entry, they can influence adaptive immune responses and they can influence DC function. For example, hCAP, the pro-peptide of the human cathelicidin LL-37, has been shown to recruit human monocytes [135], neutrophils [135-137], DCs [136] and T cells [135, 138]. Human beta defensins 1-4 are chemotactic for human macrophages [139], mast cells [139], immature DCs [140], $CD4^+$, $CD45RA^+$ naive and $CD8^+$, but not $CD4^+$, $CD45RO^+$ memory T cells [140, 141]. Human α and β defensins have also been shown to be chemotactic for human MoDCs [142]. Interspecies effects are also evident, for example, the human cathelicidin recruited rat mast cells [143] and the mouse cathelicidin CRAMP was chemotactic for human monocytes, neutrophils, and

macrophages. Transcriptional profiling demonstrated that bovine and human homologues LL-37 and BMAP-27 showed conserved trends towards profoundly suppressing many LPS-induced genes and suppression of LPS-induced translocation of NFκB subunits into the nucleus of human and bovine monocytic cells [144].

Many of the receptors used by HDPs for immune cell recruitment remain unknown. Whereas some defensins have been shown to recruit immature DCs via CCR6 [145, 146], both the human and mouse cathelicidins LL-37 and CRAMP act mostly via formyl peptide receptor like (FPRL) [135, 147-149].

HDPs influence various components of the adaptive immune system such as lymphocyte proliferation, cytokine and antibody production. This was demonstrated in different species including mice that were immunized intraperitoneally with either Keyhole Limpet Hemocyanin (KLH) alone or in combination with HDPs. Vaccinated animals displayed increased serum IgG1, IgG2a and IgG2b responses and splenocytes displayed higher proliferation and higher secretion of IL-4 and IFN-γ into supernatants [150]. In another study, mice immunized subcutaneously with CpG ODN, polyphosphazene and Indolicidin have increased IgG1 and IgG2a titres and IFN-γ secretion [151]. These results are consistent using a bovine model in which subcutaneous immunization with indolicidin, CpG ODN and hen egg lysozyme (HEL), increase serum IgG titres and IFN-γ secretion of PBMC [152].

Synthetic mimetics of HDPs known as innate defense regulator proteins (IDRs) have also been demonstrated to affect adaptive immune response. The intranasal administration to mice of a complex of CpG10101-HH2 and detoxified pertussis toxin (PTd) resulted in significantly higher levels of IgA as compared to PTd and CpG10101 alone or PTd and HH2 alone [153]. The complex of CpG10101-HH2 and PTd also resulted in higher total serum IgG titres with a balance seen between IgG1 (Th2 response) and IgG2a (Th1 response) subclasses [153]. Further evidence for the CpG-HH2 complex in modulating immune responses was seen following the intranasal administration of CpG-HH2 and examining mRNA levels in the jejunum of neonatal piglets. Both IFN-γ and IL-12

responses were enhanced, whereas IL-4 mRNA levels were reduced demonstrating the ability of IDR complexes to contribute towards Th1 type biased immunity [154, 155]. Furthermore, CpG-HH2 combined with attenuated PRV resulted in higher PRV-specific antibodies of IgG2 isotype as well as Th1 cytokines IFN-γ and IL-12 in pigs [154]. Similar results were seen in both adult and neonatal mice whereby the subcutaneous administration of a combination of the IDR HH2/CpG ODN and polyphosphazenes resulted in higher IgG2a responses [156]. Interestingly, neither of the components alone or in a double combination exerted the same effect as the triple combination suggesting the importance of synergy in responses using IDRs. The effect of a triple combination of PTd-CpG-IDR-and polyphosphazenes was demonstrated using IDR-1002 in a murine model. Garlapati et al. (2011) demonstrated that the subcutaneous administration of this combination induced higher Th1 type of responses as shown by an increase in the cytokines monocyte chemotactic protein (MCP)-1, TNF-α, IFN-α, IL-12 and IL-17 and a decrease in IL-10 concentrations [157]. In summary, these studies demonstrated the ability of HDPs and IDRs to influence humoral and cell-mediated adaptive immune responses.

1.2.4.1 Dendritic cell immunomodulation

Evidence exists demonstrating that cathelicidins and defensins can directly impact both DC phenotype and function. Stimulation of immature human MoDCs for 12 hours with 30 μg/ml of the human cathelicidin LL-37, increased the expression of both MHCII and CD86 but had no effect on cytokine production [158]. In a study by Davidson et al. (2004), instead of using immature human MoDCs, MoDCs that were still developing from monocytes into DCs were exposed to 50 μg/ml of LL-37 for 24 hours. This exposure to LL-37 resulted in an increased expression of CD86 and FITC-labeled dextran uptake. Also, stimulation of LL-37 treated MoDCs with LPS, increased IL-6 and IL-12 production, T cell proliferation and T cell production of IFN-γ production [136]. These studies demonstrated the ability of LL-37 to modulate human MoDC behavior. Notable,

is the temporal aspect of exposure i.e. the effect of pre-treatment as compared to direct stimulation.

Aside from modulation by cathelicidins, human MoDCs are influenced by stimulation with human α- and β-defensins. Presicce et al. (2009), demonstrated that stimulation of human MoDCs with human neutrophil defensin-1, an α-defensin and human β-defensin-1, increased the expression of the costimulatory molecules CD80, CD86, CD40 and MHCII, the production of TNF-α, IL-6, IL-12p70 and enhanced the proliferation of allogeneic T cells [142]. Also, related to the effects of α-defensins on human MoDCs, Rodriguez-Garcia et al. (2009), observed that the immunomodulatory effects of α-defensins1-3 were concentration dependent [159]. Stimulation of human MoDCs with low concentrations of α-defensins1-3 (0.25- 1 μg/ml) resulted in an upregulation of CD86 and MHCII expression, and an increase in TNF-α, IL-1β, IL-12p40, IL-8 and IL-10 production [159]. In contrast, high doses of α-defensins 1-3 (10-20 μg/ml) downregulated the expression of CD86 and MHCII and the production of TNF-α, IL-1β, IL-12p40 and IL-10. And low doses of α-defensins 1-3 increased T cell proliferation whereas high concentrations decreased it. Results from this study suggest that immune responses to HDP may be regulated by the dose of HDP used.

In summary, HDPs demonstrate antimicrobial activity as well as immunomodulatory functions, the latter of which is of particular interest for this project.

Chapter 2 RATIONALE, HYPOTHESIS, OBJECTIVES AND AIMS

2.1 Rationale and hypothesis

Dendritic cells are important immune cells required for uptake and presentation of foreign antigens, stimulation of both innate and acquired immunity, as well modulation of the immune response towards either a Th1, Th2, Th9, Th17, Th22 or TR. Thus, DCs are often referred to as a link between the innate and acquired immune response. The ability to immunomodulate DCs either via recruitment, antigen uptake and presentation or T cell stimulatory ability may represent a key strategy for tailoring immune responses.

While considerable research is underway in murine models to understand DC immunobiology, much remains to be examined using larger animal models such as the pig. Pigs represent an important animal model, which in many aspects closely resembles the human immune system. Therefore, the goal of this project was to characterize porcine DC subsets and subsequently determine how to immunomodulate them. Since, HDPs have been demonstrated to be immunomodulatory, my hypothesis is that HDPs can immunomodulate porcine dendritic cells towards a stimulatory immune response.

2.1 Overall Objective

Our overall objective was to characterize porcine DC subsets and to immunomodulate them with HDPs. Therefore the aims of this project were as follows:

2.2 Aims

1. To assess the immunomodulatory activity of HDPs on cultured dendritic cells.

    a.) Develop protocols to isolate and generate porcine MoDCs and BDCs
    b.) Characterize DCs functionally and phenotypically
        a. Cytokine production
        b. Endocytic ability

c. Expression of co-stimulatory molecules
d. T cell stimulatory ability
e. Cell surface marker expression

c.) Examine effects of HDPs on MoDCs and BDCs
a. Dose Response Assessment
b. Cell surface marker expression
c. Cytokine production
d. Chemokine and chemokine receptor expression
e. Endocytosis

These aims were expanded to include the following aims given the results obtained from our DC work (Appendix A).

2. To determine the immunostimulatory activity of HDPs on isolated PBMC.
   a.) *In vitro* recruitment
   b.) Cytokine production

3. To assess HDP-mediated immune cell recruitment and immune stimulation *in vivo*.
   a.) *In vivo* recruitment
   b.) Splenocyte stimulation and assessment of proliferation and cytokine production

Chapter 3 A COMPARISON BETWEEN ISOLATED BLOOD DENDRITIC CELLS AND MONOCYTE-DERIVED DENDRITIC CELLS IN PIGS (as published in Facci et al., 2010; Immunology)

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All authors participated in the design of the experiments and contributed to writing of the manuscript. Auray, Buchanan, van Kessel and Thompson assisted in sample collection. All other data presented in this manuscript are the work of the thesis author.

3.1 Abstract

Various dendritic cell (DC) populations exist that differ in their phenotype and ability to present antigen to naïve and primed T cells. For example, plasmacytoid DCs (pDCs) are less potent T cell activators compared to conventional DCs (cDCs). In the present study we compared porcine blood DCs, containing pDCs, and monocyte-derived DCs (MoDCs), consisting of cDCs, in their phenotype, ability to uptake antigen, activation and maturation and their ability to present antigen to autologous T cells. Pigs represent an important animal model, whose immune system in many respects closely resembles that of humans. For example, the distribution of toll like receptors on dendritic cells is similar to that of humans, in contrast to that of mice. Our results demonstrate that both populations were able to endocytose foreign material. Following lipopolysaccharide (LPS) stimulation, CD80/86 and chemokine receptor (CCR)7 expression was increased in both populations as was the expression of the CC chemokine ligand (CCL)-2, CCL-4, CCL-20 and CXC chemokine ligand (CXCL)-2. Basal protein concentrations of interleukins IL-6, IL-8 and tumor necrosis factor (TNF)-α were higher in MoDCs, although in response to LPS, BDCs displayed a higher fold increase. Proliferation of autologous T cells was induced in an antigen specific manner for both MoDCs and

BDCs. Interestingly, while MoDCs were able to induce stronger proliferation in naïve T cells, when compared to BDCs, no difference in proliferation was observed when primed T cells were studied. These results demonstrate that isolated porcine BDCs are highly responsive to stimulation with LPS and functionally able to increase primed T cell proliferation to the same extent as MoDCs.

3.2 Introduction

Dendritic cells (DCs) are important cells of the immune system involved in uptake and presentation of foreign antigens, stimulation of both innate and acquired immunity, as well as modulation of the immune response towards a Th1, Th2, Th17 or T regulatory type of response [160, 161]. At steady state, DC subtypes include type-1 interferon-producing plasmacytoid DCs (pDCs) and conventional DCs (cDCs), both of which are present in lymphoid and nonlymphoid tissues as well as blood [162]. In contrast, monocyte-derived DCs (MoDCs) are generated during inflammation [44, 163]. DCs have been extensively characterized in a variety of species and protocols for obtaining DC subtypes range from *in vitro* culture methods to direct isolation of DCs from blood and tissues. Isolation, however, is complicated in humans and large animal species resulting in limited availability for functional studies. In pigs, blood DCs (BDCs) have only been investigated in a few studies and very little is known about the function of these DCs in antigen presentation and T cell activation. The objectives of the present study were to compare directly isolated porcine BDCs to traditionally generated porcine MoDCs in terms of phenotype and functionality.

Various porcine DCs have been described including bone marrow-derived (BM)DCs [66], Langerhans-type cells [67] and MoDCs [66-71]. MoDCs are the most widely used subtype and can be phenotyped as $CD1^{+}$, $CD14^{+/-}$, $CD16^{+}$, $CD80/86^{+}$, $CD172a^{+}$, $MHCI^{+}$, $MHCII^{+}$, $CD4^{-}$, $CD3^{-}$, and $CD8^{-}$ [66, 67]. Initially MoDCs were generated by isolation of PBMC followed by overnight plastic adherence. Non-adherent cells were then removed and the remaining monocytes were cultured in the presence of IL-4 and GM-CSF [66].

More recent protocols, however, involve the isolation of monocytes using antibodies against CD14 [95, 164] or CD172a [165], a porcine marker known as SWC3 that is present on myeloid cells [166] including cDCs and pDCs [65]. Porcine BDCs, on the other hand, comprising of pDCs and cDCs were originally described by Summerfield et al. (2003) by flow cytometric analysis of PBMC as being $CD172a^{+}$, $MHCII^{+}$, $CD80/86^{+}$, $CD1^{+/-}$, and $CD14^{-}$ with pDCs being $CD4^{+}$ and cDCs being $CD4^{-}$ [65]. Subsequently, this approach was further developed by isolating BDCs using antibodies against CD172a. However, since CD172a is also expressed on monocytes, these enriched BDC populations contained not only different DC subtypes but monocytes as well [93]. In the present study we adapt previous protocols by initially depleting monocytes and subsequently enriching for CD172a to achieve a more pure BDC population. These BDCs were compared to MoDCs in terms of antigen uptake, activation and maturation.

DC maturation occurs upon recognition of microbe-associated molecules pattern and is characterized by upregulation of co-stimulatory molecules such as CD80/86 and MHCII, various cytokines and the chemokine receptor CCR7 [167] [168]. The process of maturation occurs as DCs migrate towards the lymph node where they encounter naïve or primed T cells. In porcine MoDCs, stimulation with LPS was demonstrated to decrease the expression of CD16, up-regulate the expression of CD80/86 [66, 94, 169] and either increase [67] or have no effect [66, 94] on expression of MHCII. Uptake of FITC-dextran [67] or DQ-OVA [169] was decreased. Expression of cytokines including IL-6 [169] and TNF-α [169] was increased. Interestingly, transcripts for IL-10, IL-13, IFN-γ and IL-12p35 were increased but no production at the protein level was detected [70, 169]. Furthermore, LPS stimulation did not induce a change in IL-4 gene expression [94]. However, T cells that had been exposed to antigen-pulsed MoDCs produced protein for both IL-4 and IFN-γ [66]. In contrast to MoDCs, however, very little information is available on maturation and activation of isolated BDCs following stimulation with LPS.

Following their activation and maturation, DCs are known to increase T cell proliferation and to modulate the immune response towards a Th1, Th2, Th17 or T regulatory type of response [160, 161]. Due to the limitations of studying T cell proliferation in outbred

species, most studies in pigs have used mixed lymphocyte reactions [66, 70, 87] and only very few have used autologous cells [65, 87, 169]. In the present study, both MoDCs and BDCs were isolated from vaccinated pigs and co-cultured with autologous T cells to assess the induction of antigen-specific T cell activation. We found that both MoDCs and BDCs were equally able to induce T cell proliferation. However, when stimulated with LPS BDCs that were directly isolated from blood showed a greater increase in cytokine and chemokine expression when compared to MoDCs. This study therefore provides further evidence that directly isolated BDCs represent an important cell population for studying DC biology in pigs. Further studies, however, are required to identify the specific role of pDCs within the BDC population.

3.3 Materials and Methods

Experimental design and animals

Eight week old Dutch Landrace pigs purchased from Saskatoon Prairie Swine Centre, University of Saskatchewan were used in this study. The goal of this study was to directly compare MoDCs to isolated BDCs both phenotypically and functionally. Phenotypically, DC morphology was examined by Giemsa staining and the expression of cell surface markers by flow cytometry. Functionally, endocytic ability was examined by flow cytometry, changes in transcript expression and the production of cytokines in response to stimulation with LPS by quantitative real-time PCR (RT-qPCR) and ELISA respectively, and lastly for their ability to stimulate autologous T cell proliferation, thymidine uptake assays were performed. Studies were performed as per the ethical guidelines of the University of Saskatchewan and the Canadian Council for Animal Care.

BDC and T cell isolation and generation of MoDCs

Blood was collected using EDTA-coated syringes and blood mononuclear cells (BMC) were isolated using a 60% Ficoll-Paque™ Plus gradient (GE Healthcare, Uppsala, Sweden). Monocytes were isolated using magnetic beads (MACS), (Miltenyi Biotec, Auburn, CA) and human anti-CD14 (TÜK4) microbeads (Miltenyi Biotec, Auburn, CA)

[95, 164]. The cross-reactivity of this antibody was confirmed by testing it against an anti-porcine CD14 (MIL-2) homologue. Flow cytometry was used to verify the purity of the separated cells.

To generate MoDCs, monocytes were cultured in RPMI 1640 (Gibco, Grand Island, NY) supplemented with 10% FBS, 0.5 mM β-Mercaptoethanol, 10% Antibiotic/Antimycotic (Gibco, Grand Island, NY), 10% HEPES (Gibco, Grand Island, NY), 10% MEM Non Essential Amino Acids (Gibco, Grand Island, NY), 100 ng/ml of recombinant porcine (rp) IL-4 (Biosource, Camarillo, CA) and 20 ng/ml of rpGM-CSF (Biosource, Camarillo, CA) for 6 days at 37°C with 5% carbon dioxide. Half of the medium was changed every 3 days. MoDCs were used between day 4 and 6, at which time non-adherent MoDCs [66, 81, 170] were washed, counted and used in subsequent assays.

To isolate BDCs, which are described to be $CD172a^{+}CD14^{-}$ [65, 81], $CD14^{-}$ cells were labeled with a CD172a antibody (Serotec, Oxford, UK) and Rat Anti-Mouse IgG1 Microbeads (Miltenyi Biotec, Auburn, CA) and positively selected using MACS. The purity of $CD172a^{+}$ expression was consistently >95%. $CD172a^{+}$ cells were rested overnight and then used in the assays. This procedure is slightly modified from Summerfield et al. (2003), in which PBMC were rested overnight and the non-adherent cells were depleted of CD3, CD8 and CD45RA, and then sorted for CD172a.

To isolate T cells, the CD172a negative population from the BDC population was positively sorted for $CD4^{+}$ and $CD8^{+}$ cells by labeling the cells with a mouse IgG2b anti-CD4 (Serotec, Oxford, UK) and a mouse IgG2a anti-CD8 antibody (Serotec, Oxford, UK) followed by incubation with Rat Anti-Mouse IgG1 Microbeads (MACS, Miltenyi Biotec, Auburn, CA).

Cell stimulation

For stimulation with LPS, day 6 MoDCs and day 1 BDCs were cultured at $1x10^{6}$ cells/ml and stimulated with 100 ng/ml of LPS (*E. coli* O55:B5, Cambrex Bioscience,

Walkersville, MD) for 6 hours for gene expression studies or for 24 hours for ELISA (unless otherwise stated) and flow cytometry.

Morphology

To evaluate morphology, $1x10^5$ cells in medium were centrifuged at 150g onto glass slides for 4 minutes, incubated with methanol for 5 minutes, air-dried and Giemsa (Sigma, Saint Louis, MO) stained for 15-60 minutes. Cells were then washed with deionised water, air dried and fixed for morphological examination by microscopy.

Antibodies for phenotyping

The following anti-porcine antibodies were used for defining the cell types: CD172a (BL1H7), CD1 (76-7-4), CD3 (PPT3), CD4 (74-12-4), CD8 (PT36B), CD14 (MIL-2), CD16 (MCA1971), CD21 (BB6-11C9.6), MHCII (K274.3G8), MHCI (SLA-I) and human CD80/86 or CD152 (CD80/86) (4 501-020, Ancell, Bayport, MN). All of the antibodies were obtained from Serotec (Oxford, UK) unless otherwise mentioned. FITC anti-mouse immunoglobulins IgG1, IgG2a and IgG2b (Southern Biotech, Birmingham, AL) were used for detection by flow cytometry.

Flow Cytometry

Immunofluorescence staining was performed by incubating $1x10^6$ cells for 20 minutes at 4°C with each antibody. Cells were washed three times with cold PBS (1X) (pH 7.2) (Gibco, Grand Island, NY) containing sodium azide (0.03%) and gelatin (0.02%) and incubated with FITC- conjugated secondary antibody for 20 minutes at 4°C, washed three times and fixed with paraformaldehyde (2%). Ten thousand events were collected and analyzed by flow cytometry (FACScalibur™, CELLQuest™ software; Becton Dickinson, BD Biosciences, Mountain View, CA).

Endocytosis by MoDCs and BDCs

To evaluate endocytosis, $2x10^5$ MoDCs or BDCs were incubated with 200 μl of FITC-dextran (1 mg/ml) (Sigma, Saint Louis, MO) or DQ™ Red BSA (1 mg/ml) (Invitrogen, Carlsbad, CA) for 1 hour at either 0° or 37°C [67]. Cells were washed three times with cold PBS and centrifuged at 350g for 5 minutes. The uptake of the labeled particles was visualized by confocal microscopy and quantified by flow cytometry using 10,000 cells/event. Since endocytosis is inhibited at 0°C, cells incubated at this temperature served as controls for non-specific fluorescence.

Lymphocyte proliferation assay

Pigs were vaccinated at 4 weeks of age with 10 μg of genetically detoxified pertussis toxoid (PTd) in 30% emulsigen (MPV Laboratories, Ohama, NE), and boosted every 2 weeks for a total of 3 vaccinations. Blood was collected from these pigs to isolate MoDCs, BDCs, and T cells.

Once generated, MoDCs and BDCs were respectively pulsed with PTd (1 μg/ml in a total of 1ml) or OVA (100 μg/ml in a total of 1 ml) for 3 hours and washed three times. $3x10^4$ MoDCs or BDCs were co-cultured in 200 μl of culture medium with a total of $3x10^5$ MACS purified CD4 and CD8 autologous T cells for 72 hours in 96-well U-bottom plates (Corning, Corning, NY). During the last 8 hours of culture 1 μCi of [^{3}H]thymidine (Amersham Pharmacia Biotech, Baie de Urfe, PQ) was added and proliferative responses were determined using standard liquid scintillation counting. Results are expressed as a stimulation index (SI) and were analyzed by Mann-Whitney tests.

RT-qPCR assay for mRNA expression

To evaluate differential mRNA expression, $1x10^6$ MoDCs or BDCs were lysed in TRIZOL (Invitrogen, Carlsbad, CA) and stored at -80C until further processing. For RNA extraction, 200 μl of chloroform was added per 1 ml of TRIZOL. The sample was

incubated at room temperature for 3 minutes and centrifuged at 12000g for 10 minutes at 4°C. The aqueous phase was collected and 500 μl of isopropanol was added. The sample was incubated for 5 minutes at room temperature and then applied to a mini-column (Qiagen RNeasy®, Mississauga, ON) and centrifuged for 15 seconds at 8000g. The sample was washed as per the manufacturer's instructions and DNase I treatment was performed. The optical density at 260 nm (OD260) was used to quantify RNA and the ratio of OD260/280 was used to determine purity. cDNA was generated and RT-PCR was performed using the SuperScript™ III Platinum® Two-Step qRT-PCR Kit as per the manufacturers recommendations (Invitrogen, Carlsbad, CA). Refer to Table 3-1. for a list of primers that were used for mRNA quantification. Samples were analyzed using a Bio-Rad iCycler iQ (Bio-Rad, Hercules, CA). Changes in gene expression were determined by calculating the Δ cycle threshold (Ct) [171] by subtracting the Ct for ribosomal protein L19 (RPL19) (housekeeping gene) from the Ct of the gene of interest for each sample. The Δ Ct of the control was subtracted from the corresponding treated sample giving rise to the ΔΔCt. The fold change was derived from the equation $2^{-[_{\Delta\Delta}]Ct}$. In order to confirm that the housekeeping gene ribosomal protein L19 is stably expressed in MoDCs and BDCs, a comparison was performed using either glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or RPL19 as the housekeeping gene. Similar trends in fold change were observed. cDNA was diluted to generate a standard curve whose correlation coefficient was >0.99. The efficiency of qPCR was determined from the slope using the equation $(10^{[-1/M]}-1)$ x100 and ranged between 90 and 110%.

Enzyme-linked immunosorbent assay (ELISA) for cytokines

To evaluate changes in cytokine secretion, $1x10^6$ MoDCs or BDCs were incubated in 1 ml of culture medium for 24 hours in 6 well plates (Corning, Corning, NY) and culture supernatants were collected. Concentrations of IL-6, IL-8 and IL-10 were assayed using commercial kits as per the manufacturer's instructions (R&D Systems, Minneapolis, MN). ELISA for IFN-α, TNF-α and IL-12 were performed as previously described [172].

Statistical analysis

Statistical analysis was performed by non-parametric Mann-Whitney t-tests (p-value < 0.05) using the statistical software program GraphPad Prism 5.

3.4 Results

Monocyte-derived DC generation and blood DC isolation

In this study, 800 ml of EDTA blood yielded approximately $2x10^9$ PBMC. Following $CD14^+$ selection, an average of $2x10^8$ cells were cultured in the presence of IL-4 and GM-CSF to generate MoDCs. On day 6, approximately $2x10^7$ MoDCs were harvested and cultured for use. The $CD14^-$ population was positively selected for cells expressing CD172a which equates to our BDC ($CD14^-CD172a^+$) population. Approximately $3x10^7$ BDCs were therefore isolated and rested overnight. In contrast to other studies [96], our protocol resulted in lower numbers of MoDCs compared to BDCs from an equal amount of blood [96].

Phenotypic characterization of porcine DCs

DC morphology is characterized by a large cytoplasmic/nuclear ratio and possession of dendrites which increase the surface area available to sample and take up antigen. In this study, the morphology of Giemsa-stained MoDCs (Figure 3-1a) and BDCs (Figure 3-1b) were compared to each other. Both DC populations displayed a typical DC morphology, characterized by an irregular cell border with a large cytoplasmic cell mass. Expression of cell surface markers CD172a, MHCII, CD16, CD1, CD80/86 and CD14 was assessed by flow cytometry in six day-old MoDCs and BDCs (3-2). Both MoDCs and BDCs expressed all of these markers, however, relative to the MoDCs, BDCs showed similar expression of CD172a and MHCII, higher expression of CD16 and lower expression of CD80/86 and CD1. CD14 was absent from the BDC population as indicated in the cell isolation procedure.

Endocytosis by MoDCs and BDCs

Central to DC functions is their ability to take up antigen. In order to directly compare the endocytic activity of MoDCs and BDCs, we examined their uptake of FITC-dextran over time from day 0-7. The ability of MoDCs to uptake FITC-dextran increased from 29 ± 30% (mean ± SD) on day 1 to 58 ± 24% on day 4 and 57 ± 27% on day 6. In contrast, 16 ± 18 % of BDCs on day 1 were endocytically active following their isolation from blood. Laser confocal microscopy confirmed uptake of particles of FITC-dextran in both MoDCs (Figure 3-2a) and BDCs (Figure 3-2b). DQ Red BSA, which only fluoresces when endocytosed, was also examined in MoDCs alone (Figure 3-2c). Taken together, these results show that BDCs were consistently less endocytic than MoDCs.

Functional characterization of DC maturation following stimulation with LPS

In general, as DCs mature, the expression of co-stimulatory molecules such as CD80 or CD86 increases giving DCs the potential to activate T cells. Furthermore, upregulation of the chemokine receptor CCR7 allows DCs to migrate to the lymph node where they encounter lymphocytes [168]. In order to compare the expression of co-stimulatory molecules and CCR7 within each DC population, MoDCs and BDCs were stimulated with LPS (100 ng/ml) for 24 hours. Flow cytometric analysis showed that CD80/86 expression increased from 43% to 65% in MoDCs (Figure 3-3a; $p<0.05$), and from 14% to 45 % in BDCs (Figure 3-3b; $p<0.05$) as determined by flow cytometry. Within 6-hours stimulation with LPS, CCR7 gene expression increased by 2.9 ± 0.6 fold in BDCs (Figure 3-4) and 2.1 ± 0.6 fold in MoDCs (Figure 3-4). In summary, in response to stimulation with LPS both MoDCs and BDCs demonstrated characteristics of mature DCs in terms of co-stimulatory molecule cell surface expression and CCR7 gene expression.

Chemokine and cytokine production by DCs

At sites of injury, DCs release chemokines that are involved in recruiting innate and adaptive immune cells. The ability of DCs to produce chemokines was examined following a 6-hour stimulation with LPS. Over 4-fold up-regulation was observed in CCL-4, CCL-20 and CXCL2 gene expression in both MoDCs and BDCs (3-4) with the up-regulation observed to be numerically higher in BDCs for all of the genes examined. In BDCs, CCL-2 was upregulated

In LNs, DCs interact with T cells by delivering different types of signals including cytokines. The expression of cytokines in MoDCs and BDCs was compared by RT-qPCR following a 6-hour stimulation with LPS. No changes were observed in IFN-α and IFN-γ, whilst a greater than 3-fold up-regulation was observed in IL-6, IL-8 and TNF-α in both MoDCs and BDCs (Figure 3-6). Greater than 2-fold induction of IL-12 expression was observed in BDCs while no change was observed in MoDCs.

Cytokine secretion was examined by ELISA following a 24-hour stimulation with LPS. Both MoDCs and BDCs showed an increase in IL-6, IL-8 and TNF-α production with no changes in IL-10, IFN-γ and IFN-α in both MoDCs and BDCs. Increased IL-12 concentrations were observed in BDCs (Table 3). Thus, there was a high correlation between the results obtained from RT-qPCR and ELISA.

Basal concentrations of IL-6, IL-8 and TNF-α were higher in MoDCs. Interestingly, when MoDCs and BDCs were stimulated with LPS, the fold increase but not the absolute concentrations, was higher in BDCs than MoDCs. The same trend was observed for changes in chemokine expression.

Stimulation of both naïve and primed T cells in an autologous proliferation assay

DCs as key antigen presenting cells are able to increase T cell proliferation. We compared the ability of MoDCs and BDCs to increase proliferation of autologous naïve T

cells compared to primed T cells. Overall, PTd or OVA-stimulated MoDCs and BDCs co-cultured at a ratio of 1 DC to 10 T cells, showed an induction of T cell proliferation (Figure 3-7). However, the stimulation index was higher in PTd compared to OVA-stimulated DCs, reflecting the difference between primed and naïve T cells. MoDCs and BDCs stimulated antigen-specific T cell proliferation in primed cells to the same extent. In contrast, MoDCs were more potent to stimulate naïve autologous T cells when pulsed with OVA. Thus, both MoDCs and BDCs differed in their ability to stimulate naïve T cell proliferation but not in their ability to stimulate proliferation of primed T cells.

Primer sequences

	Primer Sequences (5'-3')		
	Sense	Anti-sense	Accession number
CCR7	CCCTTCCCTTCTGGGCATAC	CGGTCGATGCTGATGCAGAG	AB116555
IFN-α	CCACCTCAGCCAGGACAGAAGC	GGTCACAGCCCAGAGAGCAGATG	NM_214393.1
IFN-γ	CGAAAAGCTGATTAAAATTCCGGTA	TCTTAGGTTAGATCTTGGTGACAGA	NM_213948.1
IL-12(p40)	GAAATTCAGTGTCAAAAGCAGCAG	TCCACTCTGTACTTCTTATACTCCC	NM_214013
IL-6	ACCCAGCTATGAACTCCCTCTC	GCATCACCTTTGGCATCT TCTTC	NM_214399.1
IL-8	AGAAGCAACAACAACAGCAGTAACAAC	CCAGCACAGGAATGAGGCATAGATG	AB057440.1
TNF-α	CCCTTCCACCAACGTTTTCCT	TGATGGCAGAGAGGAGGTTG	EU682384
CCL-2	GCGGCTGATGAGCTACAGAAG	CCCGCGATGGTCTTGAAG	NM_214214
CCL-4	CCTCTCCCTCCTGGTCCTG	GGCTGCTGGTCTCATAGTAATC	EF107667.1
CCL-20	TGCTCCTGGCTGCTTTGATGTC	TCATTGGCGAGCTGCTGTGTG	AJ577084.1
CXCL2	GCTGCTCCTGCTTCTAGTG	ACTTCCTGACCATTCTTGAGAG	NM_001001861.1
RPL19	AACTCCCGTCAGCAGATCC	AGTACCCTTCCGCTTACCG	AF435591
GAPDH	CTCAACGGGAAGCTCACTGG	TGATCTCATCATACTTGGCAGG	DQ845173

Table 3-1 Primer sequences for Real-time PCR analysis of changes in gene expression.

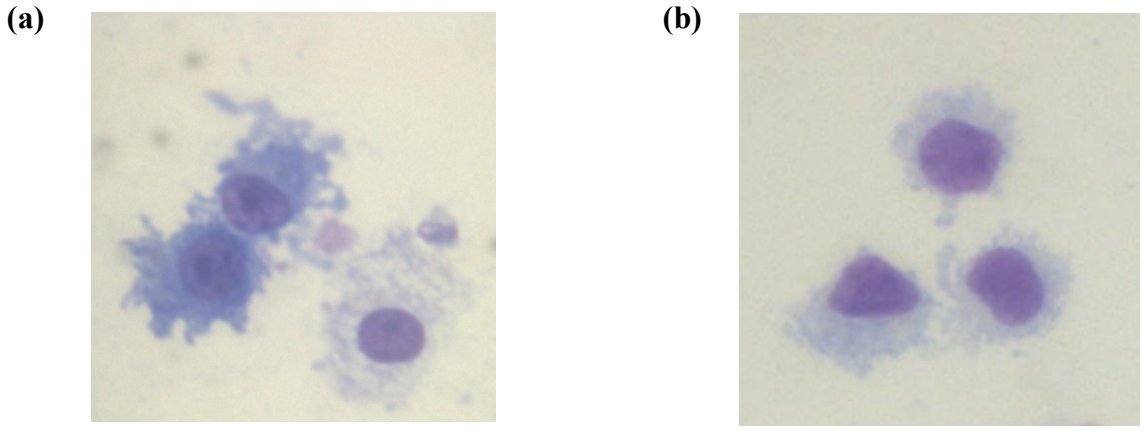


Figure 3-1 Giemsa stained pig monocyte-derived dendritic cells (MoDCs) and blood dendritic cells (BDCs).

Cell morphology of MoDCs at day 6 (a) and BDCs at day 1 (b) was examined by Giemsa staining of cytopsins. Magnification 1000x.

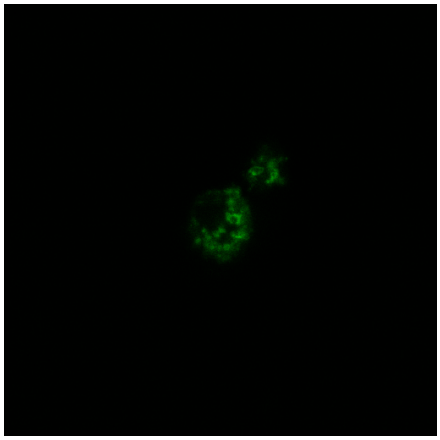
Cell surface markers	Monocyte-derived dendritic cells (MoDCs)	Isolated blood dendritic cells (BDCs)
CD172a	92 ± 3 %	96 ± 5 %
MHCII	95 ± 2 %	94 ± 10 %
CD16	85 ± 8%	92 ± 9 %
CD1	61 ± 10 %	17 ± 12%
CD80/86	43 ±10%	14 ± 7 %
CD14	81 ± 7 %	n.d.[a]

[a] n.d. The isotype of the anti-CD14 antibody was the same as that of the anti-CD172a antibody used to isolate the BDCs and therefore the % of CD14 expressing cells in the BDC population could not be determined.

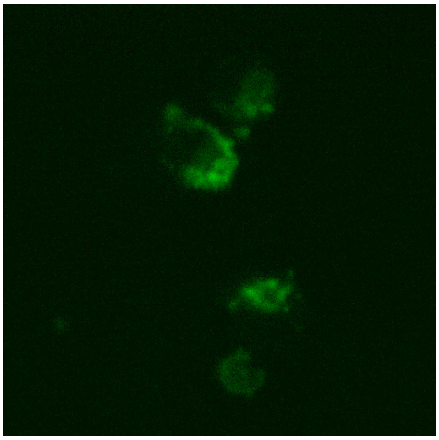
Table 3-2 Surface phenotype of MoDCs at day 6 and BDCs at day 1 analyzed by flow cytometry.

Data shown are the mean percentage ± SD of positive cells. Data for the MoDCs are representative of 6 pigs and data for the BDCs are representative of 4 pigs from a different litter.

(a)



(b)



(c)

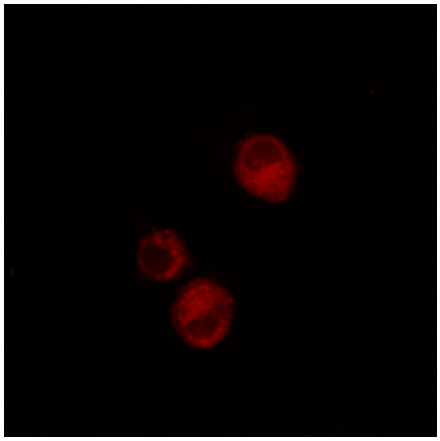
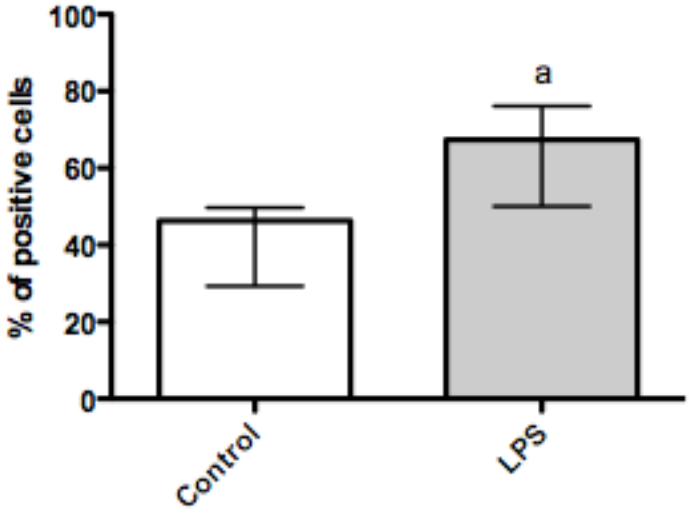


Figure 3-2 Endocytic activity of monocyte-derived dendritic cells (MoDCs) and blood dendritic cells (BDCs) by laser confocal microscopy.

MoDCs at day 3 were incubated with DQ Red BSA (a) or FITC-dextran (b). DQ Red BSA fluoresces following uptake. BDCs were examined for their ability to take up FITC-dextran (c). Images for the MoDCs are representative of 6 pigs and for the BDCs are representative of 4 pigs. Magnification 400x.

(a.) MoDCs



(b.) BDCs

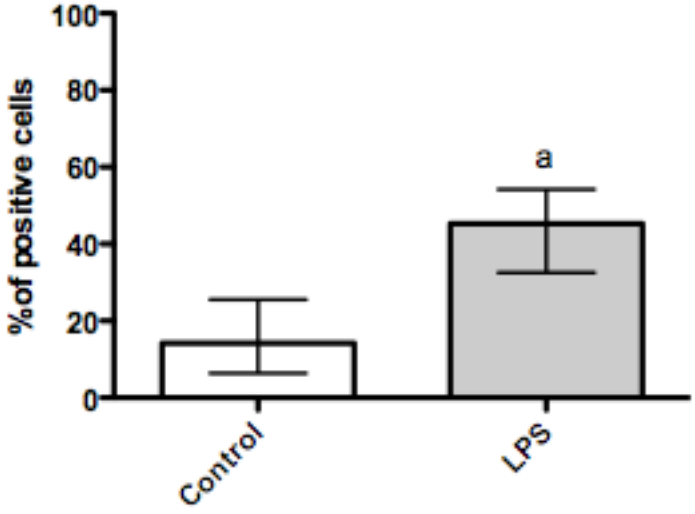


Figure 3-3 The effect of LPS stimulation on CD80/86 cell surface expression in monocyte-derived dendritic cells (MoDCs) (n=5 animals) and blood dendritic cells (BDCs) (n=6 animals).

MoDCs at day 6 (a) and BDCs (b) were isolated from blood mononuclear cells (BMCs) and rested for 4 hours before being cultured with LPS (100 ng/ml) for 24 hours. The expression of CD80/86 was determined by flow cytometry using the CD80/86 antibody to examine DCs stimulated with LPS compared to DCs in medium. Results are expressed as the median of the percentages of positive cells ± range ($p<0.05$) versus the control.

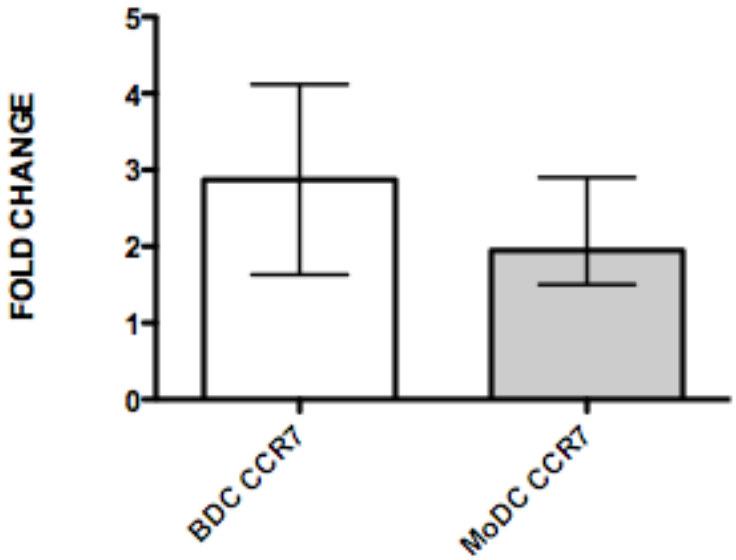


Figure 3-4 The effect of LPS stimulation on CCR7 expression in monocyte-derived dendritic cells (MoDCs) (n=4 animals) and blood dendritic cells (BDCs) (n=4 animals).

MoDCs at day 6 and BDCs were isolated from blood mononuclear cells (BMCs) and rested overnight before being cultured with LPS (100 ng/ml) for 6 hours. Samples were assessed for changes in gene expression of CCR7 by RT-qPCR using ribosomal protein L19 as the housekeeping gene. Results are shown as the median of the fold changes ± range relative to the control.

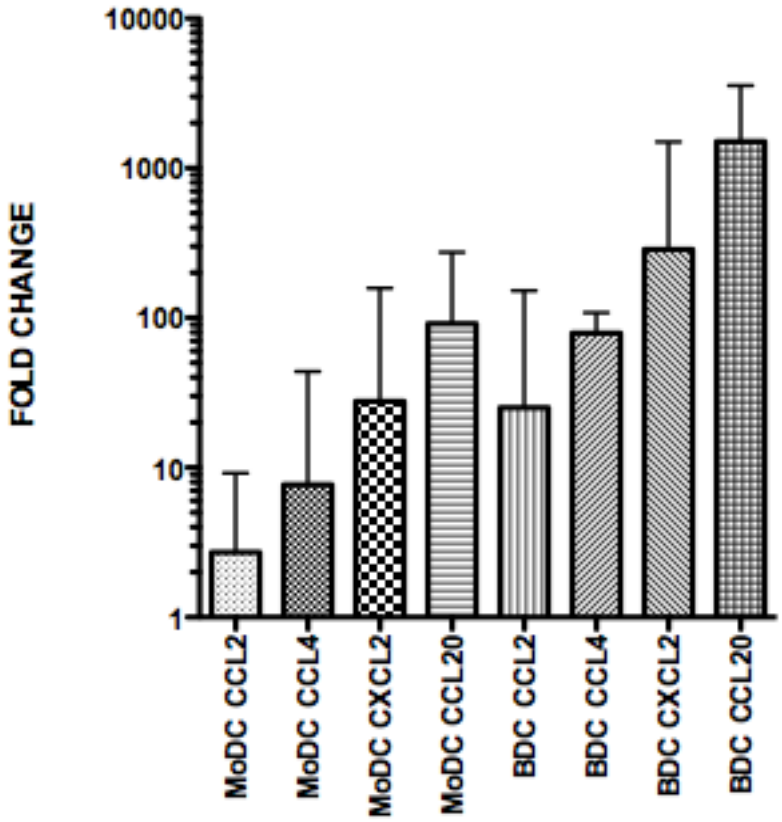


Figure 3-5 Changes in gene expression of CCL-2, CCL-4, CCL-20 and CXCL2 in monocyte-derived dendritic cells (MoDCs) and blood dendritic cells (BDCs) following a 6-hour stimulation with LPS.

MoDCs at day 6 and BDCs at day 1 were cultured with LPS (100 ng/ml). Samples were assessed for changes in gene expression by RT-qPCR using ribosomal protein L19 as the housekeeping gene. Results are shown as the median fold change ± range relative to the control (n=4 animals).

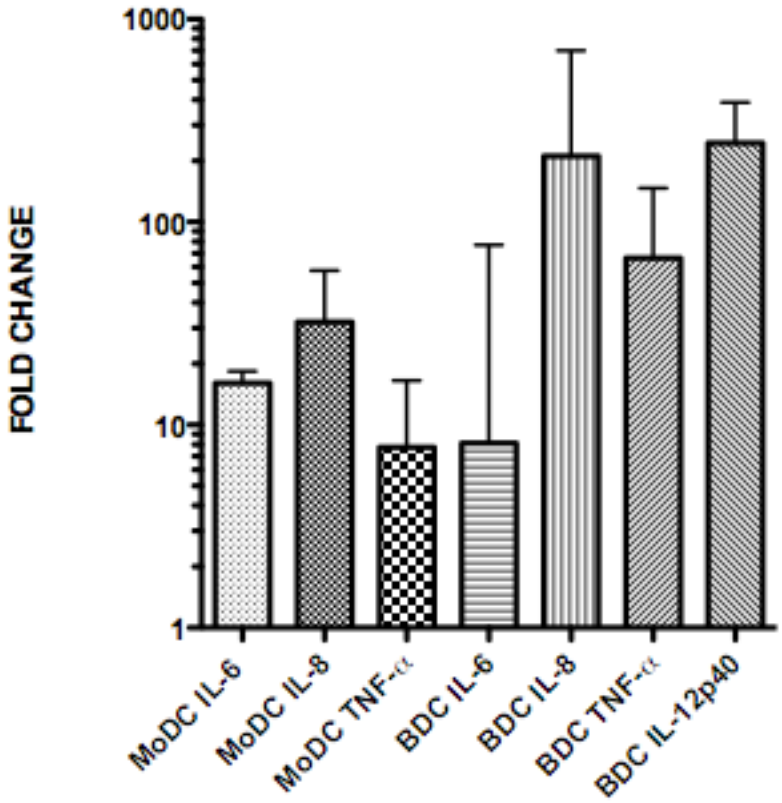


Figure 3-6. Changes in gene expression of IL-6, IL-8 and TNF-α in monocyte-derived dendritic cells (MoDCs) and of IL-6, IL-8 IL-12 and TNF-α in blood dendritic cells (BDCs) following a 6-hour stimulation with LPS.

MoDCs at day 6 and BDCs at day 1 were cultured for 6 hours with LPS (100 ng/ml). Samples were assessed for changes in gene expression by RT-qPCR using ribosomal protein L19 as the housekeeping gene. Results are shown as the median fold change ± range relative to the control (n=4 animals).

	MoDCs (pg/ml)			BDCs (pg/ml)		
	Control	LPS	P-value	Control	LPS	P-value
IL-6	426 ± 186	940 ± 277	0.2	n.d.	229 ± 260	0.03
IL-8	5261 ±5756	10586 ±1673	0.2 [a]	1114 ± 496	9557 ± 3925	0.03
TNF-α	402 ± 138	1277 ± 896	0.057[b]	277 ± 74	833 ± 511	0.057
IL-12	n.d.	n.d.		2000 ± 1000	8000 ± 3000	0.03

[a] at 16 hours of culture p= 0.057
[b] at 8 hours of culture p= 0.057
n.d., Not detectable

Table 3-3 Changes in protein IL-6, IL-8, TNF-α, and IL-12 concentrations following 24-hour LPS stimulation.

MoDCs at day 5 and BDCs at day 1 were either cultured with LPS (100 ng/ml) or were unstimulated (Control) for 24 hours (n= 4 animals). Supernatants were assayed by ELISA for protein detection. Results are expressed as mean (pg/ml) ± SD and analyzed by a Mann-Whitney test.

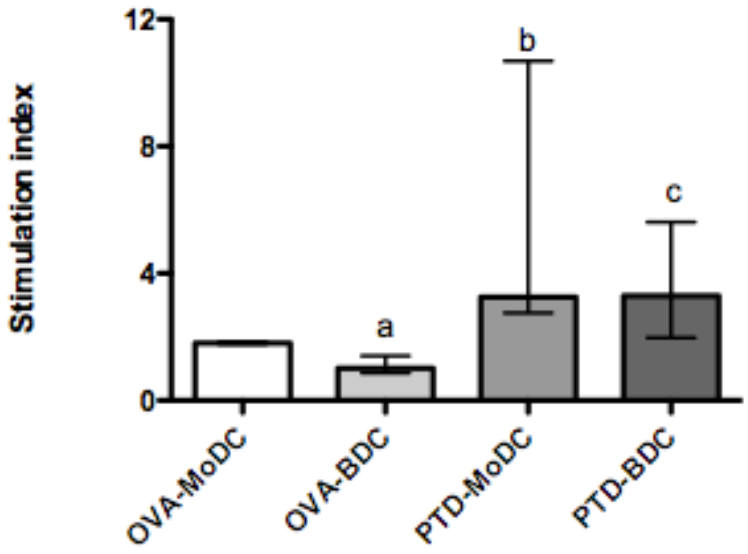


Figure 3-7 Stimulation of autologous T cells using antigen pulsed monocyte-derived dendritic cells (MoDCs) and blood dendritic cells (BDCs).

MoDCs at day 4 of culture and BDCs rested overnight were pulsed with pertussis toxoid (PTd) (1μg/ml) or OVA (100 μg/ml) for 3 hours then incubated with autologous T cells from PTd vaccinated animals from a total of 4 pigs for 72 hours at a ratio of 1 DC to 10 T cells. Results are shown as the median of the stimulation index± range and calculated as pulsed DCs/ non-pulsed DCs. ap<0.05 OVA-MoDC versus OVA-BDC, bp<0.05 OVA-MoDC versus PTd-MoDC, cp<0.05 OVA-BDC vs PTd-BDC.

3.5 Discussion

In the present study, we isolated porcine BDCs and MoDCs and demonstrated that these DC populations differ in their endocytic activity and their response to LPS with regards to cytokine and chemokine gene expression. Also, when we compared BDCs to MoDCs in autologous proliferation assays using T cells from vaccinated and non-vaccinated animals, no difference was observed in their ability to present antigen to primed T cells.

MoDCs were generated from MACS isolated monocytes that were cultured in the presence of IL-4 and GM-CSF. This isolation technique contrasts other studies which have used overnight adherence or CD172a MACS sorting [66-68, 94, 173], but is similar to the methods used in more recent studies for generating porcine MoDCs [95, 164] and is the same as that used to generate human [174] and mouse MoDCs [175]. BDCs, on the other hand, were generated using a slightly modified protocol previously described by Summerfield et al. (2003) [65]. Previously, antigen uptake by BDCs was examined by flow cytometric analysis of PBMC [65] and not on the isolated BDCs themselves. However, in contrast to this study we examined isolated BDCs using the negative fraction following CD14 MACS sorting of PBMC. Subsequently, positive selection of CD172a cells allowed us to study isolated BDCs. The $CD14^+$ fraction was used to generate MoDCs. Advantages of our isolation procedure include the isolation of a pure population of monocytes that is generated on the same day without requiring overnight adherence. For BDCs, we obtained a purity of 96% with a higher yield compared to a purity of 60-75% of CD172a cells described by others [65]. Furthermore, this population contained only very few or no contaminating monocytes in contrast to that described by others [93]. Consistent with what has been described [73], as a total percentage of PBMC, $CD14^-CD172a^+$ cells constituted approximately 2% of the population [73].

Using these isolation methods, we observed that unstimulated MoDCs displayed a more mature phenotype compared to unstimulated BDCs. While a similar percentage of

MoDCs and BDCs expressed CD172a and MHCII, BDCs showed a slightly higher expression of CD16 and a lower expression of CD80/86 and CD1. The more mature phenotype of MoDCs may be attributed to culturing artifacts such as disturbing cell-cell contact [176], the presence of serum in the culture medium [177] and the effects of IL-4 [178] and GM-CSF [179]. Compared to MoDCs, BDCs were only cultured overnight therefore culturing artifacts were expected to be minimal. This is supported by Fearnely et al. (1997), who demonstrated that when human BDCs were cultured for several days they displayed a more mature phenotype similar to that of MoDCs [177].

Despite the more mature phenotype of MoDCs, however, BDCs displayed lower endocytic activity than MoDCs. Regarding IL-6, IL-8, and TNF-α cytokine production, the basal production of cytokines by MoDCs was over 2-fold higher than that of BDCs. However, when MoDCs and BDCs were stimulated with LPS, a higher fold change of both cytokine and chemokine expression was observed in BDCs suggesting that BDCs were more responsive to LPS stimulation compared to MoDCs. Reasons for these differences remain to be examined but may be due to differences in cell signaling pathways. For example, BDCs do not express CD14 and therefore are unable to respond to LPS via a CD14 dependent-signaling pathway. However, the presence of CD14 independent signaling in porcine DCs has been previously demonstrated [66] and it is known that BDCs do respond to LPS stimulation [180] suggesting that BDCs signal via a CD14 independent pathway. Further studies are required to understand the detailed mechanisms of LPS signaling in BDCs.

Another observation in this study was that LPS-stimulated MoDCs did not produce IL-12 whereas BDCs did. This is in contrast to previous observation made by Wilkie et al. (2005), who found an increase in IL-12p35 mRNA expression in porcine MoDCs following stimulation with LPS. Possible reasons for the observed differences include cell isolation by plastic adherence, collection of both adherent and non-adherent day 8 MoDCs, and lastly a concentration of 10 g/ml of LPS for cell stimulation [94]. However, in a more recent study in which MoDCs were obtained by plastic adherence, no IL-12p40 was detected at the protein level following LPS stimulation at a concentration of 1

μg/ml [70]. Thus, there is a discrepancy in the literature regarding the ability of porcine MoDCs to produce IL-12 in response to stimulation with LPS and more studies are required to fully address these observations. For human monocytes, it was demonstrated that MoDCs generated from plastic adherence as compared to CD14 bead isolation, produced IL-12p70 [181].

We then determined if the phenotypic and endocytic differences between MoDCs and BDCs translated into differences in their ability to induce T cell proliferation using autologous T cells. To this end pigs were vaccinated with PTd and isolated cells were re-stimulated *in vitro* with two different antigens to be able to compare naïve versus primed T cells. When the antigen OVA was used to address stimulation of naïve T cells, BDCs induced less proliferation compared to MoDCs. However, when PTd was used for stimulation of autologous primed T cells, the extent of proliferation was the same between MoDCs and BDCs. Since the activation threshold for naïve T cells is higher due to an uncoupled signaling machinery [8, 182] we assume that T cells to which OVA was presented were naïve and required more signals that BDCs were less able to provide. This could be attributed to their lower endocytic ability. With respects to primed T cells, however, BDCs did not differ from MoDCs in their ability to increase T cell proliferation, which may be a result of a lesser need for additional stimulation. It has also been demonstrated that the pDC population within the BDCs is better able to induce proliferation in antigen-experienced T cells compared to naïve T cells [40]. Therefore, porcine BDCs differ from MoDCs in their ability to stimulate naïve T cell proliferation but not primed T cell proliferation. This is in contrast to observations made in mice [40] and provides further evidence that BDCs indeed are able to increase T cell activation in both naïve and memory T cells [8].

In summary, in the present study we compared two populations of DCs in their phenotype, endocytic ability, response to LPS stimulation and ability to induce antigen-specific immune response in pigs. Our findings suggest that BDCs, which contain both pDCs and cDCs, are less endocytically active than MoDCs and have a lower expression of CD80/86. They also have lower basal cytokine protein concentrations but in response

to stimulation with LPS, there is a higher fold increase in response despite the absolute amounts being lower in MoDCs. Furthermore, this is the first time in the pig that chemokines have been examined in response to LPS in both MoDCs and BDCs and allows for a more comprehensive view of DC behavior. Lastly, both MoDCs and BDCs are able to induce T cell proliferation which is in contrast to observations made in mice [40], and which will further our understanding of these important cells and their role in driving antigen-specific immune responses.

Acknowledgements

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3.6 Conclusion

Porcine DC subsets, MoDCs and BDCs were successfully generated and characterized. BDCs compared to MoDCs are less endocytically active, have a lower expression of CD80/86 and have lower basal cytokine protein concentrations. In response to stimulation with LPS there is a higher fold increase in the response of BDCs compared to MoDCs. To further characterize these DC subsets in Chapter 4 we examined the stability of expression of reference gene expression.

Chapter 4 STABILITY OF EXPRESSION OF REFERENCE GENES IN PORCINE PERIPHERAL BLOOD MONONUCLEAR AND DENDRITIC CELLS (as published in Facci et al., 2011;Vet. Immuno. Immunopath.)

Facci, M. R., Auray, G., Meurens, F., Buchanan, R., van Kessel, J. and Gerdts, V.
Vet Immunol Immunopathol **141**(1-2): 11-15.

All authors participated in the design of the experiments and contributed to writing of the manuscript. Auray, Buchanan and van Kessel assisted in sample collection. Meurens provided support in data analysis. All other data presented in this manuscript are the work of the thesis author.

4.1 Abstract

Real-time quantitative PCR (RT-qPCR) is a critical tool used to evaluate changes in gene expression. The precision of this tool is reliant upon the selection of reference genes whose expression remains unaltered in culture conditions and following stimulation. Stably expressed reference genes are used to normalize data so observed changes in expression are not due to artifacts but rather reflect physiological changes. In this study, we examined the expression stability of the porcine genes glyceraldehyde 3-phosphate dehydrogenase (GAPDH), succinate dehydrogenase complex subunit A (SDHA), eukaryotic elongation factor 1 gamma-like protein (eEF1), ribosomal protein L19 (RPL19), beta-actin (ACTB) and ATP synthase mitochondrial F0 complex (ATP5G1) in peripheral blood mononuclear cells (PBMC), monocytes, monocyte-derived dendritic cells (MoDCs), blood-derived dendritic cells (BDCs) and T cells with or without stimulation with LPS. An M value was used as a measure of gene stability as

determined using geNORM software. Recommendations for the use of reference genes include using GAPDH and B-actin in PBMC: RPL19 and SDHA and T cells; RPL19 and B-actin in monocytes; RPL-19 and SDHA in BDCs: and RPL-19 and ATP5GA in MoDCs.

4.2 Introduction

Real-time quantitative PCR (RT-qPCR) is widely used to examine changes in gene expression. Some variables encountered in using this technique include differences in starting material, enzymatic efficiency and transcriptional activity [183]. A means of controlling these variables is the use of reference genes. Ideally reference genes should be stably expressed and not change in response to stimulation with any immunostimulator. In a study by Vandesompele et al. (2002)[183], 10 reference genes were examined in 13 different human tissues. Subsequently, a method for determining the stability of expression of reference genes was devised and is the basis of the program geNORM. This program calculates a gene stability measure known as M, thereby allowing for the ranking of reference genes. The lower the M value, the more stable is the expression of the gene. Furthermore, a stability of $M < 0.5$ is desirable for homogenous samples [184].

The pig is growing in importance as a large animal model for examining and monitoring human diseases such as influenza. As part of this process, RT-qPCR is being used as a technique for examining changes in gene expression. Several genes have been studied, to assess their potential to serve as reference genes in the pig (Table 4-2). Most of these studies were performed in peripheral blood mononuclear cells (PBMC), and none have examined monocytes, monocyte-derived dendritic cells (MoDCs), blood-derived dendritic cells (BDCs) or T cells. However, these latter cell types are frequently being used to determine effector immune responses including cytokine and chemokine expression. For this reason we chose to examine the expression levels of 6 reference genes namely glyceraldehyde 3-phosphate dehydrogenase (GAPDH), succinate dehydrogenase complex subunit A (SDHA), eukaryotic elongation factor 1 gamma-like protein (eEF1), ribosomal protein L19 (RPL19), beta-actin (ACTB) and ATP synthase

mitochondrial F0 complex (ATP5G1) in these cell types using geNORM to determine M values [183]. These reference genes were chosen from different functional classes (Table 4-1). In this study, a 6-hour stimulation with lipopolysaccharide (LPS) was used to determine if the levels of expression were influenced by stimulation with LPS. At this time expression of various cytokines or chemokines has already peaked [62]. The Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines were followed to process samples and perform experiments [184].

4.3 Materials and Methods

Experimental design and animals

Eight week old Dutch Landrace pigs purchased from Saskatoon Prairie Swine Centre, University of Saskatchewan were used in this study. Studies were carried out in accordance with the ethical guidelines of the University of Saskatchewan and the Canadian Council for Animal Care.

PBMC, monocytes, T cells and BDCs were isolated. Monocytes were used to generate MoDCs. The stability of expression of the reference genes RPL19, ACTB, eEF1, SDHA, ATP5GA and GAPDH was examined in these respective cells, either cultured in medium alone for 6 hours (control cells) or following a 6-hour stimulation with 1 μg/ml LPS (stimulated cells) (*E. coli* O55:B5, Cambrex Bioscience, Walkersville, MD). Isolated cells at a concentration of $1x10^6$ cells/ml were rested overnight prior to stimulation with LPS. Subsequently, cells were lysed in TRIzol (Invitrogen, Carlsbad, CA) and stored at -80°C until further processing. Four animals per group were used in all of the stimulation assays with the exception of PBMC for which only three animals were used for the stimulation group. Studies were performed as per the ethical guidelines of the University of Saskatchewan and the Canadian Council for Animal Care.

Cell isolation and generation

Approximately 1 L of blood was collected by cardiac puncture from each pig using EDTA-coated syringes and blood mononuclear cells were isolated using a 60%

Ficoll-Paque™ Plus gradient (GE Healthcare, Uppsala, Sweden). PBMC were plated at $1x10^6$ cells/ml in 24-well plates and rested overnight before stimulation. From the remaining PBMC, monocytes were isolated using human anti-CD14 magnetic beads (MACS®; Miltenyi Biotec, Auburn, CA) [95, 164]. The cross-reactivity of this antibody was confirmed by testing it against an anti-porcine CD14 (MIL-2; Serotec, Oxford, UK) homologue. Flow cytometry was used to verify the purity of the separated cells. Monocytes were stimulated following an overnight rest.

To generate MoDCs, monocytes were cultured in RPMI 1640 (Gibco, Grand Island, NY) supplemented with 10% FBS, 0.5 mM β-Mercaptoethanol, 10% Antibiotic/Antimycotic (Gibco, Grand Island, NY), 10% HEPES (Gibco, Grand Island, NY), 10% MEM Non Essential Amino Acids (Gibco, Grand Island, NY), 100 ng/ml of recombinant porcine (rp) IL-4 (Biosource, Camarillo, CA) and 20 ng/ml of rpGM-CSF (Biosource, Camarillo, CA) for 6 days at 37°C with 5% carbon dioxide. Half of the medium was changed every 3 days. MoDCs were used at day 6, at which time non-adherent MoDCs [66, 81, 170] were washed, counted and stimulated.

To isolate BDCs, which are described to be $CD172a^+CD14^-$ [65, 81], $CD14^-$ cells were labeled with a mouse IgG1 anti-CD172a antibody (Serotec, Oxford, UK) and Rat Anti-Mouse IgG1 Microbeads (Miltenyi Biotec, Auburn, CA) and positively selected using MACS. The purity of $CD172a^+$ expression was consistently >95%. $CD172a^+$ cells were rested overnight and then stimulated with LPS.

To isolate T cells, the CD172a-negative fraction was positively sorted for $CD4^+$ and $CD8^+$ cells by labeling the cells with anti-CD4 (VMRD, Inc, Pullmann, WA) and anti-CD8 antibody (VMRD) followed by incubation with Rat Anti-Mouse IgG1 Microbeads (MACS, Miltenyi Biotec, Auburn, CA).

RT-qPCR assay for mRNA expression

For RNA extraction, per 1 ml of TRIzol, 200 μl of chloroform was added to 5-10 x 10^6 cells. Samples were incubated at room temperature for 3 minutes and centrifuged at 12000g for 10 minutes at 4°C. The aqueous phase was collected and 500 μl of isopropanol was added. The sample was incubated for 5 minutes at room temperature and then applied to a mini-column (Qiagen RNeasy®, Mississauga, ON) and centrifuged for

15 seconds at 8000g. The sample was washed and DNase I treatment was performed as per the manufacturer's instructions (Qiagen). RNA quantification and purity was obtained using an Agilent 2100 Bioanalyzer (Agilent Technologies, Mississauga, ON). cDNA was generated from 500 ng of RNA per reaction and RT-qPCR was performed using the SuperScript™ III Platinum® Two-Step RT-qPCR Kit as per the manufacturers recommendations (Invitrogen, Carlsbad, CA). Each qPCR reaction consisted of 1 µl of 500 nM of primers, 2 µl of cDNA that had been diluted 2.5X, 9.5 µl of ddH2O, and 12.5 µl of SYBR Green Supermix UDG for a final volume of 25 µl. Each sample was run in duplicate. Table 4.3 shows a list of primers that were used for mRNA quantification. Primers not listed in Table 3 were obtained from the geNORM kit and primer sequences from this kit are proprietary. The geNorm kit is a commercially available kit comprising of reference gene real time PCR assays for various species.

Samples were analyzed using a Bio-Rad iCycler iQ (Bio-Rad, Hercules, CA) at the following conditions: 95° for 3 minutes, 45 cycles each of 15 seconds at 95° (denaturation), 30 seconds at 60° (annealing) and 30 seconds at 72° (elongation). Melting curves of the products were used to evaluate the specificity of the reaction.

The quantification cycle (Cq) or cycle threshold values for all of the samples per cell type were obtained. Subsequently, the geometric mean of the Cq value is subtracted from the individual sample Cq value. The relative quantity ($2^{\Delta Cq}$; relative to other samples within the same run for the same gene) was then calculated and the file uploaded into geNORM to obtain the stability of gene expression or M value. To calculate the M value, firstly the ct value for each sample was obtained. Then for each gene the geometric average was determined and subtracted from each sample. The relative expression was then determined and this value was uploaded into geNORM. geNORM software is available at no cost and is accessible from: http://medgen.ugent.be/~jvdesomp/genorm/ Using geNORM an M value was computed (in depth calculations are described: http://medgen.ugent.be/~jvdesomp/genorm/example_calculations.xls). This M value was a measure of gene-stability derived by examining the standard deviation of control genes[185]. A cut-off value of 0.5 was used based on and any gene under this cut-off value is considered as stably expressed [184].

4.4 Results

The use of proper reference genes for RT-qPCR is important for the relevant quantification of genes of interest and takes into account variations in cDNA quantities between samples. When selecting reference genes, expression stability is critical and should be equally stable in control and stimulated cells. Given the importance of reference genes for expression analysis of immune effector functions, we examined various cell types including PBMC, T cells, monocytes, MoDCs and BDCs either alone or following stimulation with LPS. ACTB, ATP5GA, EF-1, GAPDH , RPL-19 and SDHA genes were examined to determine which genes are suitable candidates to be used as reference genes.

cDNA dilutions were used to generate a standard curve. For each gene, the correlation coefficient of the standard curve was >0.99. A PCR efficiency curve was determined from the slope using the equation $(10^{[-1/M]}-1)$ x100 and ranged from 90-110%.

When control cells were examined alone, the 2 most stably expressed reference genes for each cell type were as follows: in PBMC: GAPDH and B-actin (Fig.4-1a); in T cells: RPL19 and GAPDH (Fig.4-1b); in monocytes: RPL19 and GAPDH (Fig.4-1c); in BDCs: RPL19 and ATP5Ga (Fig.4-1d); and in MoDCs: RPL19 and B-actin (Fig.4-1e). In control cells, the only reference gene that had an M value > 0.5 was ATP5GA in monocytes, and therefore we recommended against its use.

In all of the cells types examined, stimulation with LPS affected the stability of the two most stably expressed gene in control cells (Fig. 4-1f-j). These results confirm the importance of testing the expression stability of reference genes for each stimulus used.

When control and stimulated cells were examined together, the most stably expressed reference genes (i.e. lowest M values) were as follows: in PBMC: GAPDH = B-actin < SDHA <RPL19 <ATP5GA < eEF-1 (Fig. 4-1k); in T cells: RPL19 = SDHA < GAPDH < B-actin < ATP5GA < eEF-1 (Fig. 4-1l); in monocytes: RPL19 = B-actin < GAPDH < SDHA < eEF-1 < ATP5GA (1m); in BDCs: SDHA < RPL19 < GAPDH < B-actin < ATP5GA < eEF-1 (4-1n); and in MoDCs, RPL19 = ATP5GA < SDHA < eEF-1 < GAPDH < B-actin (4-1o). Based on these findings our recommendation for LPS

stimulation is to use in PBMC: GAPDH and B-actin; in T cells: RPL19 and SDHA; in monocytes: RPL19 and B-actin; in BDCs: RPL-19 and SDHA; and in MoDCs: RPL-19 and ATP5GA. Of the cell types examined, the reference genes that do not match the required stability are eEF-1 BDCs with an M value >0.5 and ATPG5A in monocytes (M value =0.498).

In conclusion, we have demonstrated the stability of expression of the reference genes ACTB, GAPDH, RPL-19 and SDHA in porcine cell lines with recommendations of which reference genes should be used when LPS is used as a stimulus. These data should be beneficial for porcine research teams in validating their RT-qPCR data.

Table 4-1 Functional classes of reference genes

Abbreviation	Gene name	Function
ACTB	beta-actin	Cytoskeletal protein
ATP5G1	ATP synthase, mitochondrial F0 complex	Subunit of mitochondrial ATP synthase
B2M	beta-2-microglobulin	Beta-chain of major histocompatibility complex class I molecules
eEF-1	eukaryotic elongation factor 1 gamma-like protein	Role in protein translation
GAPDH	glyceraldehyde 3-phosphate dehydrogenase	Role in glycolysis and gluconeogenesis
HMBS	hydroxymethylbilane synthase	Heme synthesis
HPRT1	hypoxanthine phosphoribosyltransferase I	Purine synthesis
RPL19	Ribosomal protein L19	Component of ribosomal subunit
RPL4	ribosomal protein L4	Component of ribosomal subunit
SDHA	succinate dehydrogenase complex subunit A	Electron transport
TOP2B	topoisomerase II beta	Role in DNA transcription and replication
TPB	TATA box binding protein	RNA polymerase II transcription facots
YWHAZ	tyrosine 3-monooxygenase/tryptophan 5-monoxygenase activation protein zeta polypeptide	Involved in signal transduction

Table 4-2 Summary of studies examining porcine reference genes

References genes	Porcine tissues examined	Stimulation	Reference
ACTB, HPRT and GAPDH	Alveolar macrophages, spleen cells, bone marrow, brain, kidney, lung, Peyer's patches, ileum, mesenteric lymph node muscle, large intestine and thymus	Con A or PMA	[186]
ACTB, cyclophillin, GAPDH, and HPRT	PBMC	PMA	[187]
ACTB, HPRT, GAPDH, and cyclophilin	PBMC	LPS and Con A	[188]
ACTB, B2M, GAPDH, HMBS, HPRT1, RPL13A, SDHA, TBP, TOP2B and YWHAZ	Backfat and longissimus dorsi muscle	No stimulation	[189]
ACTB, B2, GAPDH, HMBS, HPRT1, RPL4, SDHA, TPB, and YWHAZ	Liver, kidney, thymus, brain, lymph nodes, muscle, heart, skin, pancreas, bone marrow, bladder, lung and stomach	No Stimulation	[185]

Table 4-3 Reference genes used in this study

Gene symbol	Accession Number	Amplicon length	Ta
ACTB	geNORM kit	130	60°C
ATP5G1	geNORM kit	145	60°C
eEF-1	geNORM kit	75	60°C
SDHA	geNORM kit	88	60°C
GAPDH	DQ845173	170	60°C
RPL19	AF435591	147	60°C

Primer sequences were supplied by PrimerDesign Ltd, UK, with the exception of GAPDH and RPL19 whose respective sequences('5→3') are: CTTCACGACCATGGAGAAGG; CCAAGCAGTTGGTGGTACAG; and AACTCCCGTCAGCAGATCC; AGTACCCTTCCGCTTACCG.

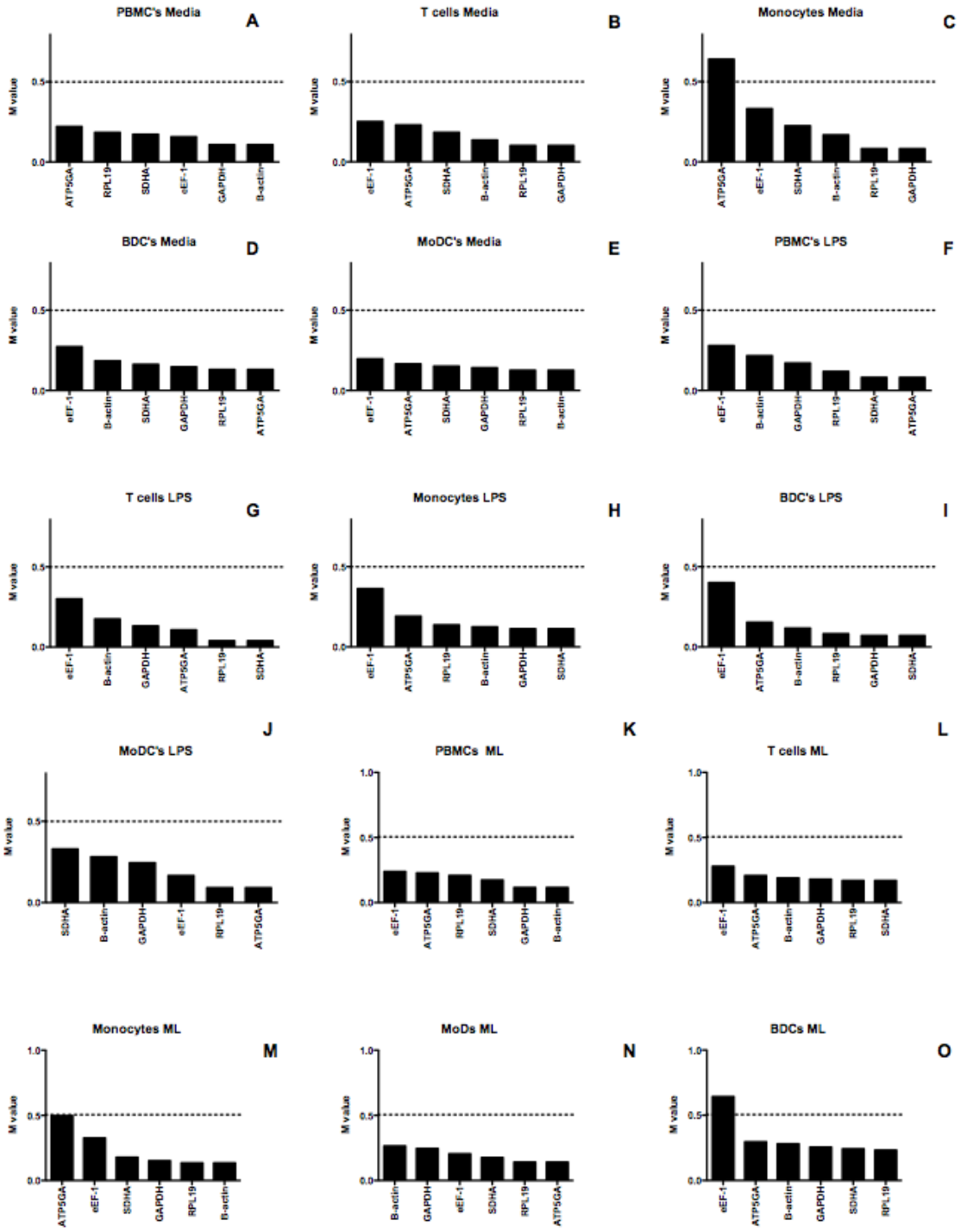
PBMC's Media
A
M value
0.5
0.0
ATP5GA
RPL19
SDHA
eEF-1
GAPDH
B-actin
T cells Media
B
eEF-1
ATP5GA
SDHA
B-actin
RPL19
GAPDH
Monocytes Media
C
ATP5GA
eEF-1
SDHA
B-actin
RPL19
GAPDH
BDC's Media
D
eEF-1
B-actin
SDHA
GAPDH
RPL19
ATP5GA
MoDC's Media
E
eEF-1
ATP5GA
SDHA
GAPDH
RPL19
B-actin
PBMC's LPS
F
eEF-1
B-actin
GAPDH
RPL19
SDHA
ATP5GA
T cells LPS
G
eEF-1
B-actin
GAPDH
ATP5GA
RPL19
SDHA
Monocytes LPS
H
eEF-1
ATP5GA
RPL19
B-actin
GAPDH
SDHA
BDC's LPS
I
eEF-1
ATP5GA
B-actin
RPL19
GAPDH
SDHA
MoDC's LPS
J
SDHA
B-actin
GAPDH
eEF-1
RPL19
ATP5GA
PBMCs ML
K
eEF-1
ATP5GA
RPL19
SDHA
GAPDH
B-actin
T cells ML
L
eEF-1
ATP5GA
B-actin
GAPDH
RPL19
SDHA
Monocytes ML
M
1.0
ATP5GA
eEF-1
SDHA
GAPDH
RPL19
B-actin
MoDs ML
N
B-actin
GAPDH
eEF-1
SDHA
RPL19
ATP5GA
BDCs ML
O
eEF-1
ATP5GA
B-actin
GAPDH
SDHA
RPL19

Figure 4-1 Stability of expression of reference genes in porcine PBMC, T cells, monoyctes, MoDCs and BDCs.

Stability of gene expression of eEF-1, ATP5GA, RPL19, SDHA, GAPDH and β-actin were examined in (a) PBMC control samples (b) T cells control samples (c) Monocytes control samples (d) MoDCs control samples (e) BDCs control samples (f) PBMC treated with LPS (g) T cells treated with LPS (h) Monocytes treated with LPS (i) BDCs treated with LPS (j) MoDCs treated with LPS (k) PBMC control and LPS treated samples (l) T cells control and LPS treated samples (m) Monocytes control and LPS treated samples (n) BDCs control and LPS treated samples (o) MoDCs control and LPS treated samples. LPS was used at a concentration of 1 μg/ml with a stimulation time of 6 hours. Samples were assessed for changes in gene expression stability by RT-qPCR and then using the software geNORM. Results are shown as an M value (n=4 animals for controls and treatment group except for the PBMC treatment group in which n=3 animals). M values < 0.5 indicate stable expression with a lower value being ideal.

4.5 Conclusion

In Chapter four, we describe which genes are stably expressed in porcine MoDC and BDC subsets. With the successful generation and characterization of porcine MoDCs and BDCs [82, 190], the next objective was to determine the *in vitro* effects of HDPs on DC cytokine production. For this purpose 3 peptides namely HH2 (N-VQLRIRVAVIRA-C),, HH17 (N-KIWVRWK-C) and HH18 (N-RLCRIVVIRVCR-C) were initially selected. Preliminary experiments examined peptide safety, effects on DC cell surface marker expression, maturity and cytokine production. The effects of the HH peptides were examined using a low dose of 5 μg/ml and a higher dose of 133 μg/ml. No differences were seen in flow cytometry for the percent of cells positive for CD172a, MHCII, CD16, MHCI, CTLA, CD1 and CD14 expression as well as for their mean fluorescence intensity (Appendix A). Also, no changes were observed in the expression of CD80, CD86, CCR7, MHCI and MHC2 by RT-qPCR in MoDCs and BDCs (Appendix A). The production of various cytokines was examined in both MoDCs and BDCs with minimal changes observed. Given the results obtained by stimulating DCs with HH2, HH17 and HH18 at 133 μg/ml it was not possible to detect a clear a Th1 type response (i.e. IL-12 production as detected by ELISA and confirmed by RT-qPCR).

At this stage we postulated that the lack of an effect attributed to the peptides might have been attributed to either an inability of our porcine DC populations to be challenged, to the nature of the selected HH peptides or to a need for the peptides to act synergistically with TLR ligands. The functionality of the DC populations did not appear to be factor contributing to the lack of effect that we were observing. Experiments in our lab demonstrated that porcine MoDCs and pDCs could be modulated by various TLR ligands including poly I:C (TLR3 ligand), LPS (TLR4 ligand), flagellin (TLR5 ligand), Imiquimod (TLR7 ligand), class A (8954) and class C (10103) CpG ODN (TLR9 ligand)[92]. Results showed that Poly I:C, LPS, flagellin and Imiquimod induced a significant increase in the percentage of MoDCs expressing CD80/86 and that Flagellin, LPS, poly I:C, and imiquimod but not CpG ODNs induce a significant increase in the percentage of BDCs expressing CD80/86. No changes were observed in the expression of MHCII. Furthermore some of these ligands were able to increase CCR7 in both MoDCs and BDCs as well as influence cytokine production [92].

In order to determine if the HH peptides selected did not immunomodulate porcine DCs, a wider array of natural peptides was selected. This led to subsequent studies (Chapters 5 and 6) in which a panel of naturally-occurring peptides was examined for their ability to influence immune functioning in PBMC. Furthermore, in-house vaccination experiments had demonstrated that the peptide IDR-1002 had significant potential as an adjuvant [156]. Therefore, we focused on the peptide IDR-1002 and naturally-occurring peptides and screened them for their effects in PBMC. The switch in cell type from DCs to PBMC was made in order to ensure that more cell types were available to respond to HDPs either via cell-cell interactions or via the production of modulatory factors.

Chapter 5 CHEMOATTRACTIVE ACTIVITY OF HDPS ON PORCINE IMMUNE CELLS (manuscript in preparation)

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All authors participated in the design of the experiments and contributed to writing of the manuscript. Auray, Buchanan and van Kessel assisted in sample collection. Uwiera analyzed slides for the histological examination of cell recruitment. All other data presented in this manuscript are the work of the thesis author.

5.1 Abstract

Host defense peptides (HDPs) are potent immunomodulators that can induce cytokine and chemokine production, recruit dendritic cells, as well as stimulate and modulate antigen-specific T cell responses. In order to detect if selected HDPs have an effect on porcine immune cells, we tested the ability of HDPs from various species to recruit porcine PBMC both *in vitro* and *in vivo* and to induce cytokine and chemokine expression in these cells. The HDPs examined included a synthetic innate defense regulator (IDR) peptide 1002, the porcine cathelicidins PR39 and Protegrin-1 (PG-1), the human cathelicidin LL-37, the bovine cathelicidin Indolicidin and the porcine beta-defensin (pBD)-1 and pBD-2. Our results demonstrate that IDR-1002, PG-1, Indolicidin, pBD-2, LL-37 enhanced IL-8 production in porcine PBMC, while IL-12 production levels were increased by PG-1. The peptides IDR-1002 and LL-37 both were able to chemoattract porcine PBMC *in vitro* however, this effect was not observed following the intradermal administration of HDPs *in vivo*.

5.2 Introduction

Host defense peptides (HDPs) are small peptides ranging in size from 5-60 amino acids in length. Cationic HDPs can be divided into two main families, namely, defensins and cathelicidins based on the respective presence of β-sheets and α-helices [191]. HDPs display heterogeneity in their structure, regulation, function, and site of action, and are present in both plants and mammals [192]. Also known as antimicrobial peptides, many of these cationic peptides are able to bind to the negative charge found on bacterial cells walls [193], thereby allowing them to have a wide spectrum of anti-bacterial activity. More importantly, HDPs have a number of immunomodulatory functions including induction of cytokine and chemokine production, recruitment and activation of important immune cells such as dendritic cells, monocytes, neutrophils to name a few [194].

Recruitment occurs either directly or indirectly by inducing chemokine production. Both cathelicidins and defensins recruit a wide array of immune cells. For example, hCAP, the propeptide of the human cathelicidin LL-37 has been shown to recruit human monocytes [135], neutrophils [135-137], dendritic cells (DCs) [136] and T cells [135, 138]. The human beta defensins 1-4 were chemotactic for human macrophages [139], mast cells [139], immature DCs [140], $CD4^+/CD45RA^+$ naive and $CD8^+$, but not $CD4^+/CD45RO^+$ memory T cells [140, 141]. Human α- and β-defensins were also chemotactic for human monocyte-derived DCs [142]. In cattle, bovine β-defensins recruited monocyte-derived DCs (MoDCs) [195]. Interestingly, the ability to recruit immune cells is highly conserved amongst some HDPs and in fact may work across species. For example, the human cathelicidin has been shown to recruit rat mast cells [143] and the mouse cathelicidin CRAMP was chemotactic for human monocytes, neutrophils and macrophages [149].

Cell recruitment is an important feature of adjuvants and critical for their ability to propagate immune responses. For example, MF59, the adjuvant used in several H1N1 influenza vaccines, exerts adjuvanticity by recruiting macrophages to the site of antigen deposition [196, 197]. Therefore, the ability of HDPs to directly recruit immune cells may represent a key strategy for the proposed adjuvant effects of HDPs. In addition,

HDPs have been shown to increase chemokine production, which indirectly results in the recruitment of immune cells. For example, LL-37 increased IL-8 production in human airway smooth muscle cells [198], human epithelial cells [199], murine macrophages [199], monocytes [200] and neutrophils [201]. Similar to LL-37, defensins also induced similar increases on IL-8 production in lung epithelial cells [202], which subsequently can lead to the recruitment of neutrophils and subsequent propagation of immune responses. In the present study we assessed the ability of selected HDPs to stimulate porcine dendritic cells *in vitro* and *in vivo*. The pig is a good research model due to its immunophysiological similarities to humans [64]. Results obtained in the pig model are often more predictive of outcomes in humans and therefore more relevant in the development of novel therapeutics [203]. For example, due to structural similarities with human skin and in particular the ratio of the epidermis and the ratio of dermal-epidermal thickness [203], we are studying the *in vivo* recruitment of porcine immune cells into the skin following intradermal administration of HDPs.

IDRs have been shown to be effective adjuvants when used in combination with various vaccine formulations in pigs [154-156, 204]. However, little is known about the ability of HDPs to modulate porcine immune cells, therefore the goal of this study was to examine the immunomodulatory functions of HDPs from various species on pig immune cells. These functions included the ability to induce cytokine production as well as the recruitment of porcine immune cells both *in vitro* and *in vivo*. Pigs express a variety of HDPs including 13 β-defensins but no α-defensins [113], hepcidin, liver-expressed antimicrobial peptide -2, PGRP-S, NK-lysins, proline-rich lactoferricins and mucosal SP-B to name a few. There are 11 porcine cathelicidins which comprise of the proline-arginine-rich 39-amino-acid peptide (PR-39), proline-phenylalanine-rich prophenin-1 (PF-1) and PF-2, cysteine-rich PG 1 (PG-1) to PG-5, and three porcine myeloid antimicrobial peptides (PMAP)-23, PMAP-36 and PMAP-37 [113]. The HDPs selected in this study include the porcine cathelicidins PG-1 and PR-39 and pBD-1; the innate defense regulator (IDR)-1002; the human cathelicidin LL-37 and the bovine cathelicidin indolicidin. Of these peptides, only PR-39 has been examined and demonstrated to recruit porcine neutrophils *in vitro* [205]. Our goal was therefore to characterize the effects of

these HDPs and examine if *in vitro* production of IL-8 and recruitment translates into *in vivo* recruitment.

5.3 Materials and Methods

Animals

Eight-week old Dutch Landrace pigs were purchased from Saskatoon Prairie Swine Centre, University of Saskatchewan. Experiments were performed in accordance with the ethical standards set by the University of Saskatchewan and the Canadian Council for Animal Care.

Isolation and stimulation of PBMC

Blood was collected by cardiac puncture from each pig using EDTA-coated syringes and blood mononuclear cells were isolated using a 60% Ficoll-Paque™ Plus gradient (GE Healthcare, Uppsala, Sweden). PBMC were maintained in RPMI 1640 (Gibco, Grand Island, NY) supplemented with 10% FBS, 0.5 mM β-Mercaptoethanol, 10% Antibiotic/Antimycotic (Gibco, Grand Island, NY), 10% HEPES (Gibco, Grand Island, NY) and 10% MEM Non Essential Amino Acids (Gibco, Grand Island, NY) and incubated in humidified air containing 5% CO_2.

Peptides

PR39, pBD-1, pBD-2 were synthesized at the Vaccine and Infectious Disease Organization, Saskatoon, SK, Canada as described by Elahi et al. (2006) [122], PG-1 and LL-37 were purchased from ChemPep Inc (Miami, Florida) and Indolicidin and IDR-1002 were synthesized by using F-moc chemistry at the Nucleic Acid/Protein Synthesis Unit of University of British Columbia, Vancouver, BC, Canada. For each HDP, its family and the species from which it is derived are listed in Table 5-1.

Table 5-1 Peptide family and species

Peptide	Family	Species
IDR-1002	Newly synthesized peptide	Not applicable
PR-39	Cathelicidin	Porcine
Indolicidin	Cathelicidin	Bovine
PG-1	Cathelicidin	Porcine
pBD-1	Defensin	Porcine
pBD-2	Defensin	Porcine
LL-37	Cathelicidin	Human

HDP cytotoxicity

Cell viability in response to HDPs was evaluated by quantifying the amount of adenosine triphosphate (ATP) present, relative to cells in media using Cell Titre-Glo™ luminescent cell viability assay (Promega, Madison, WI, USA) as per the manufacturers protocol. The assay used to examine cytotoxicity is based on ATP production since non-viable cells do not produce ATP and have enzymes to degrade existing ATP stores. In order to determine at what concentrations the various HDPs are toxic to PBMC, a dose response curve was performed. Various peptides were used in a 24-hour incubation at concentrations of 50, 100 and 200 μg/ml, except for LL-37 which was used at concentrations of 1, 50 and 100 μg/ml. Briefly, 100 μl of stimulated cells (2 $x10^6$ cells/ml) were incubated with 100 μl of mixed Glo reagent, cells were placed on shaker for lysis to occur and the released luminescent was read on a TD21 Luminometer (Turner Designs, Sunnyvale, CA, USA). Results are expressed as a stimulation index (SI) relative to the medium.

Enzyme-linked immunosorbent assay (ELISA) for cytokines

Cytokine secretion were evaluated by incubating $1x10^6$ PBMC in 1 ml of culture medium for 24 hours in 6 well plates (Corning, Corning, NY) with the following peptides: IDR-1002, PR-39, Indolicidin, PG-1, pBD-1, pBD-2 and LL-37 at concentrations of 1, 5, 10, 25, 50 or 100 μg/ml. LPS (100 ng/ml) was also used as a control. Culture supernatants

were collected and concentrations of IL-6, IL-8, IL-12 and TNF-α were assayed using porcine DUOSET® commercial kits as per the manufacturer's instructions (R&D Systems, Minneapolis, MN).

Chemotaxis assay

Migration of PBMC in response to HDPs was assessed using 24-well TRANSWELL® chamber plates (5 μm pore size, Corning) as described [92]. Briefly 600 μl of media contained the peptides was added in the lower chamber of the TRANSWELL® and 5×10^5 PBMC in 100 μl of culture medium were added to the upper chamber. Following a 2-hour incubation at 37 °C in humidified air with 5% CO_2 cells in the lower chamber were harvested and counted on a hemocytometer using Trypan Blue exclusion. Results are expressed as the number of cells that migrated into the lower chamber containing peptides/ by the number for cells that migrated into the lower chamber containing media alone

Immune cell recruitment following intradermal peptide administration

HDPs (LL-37, PG-1, PR-39, pBD-1, Indolicidin and IDR-1002) were intradermally administered to 4 pigs at concentrations of 10 and 100 μg per site in 100 μl of PBS. Saline was used as a negative control and IL-8 ($2*10^{-7}$ M) and LPS (2.5 mg/ml) were used as positive controls. Each pig flank received the same treatments (Figure 5-1) and was used for a time point, namely, 30 minutes and 3 hours. Another experiment was set up in a similar manner except the samples were taken after 17 hours. Samples were collected in formalin, paraffin-embedded and stained with Hematoxylin and Eosin (H and E). Slides were examined by Dr. Richard Uwiera (DVM, PhD., University of Alberta) for immune cell recruitment.

Statistical analysis

Statistical analysis was performed by non-parametric Wilcoxon signed rank test for the cytokine data and single column t-test for chemotaxis (p-value < 0.05). Analysis was performed using the statistical software program GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA).

A B C

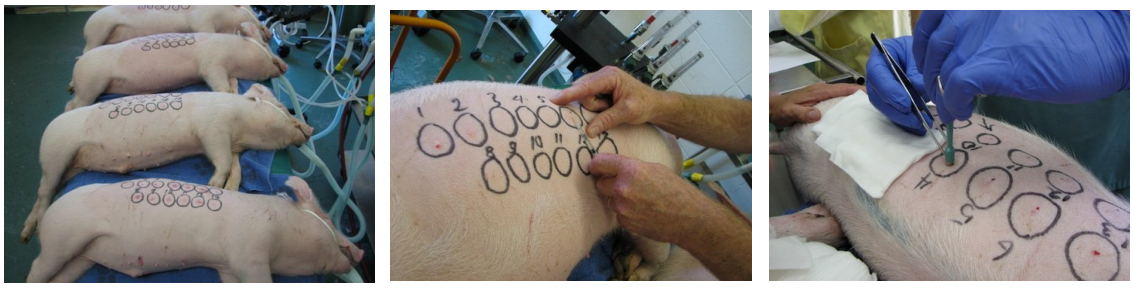

Figure 5-1 Porcine model for intradermal administration of HDPs.

Pigs were anesthetized for 3 hours (A) and intradermally administered HDPs into various sites (B) on either flank. Each flank received the same treatments and was stimulated for 30 minutes on one side and 3 hours on the opposite side. Punch biopsies (5 mm) were taken of the injection site (C).

5.4 Results

LL-37 and PG-1 decrease PBMC viability at high concentrations

The cytotoxic effect of various peptides at different concentrations was examined by measuring ATP production (Figure 5-2). Stimulations were compared to cells in media alone and expressed as a stimulation index (SI). Decreases in ATP production indicated compromised cell viability. Following a 24-hour incubation, the peptides LL-37 at a concentration of 100 μg/ml and PG-1 at concentrations of 100 and 200 μg/ml were cytotoxic to PBMC. The peptides IDR-1002, PR-39, pBD-1, pBD-2 and Indolicidin at concentrations ranging from 50 μg/ml to 200 μg/ml did not decrease PBMC viability and were therefore used at these concentrations in further experiments. The concentrations of

peptides used in this study were comparable to those used in other studies for LL-37 [206], IDR-1002 [206], PG-1 [207], PR-39 [208], pBD-1 [209]and pBD-2 [209].

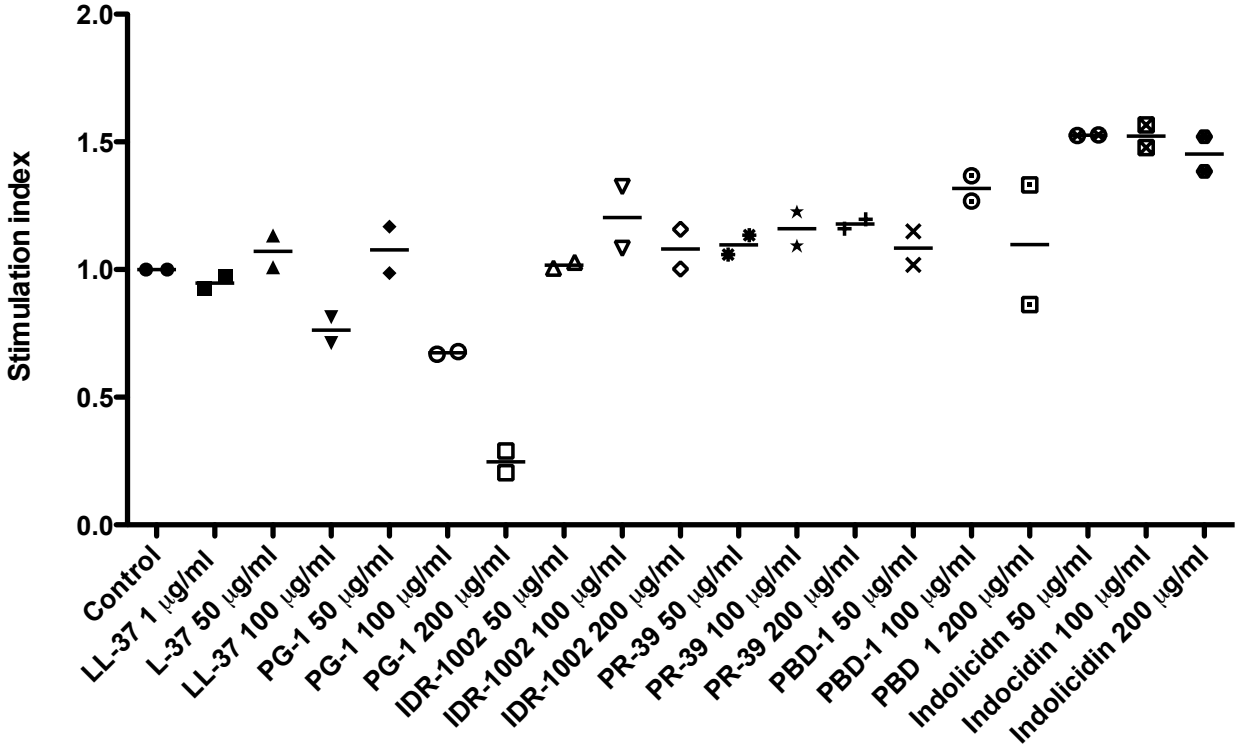

Figure 5-2 Cytotoxicity of HDPs on Porcine PBMC

Cytotoxicity assay following a 24 hour stimulation of PBMC with peptides: IDR-1002, PR-39, Indolicidin, PG-1, pBD-1, pBD-2 and LL-37 at concentrations of (1, 50 and 100 μg/ml). The experiments were performed using 2 pigs and results are shown as the median ± range and expressed as a stimulation index (ratio of stimulated cells/unstimulated cells). Technical triplicates were used.

HDPs increase IL-8 cytokine release

Few studies have examined cytokine production in porcine PBMC following HDP stimulation. Here we looked at the ability of various HDPs to induce production of IL-6,

IL-8, IL-12 and TNF-α in PBMC (Figure 5-3). Using ELISA we measured the presence of cytokines in the supernatant following 24 hours of stimulation with the HDPs. Overall, very low levels of cytokines were detected after stimulations with peptides. However, stimulation with the synthetic IDR-1002 at concentrations of 1 μg/ml and 100μg/ml significantly induced IL-8 production. Interestingly, at concentrations of 5 μg/ml, 25 μg/ml and 50 μg/ml there were no statistically significant difference detectable, although a trend (p=0.057) was evident. IDR-1002 did not induce the production of IL-12 or TNF-α in these cells. Similar to IDR-1002, the peptide PR-39 increased the production of IL-8 at concentrations of 25 μg/ml and 100 μg/ml but had no effect on IL-12 or TNF-α production. The peptide Indolicidin increased IL-8 production at 50 μg/ml and 100 μg/ml with no effect observed for the other cytokines examined. PG-1 at concentrations of 5 μg/ml, 50 μg/ml and 100 μg/ml increased IL-8 protein with the higher concentrations also increasing IL-12 production (Figure 5-4) but no effect on TNF-α production. Interestingly pBD-1 had no effect on the cytokines examined at all, whereas pBD-2 increased IL-8 concentrations at 1 μg/ml, 5 μg/ml and 100 μg/ml.

In summary we found that at concentrations of 100 μg/ml, with the exception of pBD-1, the peptides IDR-1002, PG-1, Indolicidin, pBD-2, PR-39, LL-37 and the lipoglycan LPS statistically increased IL-8 protein concentrations. Of the other cytokines examined, PG-1 induced a statistically significant increase in IL-12 production at a concentration of 100 μg/ml. None of the peptides examined had any effect on TNF-α production but the lipoglycan LPS, stimulated its increase (data not shown).

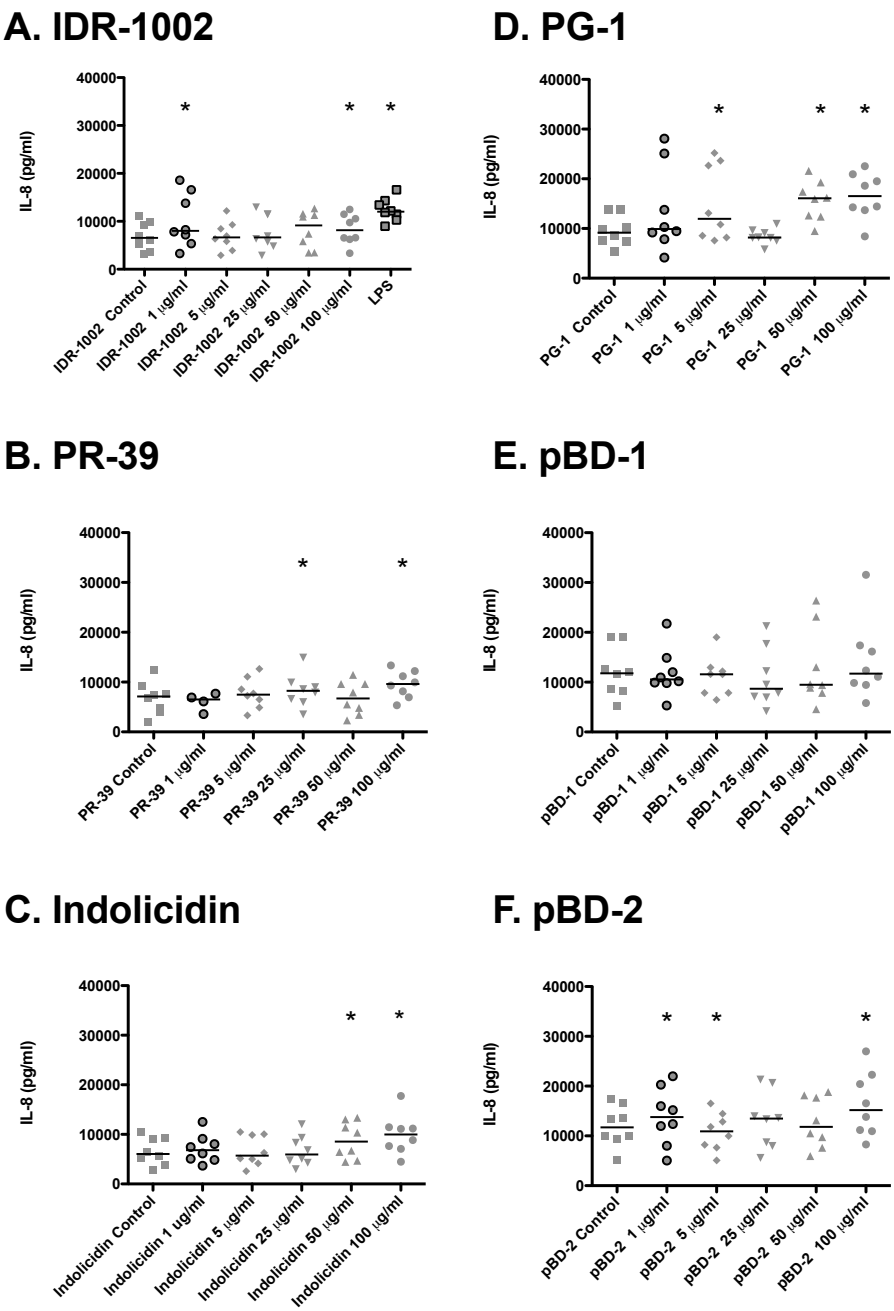

Figure 5-3 HDPs increase IL-8 release in PBMC.

IL-8 production by PBMC following a 24 hour stimulation with peptides and LPS. The following peptides at concentrations of 1, 5, 10, 25, 50 and 100 μg/ml were used: (a) IDR-1002, (b) PR-39 (c) Indolicidin and (d) PG-1 (e) pBD-1 and (f) pBD-2. LPS was used at a concentration of 100 ng/ml. Results are shown as the median and were analyzed by a non-parametric Wilcoxon signed rank test (*$p<0.05$). The experiments were performed using 8 pigs and each data point represents a pig.

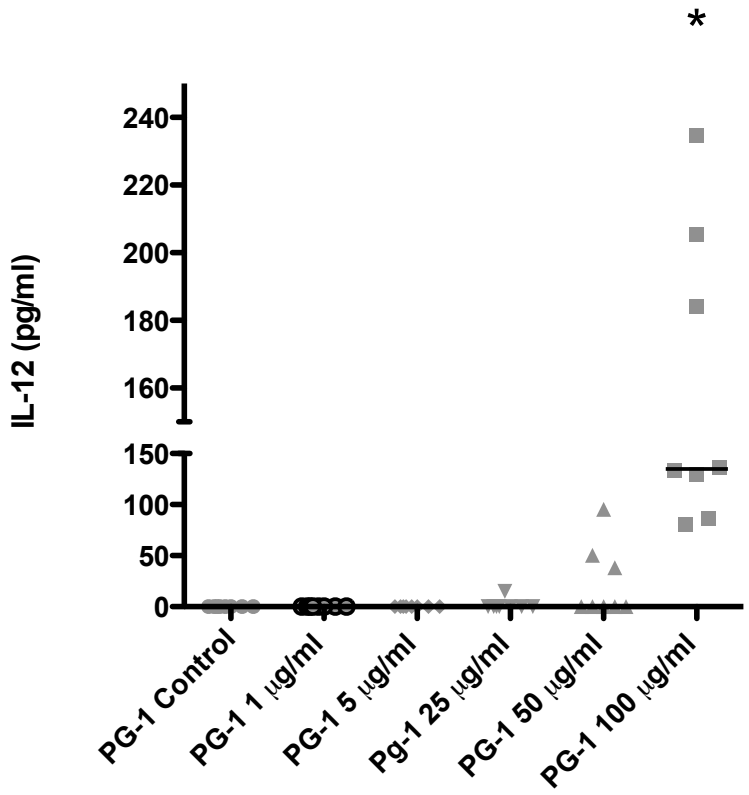

Figure 5-4 PG-1 induces IL-12 production in PBMC

IL-12 production by PBMC following a 24 hour stimulation with the peptide PG-1 at concentrations of 1, 5, 10, 25, 50 and 100 μg/ml. Results are shown as the median value and were analyzed by a non-parametric Wilcoxon signed rank test (*$p<0.05$). The experiments were performed using 8 pigs and each data point represents a pig.

IDR-1002 and LL-37 chemoattract porcine PBMC

Given that various HDPs are known to chemoattract immune cells, we tested the ability of HDPs to chemoattract porcine PBMC by incubating PBMC with various concentrations of HDPs for three hours in a transwell assay system. Results demonstrate that LL-37 (1 μg/ml) and IDR-1002 (100 μg/ml) were able to attract significant number of cells (Figure 5-5). PG-1 (100 μg/ml) (n=12) was also able to significantly chemoattract PBMC (Figure 5-5). Given that at this concentration PG-1 decreased cell viability, a dose response curve with concentrations ranging from 0-200 μg/ml was performed using four

pigs (data not shown). Only at concentrations of 100 μg/ml was PG-1 able to chemoattract PBMC. The peptides PR-39, pBD-1, pBD-2 and Indolicidin had no effect on PBMC recruitment.

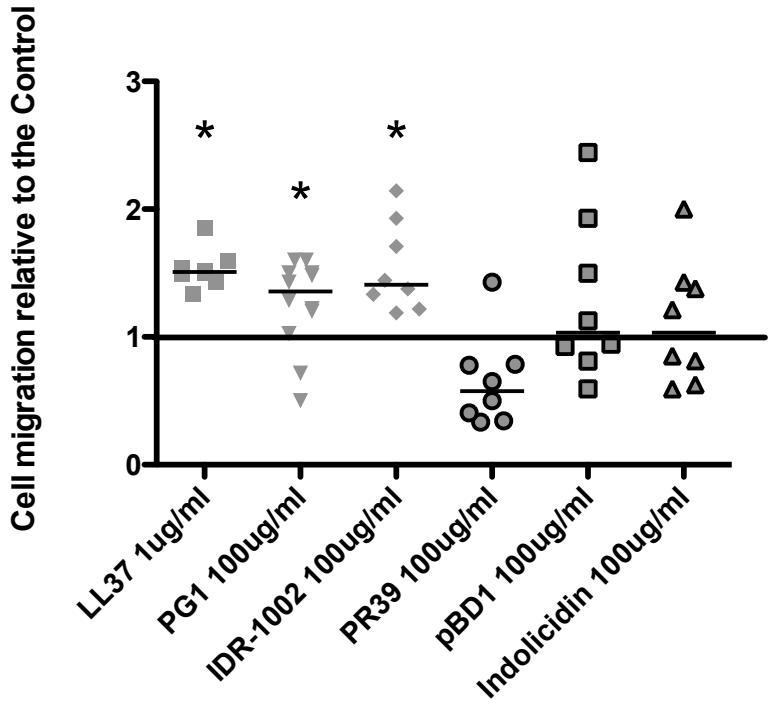

Figure 5-5 HDP chemoattraction of PBMC

The ability of the HDPs IDR-1002 (100 μg/ml), PR-39 (100 μg/ml), Indolicidin (100 μg/ml) and PG-1 (100 μg/ml) to recruit PBMC was examined using a transwell assay system following a 2 hour incubation. Cells that migrated into the lower chamber were counted using trypan blue dye exclusion on a hemocytometer. Results are expressed as the number of cells that migrated into the lower chamber containing peptides/ by the number for cells that migrated into the lower chamber containing media alone and are shown as the median (n= 8 pigs). Analysis was performed using a single column t-test (*p-value < 0.05).

Immune cell recruitment following intradermal peptide administration

Having demonstrated that the peptides IDR-1002, LL-37 and PG-1 were able to recruit immune cells *in vitro* we decided to study their effect *in vivo*. Following the intradermal administration of HDPs skin biopsies were obtained after 30 minutes, 3 hours and 17

hours and stained with H and E (Figure 5-1). Slides were examined for immune cell recruitment to the site of administration. LPS was included as positive control, as it had been previously demonstrated that injection of LPS results in recruitment of neutrophils [210]. Neutrophils were present in the IL-8 and LPS treated samples at 30 minutes (data not shown), 3 hours (Figure 5-6) and 17 hours. These results demonstrate that despite the *in vitro* ability of HDPs to increase the production of IL-8 in PBMCs and their chemotactic ability in PBMC, *in vivo*, HDPs did not recruit neutrophils into the pig dermis.

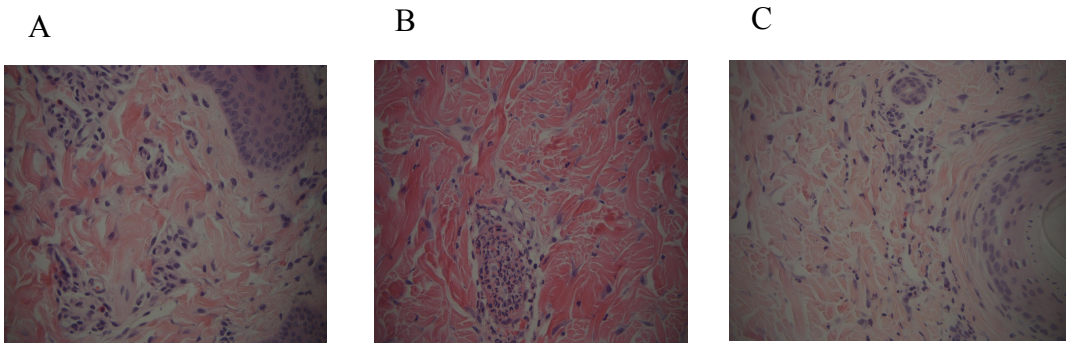

Figure 5-6 Porcine intradermal administration of HDPs.

Pigs were anesthetized for 3 hours and HDPs intradermally administered into various sites on the flank. Each flank received identical treatments and represented a different time point i.e. 30 minutes or 3 hours. Punch biopsies (5 mm) were taken of the injection site. Results are shown for the 3 hour time period: Saline (A) was used as a negative control LPS (2.5 mg/ml) (B) and IL-8 (C) were each used respectively as positive controls. Sections were paraffin embedded and H and E stained. Magnification is 400x. (n=four pigs per treatment with each pig serving as its own control).

5.5 Discussion

Aluminum salts have been the predominant adjuvant present in licensed vaccines until the approval of MF59 as an adjuvant in an influenza vaccine [211]. Important features of adjuvants include both the ability to retain antigen at the site of injection and to recruit

and activate antigen presenting cells [212]. HDPs are suitable candidates for vaccine adjuvants as they induce cytokine production, recruit immune cells and modulate T_H cell polarization [194]. Several studies have demonstrated that HDPs or IDRs co-formulated with vaccine formulations enhanced the immune response to a variety of antigens [151-153, 156, 157]. The goal of the present study was to characterize some of the mechanisms by which HDPs or IDRs modulate immune responses using the porcine model. We demonstrated that HDPs and IDR-1002 induce IL-8 production and recruit PBMC *in vitro*. However, this IL-8 production did not result in the recruitment of neutrophils *in vivo*.

The peptides IDR-1002, PG-1, Indolicidin, pBD-2, PR-39, LL-37 and the lipoglycan LPS increased IL-8 protein concentrations *in vitro*. This is the first study to describe the ability of PR-39, IDR-1002 and PG-1, to produce IL-8 in porcine PBMC. Similar to these results, stimulation of human bronchial epithelial cells and human airway smooth cells with LL-37, pBD-1, pBD-2 and pBD-3 increased IL-8 protein concentrations [198, 202, 209, 213]. We hypothesized that this ability to enhance IL-8 production would translate into increased neutrophil recruitment *in vivo.*

Following the intradermal administration of peptides in this study, no neutrophil recruitment was observed. This observation was surprising given that there is evidence that HDPs, both natural and synthetic, recruit neutrophils both *in vivo* and *in vitro*. For example, it has been demonstrated that the peptide PR-39 recruits porcine neutrophils *in vitro* [205]. Using a mouse model, Nijnik et al. (2010) demonstrated that following the administration of IDR-1002, neutrophils are present in the intraperitoneal lavage within one hour [214]. Also using a mouse model, Chertov et al. (1997) have shown that following the subcutaneous injection of human defensins and CAP37/azurocidin both neutrophil and mononuclear cell infiltrates were present at four and 24 hours [215]. However, it is possible that the time intervals that were used to examine recruitment, the persistence of the peptide *in vivo*, or possibly the nature of the immunization route resulted in less recruitment.

Regarding the time intervals used, in this study we chose a 30 minute, 3 and 17 hour timeframe. At 30 minutes we expected that if the peptides were directly chemotactic then we would observe neutrophils at this timepoint. At 3 hours and 17 hours we anticipated that the peptides might be acting on local or recruited cells that in turn would release IL-8 and recruit neutrophils. If this latter scenario was true then it would have been possible that later time points may have been more suitable. Future studies will examine timepoints extending up to 48 hours. Another consideration is that immune cells such as macrophages or dendritic cells may have been recruited to the injection site at the timepoints used in this study. This limitation could be addressed in future studies by performing immunocytochemistry and using a panel of markers specific to other cells such as DCs and macrophages.

Another reason for the lack of neutrophil recruitment could be that the HDPs did not persist at the immunization site. This seems unlikely given that a recent study demonstrated that when ovalbumin (OVA) and indolicidin were subcutaneously administered in mice, OVA and indolicidin were retained at the immunization site for up to 48 hours [216]. However, it is possible that the chemistry and interaction between OVA and the HDP may contribute to site retention. Further studies are needed to address this interaction.

It is plausible that the nature of the immunization route impacts on the ability of HDPs to recruit immune cells. In previous studies demonstrating the recruitment of immune cells by peptides, either an intraperitoneal route or a subcutaneous route were used. The route of administration may impact on the nature of the immune response demonstrated. For example, in the skin, keratinocytes produce innate immune regulators such as HDPs [217]. Since HDPs are already present in the dermis, further introduction of them alone may not have an effect. It is possible though that in the presence of an antigen that an immune response would then be elicited. Another important observation is that certain skin conditions such as psoriasis are characterized by high levels of HDPs. Therefore during normal conditions it is conceivable, that the skin has a means of regulating the production of HDPs to maintain homeostasis. This postulate may be addressed in future

studies by examining cytokines that are produced around the injection site to determine what type of inflammatory milieu is created if any.

In summary findings from this study demonstrate the interspecies ability of HDPs to increase IL-8 production in PBMC, recruit PBMC *in vitro* but not recruit neutrophils following their intradermal administration of HDPs.

Acknowledgments:

We are grateful to all members of the Animal Care Unit at VIDO for their help in isolating large amounts of blood and for housing the piglets. We are especially thankful to Stewart Walker for his help collecting pig skin biopsies. This work was supported by the Natural Science and Engineering Research Council, the Alberta Agriculture Funding Consortium, CIHR and the Bill and Melinda Gates Foundation through a grant through the Grand Challenges in Global Health Initiative.

5.6 Conclusion

In Chapter five we describe the ability of HDPs to attract PBMC *in vitro* and neutrophils *in vivo*. We demonstrate that the HDPs, LL-37, PG-1 and IDR-1002 recruit PBMC and induce IL-8 production *in vitro*, but are not able to recruit neutrophils *in vivo.* While undertaking these studies we were also examining the effects of intradermally administered IDR-1002 on antigen-specific proliferation. Results from this study are presented in Chapter six.

Chapter 6 DECREASED LYPHOCYTE PROLIFERATION IN PIGS FOLLOWING INTRADERMAL APPLICATION OF THE INNATE DEFENSE REGULATOR-1002 CO-FORMULATED WITH KEYHOLE LIMPET HEMOCYANIN (manuscript in preparation)

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All authors participated in the design of the experiments and contributed to writing of the manuscript. Auray, Buchanan and van Kessel assisted in sample collection. All other data presented in this manuscript are the work of the thesis author.

6.1 Abstract

Host defense peptides (HDPs) demonstrate significant promise as potential vaccine adjuvants. HDPs have a plethora of immunomodulatory functions including the induction of cytokine and chemokine production, recruitment of dendritic cells, as well as stimulation and modulation of antigen-specific T cell responses. In this study, the novel innate defense regulator (IDR)-1002, was examined for its immunomodulatory characteristics in a porcine model. Previously, IDR-1002 was demonstrated to enhance IL-8 protein production and increased leukocyte recruitment *in vitro*. Here, to assess its immunomodulatory effects on antigen-specific proliferation, IDR-1002 was intradermally administered to pigs in combination with Keyhole Limpet Haemocyanin (KLH) antigen and/or CpG oligodeoxynucelotides (ODN). Pigs were vaccinated, boosted after 10 days, and sacrificed 24 days later. IgG serum antibody titres did not differ between the groups

throughout the experiment. At the time of sacrifice spleen cells were isolated and co-incubated with either media, KLH, ovalbumin (OVA) (irrelevant antigen) or Con A. Interestingly, both antigen-specific and mitogen-induced proliferation were lowered in the KLH/IDR-1002 vaccinated animals, even at four weeks post-treatment. These results demonstrate that specific host defense peptides can suppress certain aspects of the pro-inflammatory immune response, making them highly versatile tools to modulate and tailor the immune response to vaccination. Flow cytometry was used to determine if the percentage or constituency of various immune cell populations was altered by IDR-1002. No changes in either the total percentage or mean fluorescent intensity (MFI) of CD3, CD4, CD8, CD21, CD25, CD172a or MHCII were evident. These results suggest that the suppressive effects of IDR-1002 are not attributed to its ability to modify immune cell populations under the conditions used in this study.

6.2 Introduction

Initially host defense peptides (HDPs) were recognized for their microbicidal activity. More recently, research efforts have identified various immunomodulatory functions ranging from protection against pathogens, anti-endotoxic effects, cytokine production, immune cell recruitment and polarization of adaptive immune responses [105]. The ability to tailor adaptive immune responses is a key function that is highly relevant for the development of novel vaccines as well as the development of therapeutic approaches for various diseases. Adaptive immune responses are characterized by several T helper (Th) subsets including Th1, Th2, Th0, Th17 and regulatory T cells (TR). Each of these subsets is categorized by the production of certain cytokines: amongst others Th1 cells produce interferon (IFN)-γ, a cytokine involved in the clearance of intracellular pathogens; Th2 cells produce interleukin (IL)-4, IL-5 and IL-13 and are involved in the clearance extracellular pathogens; Th17 cells produce IL-17 and IL-22 and are implicated in the clearance of fungi; Th0 subsets are known for their ability to produce IL-4 and IFN-γ; and TR cells are known for their production of TGF-β and IL-10 [218].

Various studies have described the role of HDPs in modulating these various types of adaptive immune responses [194]. HDPs can be grouped into two main families of HDPs, which comprise of defensins and cathelicidins, both of which modulate aspects of immune functioning. Using a mouse model, it has been demonstrated that the administration Keyhole Limpet Hemocyanin (KLH) in combination with defensins resulted in higher KLH-specific IgG1, IgG2a and IgG2b antibodies [150]. Also, spleen isolates from defensin treated mice had higher concentrations of IL-4 and IFN-γ as well increased lymphocyte proliferation [150]. The subcutaneous immunization of a formulation comprising of CpG ODNs, polyphosphazene and indolicidin (a bovine host defense peptide), gave rise to higher IgG1 and IgG2a titres and IFN-γ secretion in a mouse model [151]. This group also demonstrated increased IgG titres and IFN-γ secretion using a bovine model, and subcutaneously administering indolicidin, CpG ODN and hen egg lysozyme (HEL), [152]. These studies demonstrate the immunomodulatory capability of HDPs on immune responses.

Interestingly, specific immunomodulatory functions can also be attributed to synthetic HDP derivatives, known as IDRs. The molecules have demonstrated substantial potential as immunomodulators. For example, the intranasal administration to mice of a complex of CpG ODN 10101, IDR-HH2 and detoxified pertussis toxin (PTd) resulted in significantly higher IgA and total IgG titres compared to PTd and CpG ODN 10101 alone or PTd and IDR-HH2 alone [153]. Furthermore, following the intranasal administration of CpG ODN and IDR-HH2, IFN-γ and IL-12 mRNA levels were increased while IL-4 mRNA levels were decreased in the jejunum of neonatal piglets [145]. Another study also demonstrated that CpG ODN and IDR-HH2 combined with attenuated Pseudorabies virus (PRV) vaccine resulted in higher PRV-specific antibodies of IgG2 isotype as well the Th1 cytokines IFN-γ and IL-12 [154]. Similar results were seen in both adult and neonatal mice, whereby the subcutaneous administration of a combination of the IDR-HH2, CpG ODN and polyphosphazenes resulted in higher IgG2a responses [156]. In addition to the effects seen with IDR-HH2, the novel IDR-1002 also demonstrates immunomodulatory capacity. IDR-1002 increased IL-8 cytokine production in pigs [219], recruited porcine PBMC *in vitro* [219], recruited mouse immune cells [214] and

decreased bacterial load in a mouse model of *Staphylococcus aureus* [214]. The effect of a triple combination of PTd, CpG ODN, an IDR and polyphosphazenes was also demonstrated using IDR-1002 in a murine model. Garlapati et al. (2011) demonstrated that the subcutaneous administration of this combination induced higher Th1 type of responses as shown by an increase in the cytokines, monocyte chemotactic protein (MCP)-1, tumor necrosis factor (TNF)-α, IFN-α, IL-12 and IL-17 and decrease in IL-10 concentrations [157].

In this study the effects of intradermal administration of IDR-1002 and KLH to pigs was examined for its potency to modulate antigen-specific proliferation, antibody responses and changes in the constituency of various immune cell populations. While no changes in antibody titres were observed between different vaccination groups, in the KLH-IDR-1002 vaccinated animals antigen-specific and mitogen-induced proliferation was lowered and this was not attributed to changes in the constituency of immune cell populations.

6.3 Materials and Methods

Animals

In this study eight week old Dutch Landrace pigs purchased from Saskatoon Prairie Swine Centre, University of Saskatchewan were used. Experiments were performed in accordance with the ethical standards set by the University of Saskatchewan and the Canadian Council for Animal Care.

Intradermal vaccination of IDR-1002

Pigs at four weeks of age were intradermally vaccinated with 1 mg of Keyhole Limpet Hemocyanin *(*KLH) from *Megathura crenulata* (Sigma Aldrich**)** in 30% emulsigen either alone or with the class C CpG ODN 10101 (TCGTCGTTTTCGGCGCGCGCCG) (150 μg) or IDR-1002 (300 μg). IDR-1002 (VQRWLIVWRIRK-NH_2), was synthesized by F-

moc chemistry at the Nucleic Acid/Protein Acid/Synthesis Unit at the University of British Columbia (Vancouver, British Columbia, Canada) and was resuspended in endotoxin-free water. Every two weeks pigs were boosted for a total of two vaccinations. At the time of sacrifice spleen cells and PBMC were isolated.

Isolation and stimulation of PBMC

Blood was collected by venipuncture from each pig using EDTA-coated syringes for flow cytometric analysis at the time of the first bleed (day0), day two, day 22 and at the time of sacrifice. Blood mononuclear cells were isolated using a 60% Ficoll-Paque™ Plus gradient (GE Healthcare, Uppsala, Sweden). PBMC were maintained in RPMI 1640 (Gibco, Grand Island, NY) supplemented with 10% FBS, 0.5 mM β-Mercaptoethanol, 10% Antibiotic/Antimycotic (Gibco, Grand Island, NY), 10% HEPES (Gibco, Grand Island, NY) and 10% MEM Non Essential Amino Acids (Gibco, Grand Island, NY) and incubated in humidified air containing 5% CO_2.

Lymphocyte proliferation assay

Spleen cells were pulsed with OVA, KLH or Con A for 72 hours in 96-well U-bottom plates (Corning, Corning, NY). During the last eight hours of culture 1 μCi of [^{3}H]thymidine (Amersham Pharmacia Biotech, Baie de Urfe, PQ) was added and proliferative responses were determined. Results are expressed as a stimulation index (SI).

Antibody titres

Microtiter plates (Immulon 2 HB; Dynex Technologies, Chantilly, VA) were coated with KLH 10 μg/ml (100 μl per well) and incubated with sera that were serially diluted. KLH specific IgG was detected using alkaline phosphatase-conjugated goat anti-pig immunoglobulin G (IgG; 1:5,000 dilution; Kirkegaard & Perry Laboratories, Gaithersburg, MD). Biotinylated goat anti-mouse IgG (1:5,000 dilution; Zymed) was

used for reaction amplification. Streptavidin peroxidase (1:5,000 dilution; Jackson Laboratories) *p*-nitrophenylphosphate (Sigma-Aldrich) were used to detected the reaction at 450 nm on a microplate reader (Bio-Rad Laboratories).

Flow Cytometry

The following anti-porcine antibodies were used for assessing immune cell types: CD172a (BL1H7), CD1 (76-7-4), CD3 (PPT3), CD4 (74-12-4), CD8 (PT36B), CD14 (MIL-2), CD16 (MCA1971), CD21 (BB6-11C9.6), MHCII (K274.3G8) and MHCI (SLA-I). All of the antibodies were obtained from Serotec (Oxford, UK) unless otherwise mentioned. Fluorescein isothiocyanate (FITC) anti-mouse immunoglobulins IgG1, IgG2a and IgG2b (Southern Biotech, Birmingham, AL) were used for detection by flow cytometry.

Immunofluorescence staining was performed by incubating $1x10^6$ cells for 20 minutes at 4°C with each antibody. Cells were washed three times with cold PBS (1X) (pH 7.2) (Gibco, Grand Island, NY) containing sodium azide (0.03%) and gelatin (0.02%) and incubated with FITC- conjugated secondary antibody for 20 minutes at 4°C, washed three times and fixed with paraformaldehyde (2%). Ten thousand events were collected and analyzed by flow cytometry (FACScalibur™, CELLQuest™ software; Becton Dickinson, BD Biosciences, Mountain View, CA).

Statistical analysis

Statistical analysis was performed using the statistical software program GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA). For lymphocyte proliferation, results are expressed as a stimulation index (SI) and analyzed by Kruskal-Wallis test. For flow cytometry, to determine treatment effects over time in PBMC, the non-normally distributed data were rank transformed and then analyzed by a 1-way ANOVA (p-value < 0.05). Splenocytes were analyzed by a 1-way ANOVA (p-value < 0.05).

6.4 Results

KLH specific lymphocyte proliferation

KLH-specific *in vitro* proliferation of spleen cells from immunized pigs was determined by examining the uptake of [^{3}H]thymidine. The four vaccinations groups comprised of animals immunized with KLH alone or a co-formulated with either KLH/IDR-1002, KLH/CpG ODN or KLH/ IDR-1002/CpG ODN. Splenocytes from these different vaccination groups were stimulated with either media alone, KLH, OVA or Con A to determine an effect of the treatment.

Splenocytes from animals immunized with KLH, and stimulated with KLH (Figure 6-1A) compared to stimulation with OVA (Figure 6-1B) showed statistically higher proliferation ($p<0.05$). Splenocyte median stimulation indices of KLH stimulation versus OVA stimulation ranged for KLH alone from 16.8 to 1.2; for KLH/ IDR-1002 from 4.2 to 1.1; for KLH/CpG ODN from 11.2 to 1.3; and for KLH/IDR-1002/CpG ODN from 7.9 to 1.1. These results demonstrate that proliferation occurred in an antigen-specific manner.

The effects of stimulation with KLH in the four vaccination groups were as follows: Splenocytes from the KLH immunized animals had a median SI of 16.8 compared to 4.2 in the KLH/IDR- 1002 group, 11.2 in the KLH/CpG ODN group and 7.9 in the KLH/IDR- 1002/CpG ODN group ((Figure 6-1A). A similar trend was observed with Con A stimulation (Figure 6-1C), with SIs for the respective vaccination groups KLH, KLH/ IDR-1002, KLH/CpG ODN and KLH/ IDR-1002/CpG ODN as such: 139.4, 14.2, 42.5, and 33.4. Vaccination groups comprising of IDR-1002 in the formulation had lower SIs compared to KLH alone when stimulated with either KLH or Con A respectively.

In summary, when KLH immunized animals and KLH/ IDR-1002 immunized animals were stimulated with the mitogen Con A, there was a statistically significant decrease in

splenocyte proliferation in the KLH/IDR-1002 immunized animals. The same trend was observed when KLH was used as an antigen.

In KLH immunized pigs, stimulation with the mitogen ConA as compared to KLH, induced a statistically higher amount of proliferation, demonstrating the viability of the assay.

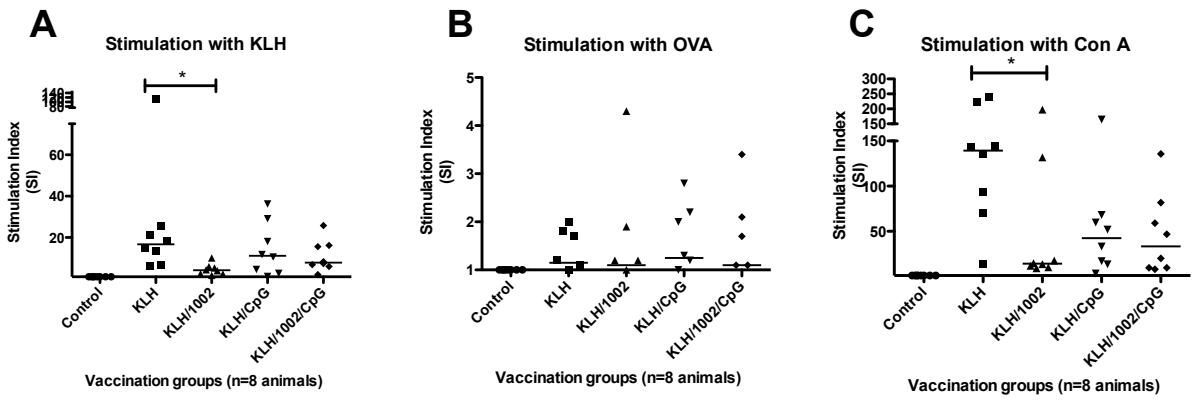


Figure 6-1 Effect of intradermal vaccination with IDR-1002 on antigen specific and mitogen induced lymphocyte proliferation

Splenic lymphocyte proliferation following the intradermal administration of KLH was observed in four vaccination groups namely, KLH, KLH/IDR-1002, KLH/CpG ODN and KLH/IDR-1002/CpG. Eight pigs were used per group, spleens were isolated and then stimulated with either KLH (A), OVA (B) (irrelevant antigen) or *concanavalin (*Con A) (C) (control) for 72 hours.

Antibody titres

To determine if the vaccination groups influenced overall IgG antibody titres, at two weeks post vaccination at the time of the boost, sera was collected from immunized animals and examined for serum IgG levels (Figure 6-2). Animals immunized with KLH and KLH/IDR-1002 had similar antibody titres to animals immunized with KLH/ CpG ODN and KLH/IDR -1002/CpG ODN. Levels of IgG were numerically lower in animals immunized with KLH and KLH/IDR-1002 compared to animals immunized with

KLH/CPG ODN and KLH/IDR-1002/CpG ODN. At the time of sacrifice, this effect was absent and no differences in antibody titres were observed (Figure 6-2).

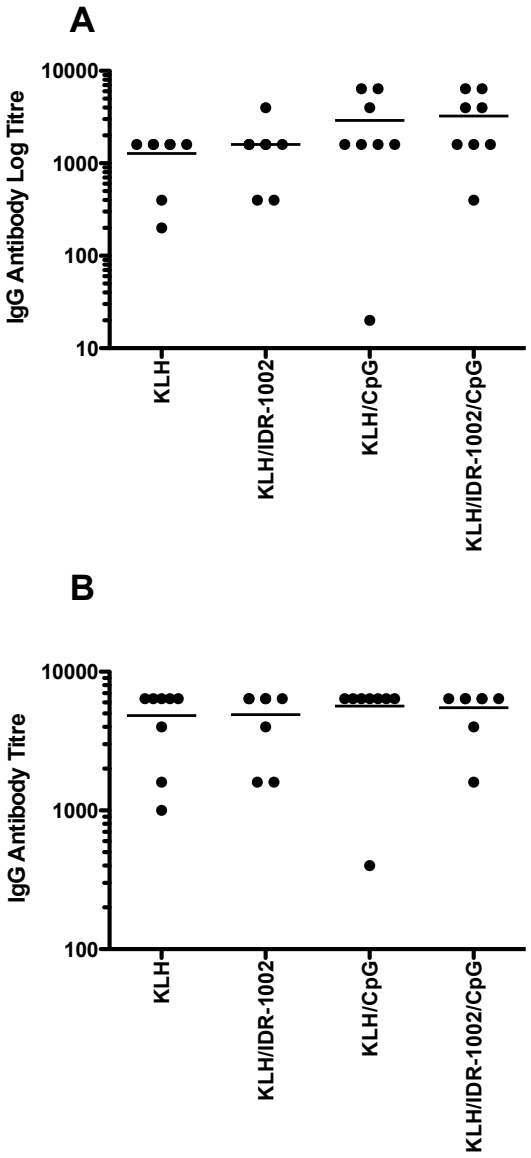
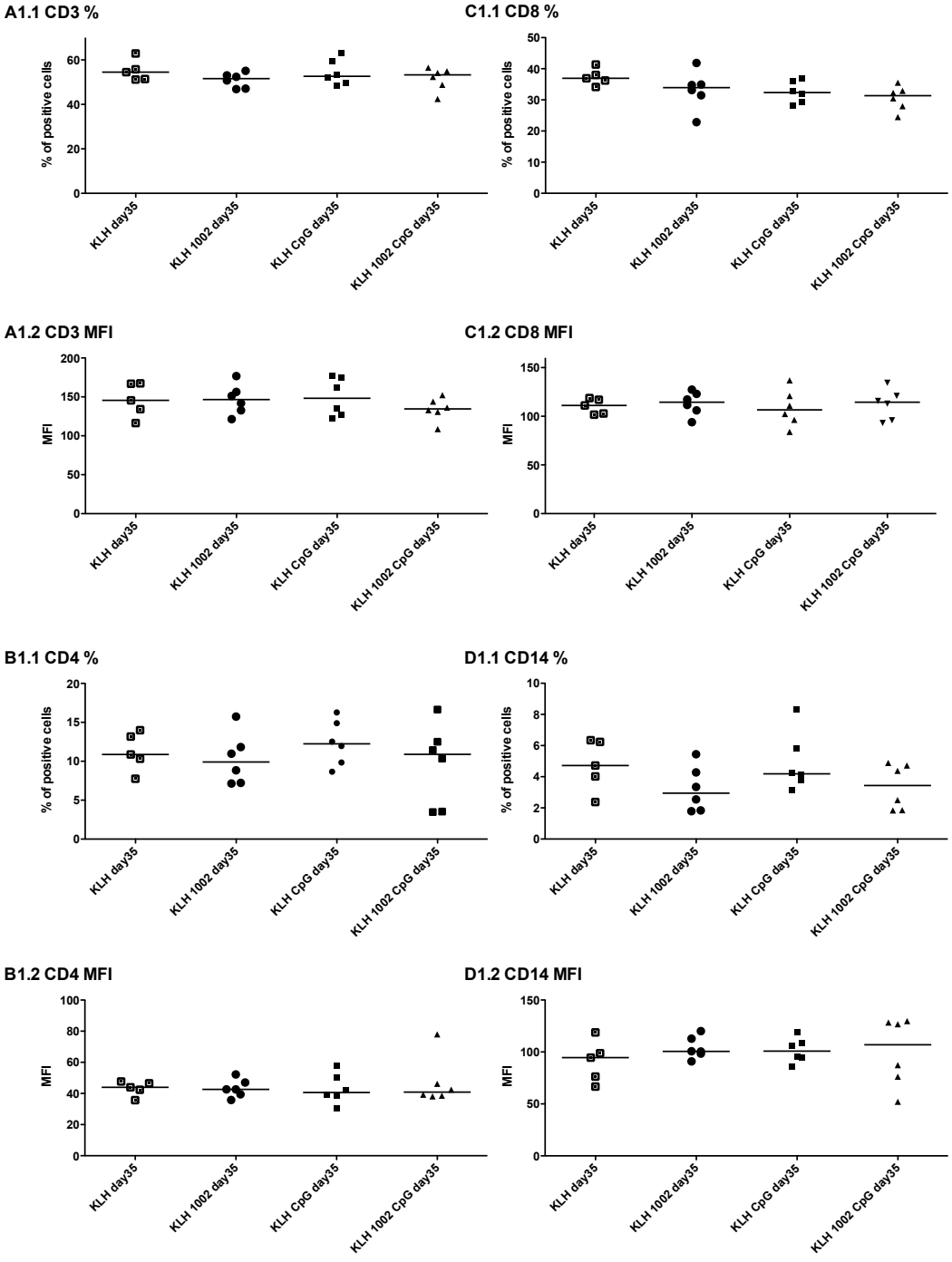


Figure 6-2 IgG antibody titres to immunization with various adjuvants.

Pigs (n=8 per group) were immunized intradermally with KLH, KLH/IDR-1002, KLH /CpG ODN or KLH, IDR-1002/CpG ODN at four weeks of age with an identical immunization given at six weeks. Sera were collected before the vaccination, at the time of the first boost which was 2 weeks later. Results are demonstrated as IgG antibody log titres following the first vaccination (A) and the boost (B).

Flow Cytometry

To determine if the ability of IDR-1002 to lower lymphocyte proliferation was attributed to immune cell populations, we examined the cell surface marker expression of CD3, CD4, CD8, CD14, CD21, CD25, CD172a, MHCII and MHCI in splenocytes from immunized animals. No differences in both overall percentage and mean fluorescent intensity were observed in PBMC (data not shown) or splenocytes (Figure 6-3).


A1.1 CD3 %
% of positive cells
KLH day35
KLH 1002 day35
KLH CpG day35
KLH 1002 CpG day35
C1.1 CD8 %
% of positive cells
KLH day35
KLH 1002 day35
KLH CpG day35
KLH 1002 CpG day35
A1.2 CD3 MFI
MFI
KLH day35
KLH 1002 day35
KLH CpG day35
KLH 1002 CpG day35
C1.2 CD8 MFI
MFI
KLH day35
KLH 1002 day35
KLH CpG day35
KLH 1002 CpG day35
B1.1 CD4 %
% of positive cells
KLH day35
KLH 1002 day35
KLH CpG day35
KLH 1002 CpG day35
D1.1 CD14 %
% of positive cells
KLH day35
KLH 1002 day35
KLH CpG day35
KLH 1002 CpG day35
B1.2 CD4 MFI
MFI
KLH day35
KLH 1002 day35
KLH CpG day35
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D1.2 CD14 MFI
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KLH CpG day35
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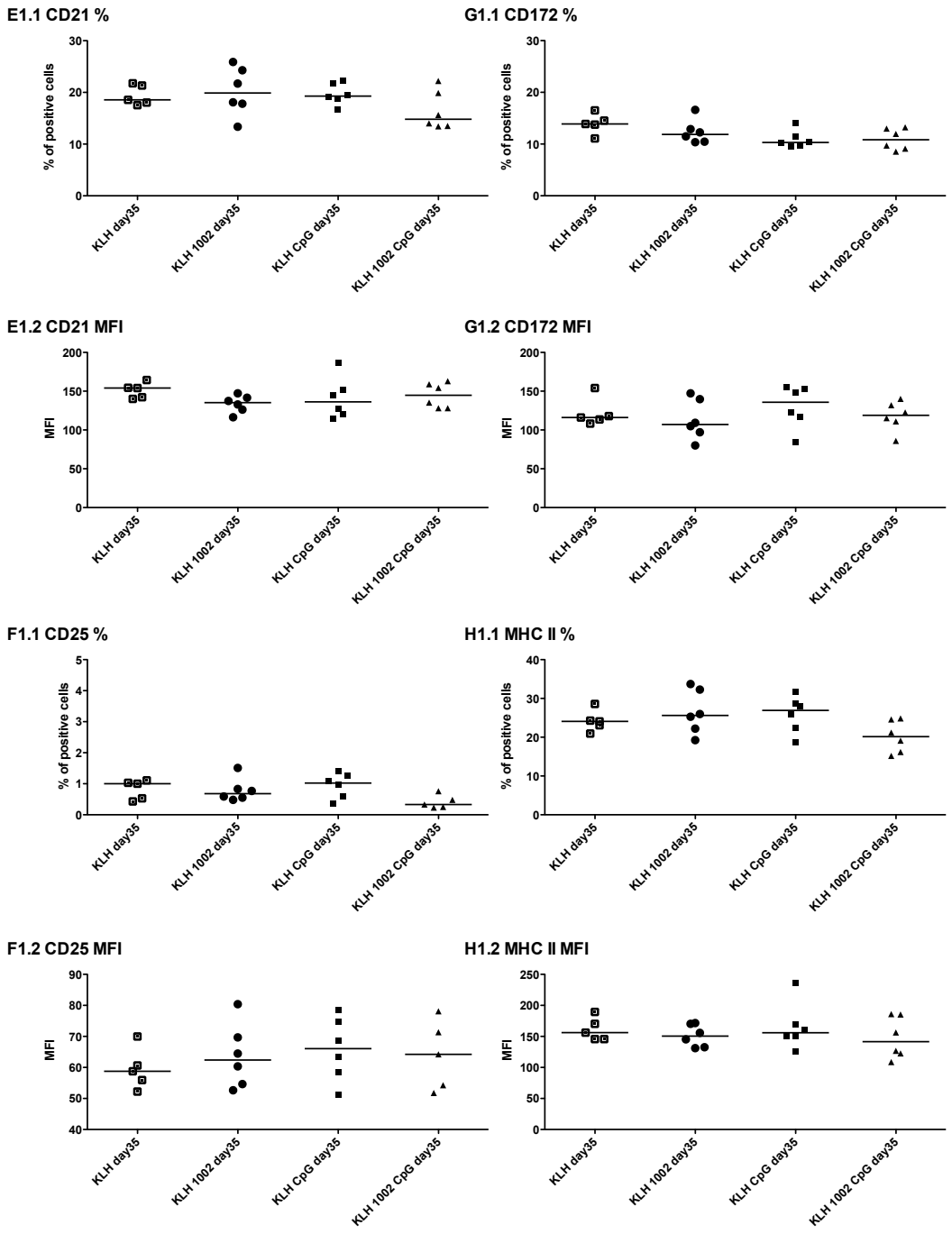

Figure 6-3 Surface phenotype of porcine splenocytes.

At day 35 splenocytes were analyzed by flow cytometry for CD3 (A), CD4 (B), CD8 (C), CD14 (D), CD21 (E), CD25 (F), CD172a (G) and MHCII (H). Data are shown as a median percentage of positive cells (1.1) and mean fluorescent intensity (1.2) for each marker examined. Data for the splenocytes are representative of six pigs from four different vaccination groups of KLH, KLH/IDR-1002, KLH/CpG ODN and KLH/IDR-1002/CpG. Data were analyzed by a 1-way ANOVA ($p>0.05$).

6.5 Discussion

Vaccine research is driven by the need to develop novel vaccines and improve the efficacy of currently existing vaccines via the use of novel adjuvants. Candidates for novel adjuvants include HDPs and IDRs, which have been shown to modulate immune responses [151-153, 156]. We demonstrated that IDR-1002 did not alter overall IgG titres but decreased antigen-specific lymphocyte proliferation. Moreover, we have shown that this effect was not due to changes in the proportion of immune cell populations present. In summary the IDR-1002 demonstrated promise as an immunosuppressive agent.

No other study has examined the effect of the IDR-1002 on antigen specific proliferation. Here we demonstrate that the IDR-1002 decreased antigen specific proliferation. It is important to note that the decreased proliferation was not due to cell death induced by IDR-1002. In the proliferation assay, splenocytes were exposed to either KLH, OVA or Con A and not IDR-1002. Exposure to IDR-1002 only occurred during the vaccinations.

Explanations for this decreased proliferation may include the following: neutrophil uptake of antigen at the injection site, the generation of tolerogenic DCs or the direct action of the IDR-1002 on T cells. In PBMC we demonstrate that the IDR-1002 induces IL-8 production. Therefore, it is conceivable that *in vivo* neutrophils are recruited by the IDR-1002 to the injection site where they might compete for antigen with antigen presenting cells [220]. By decreasing the amount of antigen available, T cell responsiveness may be affected [221]. While this explanation is plausible, it seems unlikely since intradermal administration of the IDR-1002 did not results in neutrophil recruitment at 30 minutes, 3 hours and 17 hours [219].

Another theory for decreased lymphocyte proliferation is the generation of tolerogenic DCs. Tolerogenic DCs can be induced by IL-10 [222]. Stimulation of human monocytes with IDR-1002 gives rise to higher levels of IL-10 [214]. Therefore, it is possible that following intradermal administration of 1002, IL-10 was produced and subsequently induced tolerogenic DCs. Also, skin Langerhans cells have been demonstrated to be

tolerogenic [223]. Thus, it is possible that during intradermal administration of KLH and the IDR-1002 Langerhans cells were rendered tolerogenic. Future experiments to address this possibility would need to address which immune cell type is responding to the vaccination as well as what assessment of the cytokine milieu at the injection site.

A final explanation for lowered lymphocyte proliferation might be that the IDR-1002 acts directly on T cells making them less responsive to proliferative stimuli. Evidence for this explanation arises from studies whereby HDPs decreased lymphocyte proliferation [224]. Schluesener et al. (1993) observed that at doses of 10 μg/ml Indolicidin decreased [^{3}H]thymidine incorporation into T cells and was cytotoxic using a model of rat autoimmunity [225]. In another study, proliferation was reduced in porcine PBMC, which in the presence of Con A, were stimulated with BMAP-27, BMAP-28, or Indolicidin [224]. In the latter study, isolation of porcine $CD4^{+}$/$CD8^{+}$ T cells demonstrated that T cell proliferation was significantly lowered following stimulation with HDPs [224]. This decrease in proliferation was not attributed to cell necrosis but to anergy as ATP production was reduced and CTLA expression increased [224]. Using flow cytometry we examined different immune cell populations. We did not, however, examine CTLA expression, which could have provided more evidence that the immune suppression that we observed was due to anergy as was the case with BMAP-27 and BMAP-28. In Dybvig's study the addition of exogenous IL-2 reversed anergy. To determine if decrease lymphocyte proliferation was due to IDR-1002 inducing T cell anergy, future studies will involve the addition of IL-2 to proliferation assays.

The ability of HDPs to induce regulatory T cells (TR) is demonstrated in a study by Navid et al. (2012), whereby murine β-defensin-14 (mBD-14) induced antigen specific TR [226]. In this study, mice that had been exposed to ultraviolet radiation were sensitized with 2,4-dinitro-1-fluorobenzene (DNFB). In these mice the contact allergen DNFB did not induce sensitization but instead immunotolerance. Ultraviolet radiation induced the expression of mBD-14, which was demonstrated to induce $FOXP3^{+}$ in $CD4^{+}CD25^{-}$ cells. The effects of mBD-14 were demonstrated to be APC independent and IL-10 dependent [226]. This study demonstrates the potential of HDPs to induce TR. In

this context, it would have been interesting to examine the effects of IDR-1002 on T cell phenotype to determine if IDR-1002 induced $FOXP3^+$ expression as well as IL-10 cytokine production.

We did not observe a change in overall IgG titres. Results from other studies support this observation. In mice, it has been demonstrated that following the subcutaneous administration of OVA and the adjuvant indolicidin there was no change on overall IgG titres [151]. Similar results were shown in cattle, using the antigen HEL and the HDP Indolicidin [152]. Contrary to the lack of effect on IgG titres that we observed, in studies where the IDR-HH2 was used, overall IgG titres increased. It was demonstrated that the intranasal delivery of PRV alone or PRV and IDR-HH2 in pigs induced higher overall IgG titres [154]. A similar effect on IgG titres was observed in mice, following the intranasal delivery of PTd alone or in combination with HH2 [153].

Reasons for the discrepancies observed in changes in IgG titres may be several-fold. The ability to induce higher overall IgG titres maybe a function of the HDP or IDR used, the antigen, animal age, animal species and/or route of immunization. Regarding the HDP or IDR used, it appears that HH2 as compared to the IDR-1002 or Indolicidin may have the ability to enhance overall IgG titres. It is important to note that we did not assess IgG1 and IgG2 titres to determine if there was a change in the respective titres. In studies demonstrating an overall change in IgG titres in pigs, the peptide HH2 skewed the immune response towards a Th2 type of response (higher IgG1 titres) [154]. Since we did not see a change in overall IgG titres, it is not likely that there was a change in IgG1 or IgG2 shifting. The antigen used and animal age also impact on the production of IgG titres. In studies using CpG as an adjuvant both of these factors play a contributing role.
In three to seven day old piglets, the use of CpG ODN increased IgG titres to PRV following subcutaneous immunization [154, 227]. In our study, the intradermal administration of CpG ODN with KLH to eight week-old pigs did not enhance overall IgG titres. Animal species also impacts on the production of IgG titres. In contrast to some of the pig studies, in cattle, CpG ODN in combination with HEL did not give rise to statistically significantly higher IgG titres [152]. This effect of CpG ODN on IgG titres

was also not observed in BALB/c mice, where the intranasal immunization of PTd alone or in combination with CpG ODN had no effect on IgG, IgG1 or IgG2a titres [153]. Differences in the effect of HDPs or IDRs on overall IgG titres may also be impacted by the route of immunization used i.e. subcutaneous as compared to intranasal or intradermal. Studies demonstrate that the route of vaccination contributes to the nature of the immune response generated. For example, Kaur et al. (2008) demonstrated that inoculation with leishmania promastigotes via the subcutaneous route elicited a Th1 response while the intracardiac route elicited a Th2 response [228]. Using a pig model, Cao et al. (2011) demonstrate that while there was no significant difference in cytokine production between intranasal and subcutaneous route, the latter route induced a stronger IFN-γ and IL-12 response than the intranasal route [154]. Therefore, it is plausible that in our study we are observing an effect related to the intradermal route of immunization. Future studies exploring different routes of immunization will allow us to understand if the immunosuppressive effect of IDR-1002 is related to immunization route.

In conclusion we demonstrate that the IDR-1002 has potential to act in an immunosuppressant manner. Certain diseases such as arthritis are characterized by a pro-inflammatory state. Agents that suppress pro-inflammatory components of the immune system serve as potential immunotherapeutic agents. In summary, using this model of intradermal administration, IDR-1002 demonstrates merit as an immunosuppressive agent and may be useful in the treatment of conditions characterized by pro-inflammatory states.

Acknowledgments:

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Chapter 7 GENERAL DISCUSSION

Dendritic cells are important surveillance cells in the immune system. They are located at sites of pathogen entry where they can sample and process antigens. The presentation of antigens by DCs can elicit potent T cell responses, which in turn links the innate and adaptive immune system [4]. Targeting of DCs and immunomodulation of their function is a key strategy in the design of more effective and novel vaccines [229]. HDPs and IDRs show great promise as vaccine adjuvants. As part of a larger multinational research effort to develop a novel adjuvant platform for neonates, we screened a number of HDPs in porcine, murine and human cells. We hypothesized that selected HDPs could immunomodulate porcine DCs. To this end we characterized two subsets of porcine DCs, namely MoDCs and BDCs. In chapter three we compared these two DC subsets and also described how they responded to stimulation by LPS [82]. Phenotypically MoDCs had a higher expression of CD80/86 and demonstrated higher endocytic activity. Interestingly, MoDCs had higher base concentrations of IL-6, IL-8 and TNF-α proteins than BDCs, but were less responsive to stimulation with LPS. MoDCs were better able to stimulate the proliferation of both naïve and primed T cells whereas BDCs were better able to stimulate the proliferation of primed T cells. Following the successful generation of porcine MoDCs and isolation of BDCs, we further characterized these subsets by examining the stability of reference gene expression in chapter four. This characterization arose from the importance of knowing which reference genes are stably expressed and therefore could be used for expression analysis studies in porcine immune cells. In chapter four, we described our recommendations for which reference genes could be used for expression analysis studies in porcine PBMC, T cells, monocytes, MoDC and BDCs, either alone or following stimulation with LPS.

Following the successful generation and isolation of MoDCs and BDCs, we investigated the effects of HDP treatment on DC cytokine production and chemokine expression using the IDRs HH2, HH17 and HH18. Surprisingly, we were unable to demonstrate any effects of stimulation of the DCs with these peptides. This could be due to a number of reasons including the peptides that were selected, their inability to react with porcine

cells, the cell type being used, or compromised function of the isolated DCs due to the isolation procedure itself. I was involved in some studies with Dr. Auray involving the characterization of porcine MoDCs and BDCs in response to various TLR ligands. We demonstrated that both MoDCs and BDCs responded to TLR ligands [92]. These results showed that it was unlikely that the lack of effect that we were observing using HH peptides was due to compromised function of the DCs. It is possible that TLR ligands are able to modulate DCs in the absence of antigen, whereas HDPs, which are naturally present in many sites in the body, may require extra signals to modulate DCs. We did not explore this possibility with the HH peptides and DCs. To ensure that the lack of effect that we observed was not due to the HH peptides that we had selected, we decided to use different HDPs and to include a variety of naturally-occurring peptides from different species that included the bovine cathelicidin indolicidin, the human cathelicidin, LL-37, porcine defensin pBD-1 and the porcine cathelicidins PG-1 and PR-39. We also included the synthetic peptide, IDR-1002, which by this time was showing promise as an immunomodulater from in-house vaccine trials. We also decided to screen PBMC and not DCs, which are an isolated cell population. This allowed us to assess the effect of HDPs and the IDR-1002 in a mixed cell population containing monocytes, DCs and leukocytes. In chapter five, the ability of these HDPs and the IDR-1002 to induce cytokine production and recruit porcine PBMC was examined [219]. We assessed the expression of cytokines IL-6, IL-8, IL-12, TNF-α. We were the first group to show that the peptides IDR-1002, PR39, Indolicidin, PG-1, pBD-1, pBD-2 and LL-37 enhanced IL-8 production. The peptide PG-1 also increased IL-12 cytokine production. Of the peptides examined we demonstrated that IDR-1002, LL-37 and PG-1 also recruited porcine PBMC *in vitro*. These observations led us to evaluate the *in vivo* effects of intradermal administration of HDPs. While both LPS and IL-8 demonstrated a strong recruitment of neutrophils, none of the peptides had an effect on neutrophil recruitment at the time points examined. It is possible, that the peptides examined might not recruit neutrophils in the absence of antigens. The presence of antigens may provide additional signals, which in the presence of the peptides may signal the immune system's ability to respond. More time points may have been required to examine neutrophil recruitment. Alternatively, the peptides may recruit immune cells other than neutrophils to the

immunization site, such as macrophages or DCs. A final consideration is that the peptides may have been degraded. This issue can be addressed by covalently attaching a tracer to the peptides to detect their stability following immunization.

A major focus of this thesis was to examine the immunomodulatory effect of IDRs. Therefore, in chapter 6 we examined the effects of vaccinating pigs intradermally with KLH alone or in combination with CpG ODN, IDR-1002 or CpG ODN and IDR-1002. We observed a decrease in lymphocyte proliferation with the IDR-1002 and no effect on overall antibody titres or immune cell constituency. Further studies are required to examine the mechanism by which IDR-1002 is able to exert this effect. Reasons for which IDR-1002 decreases lymphocyte proliferation may be as follows: Neutrophil recruitment to the injection site. These neutrophils may compete with APCs for antigen, therefore making fewer antigens available for presentation. Examining the immune cells that are recruited to the immunization site would be important in determining if neutrophils play a role in the effects that we observe. Another reason may be that IDR-1002 renders DCs tolerogenic. These DCs would be unable to stimulate T cell proliferation. This explanation seems unlikely as no changes in the percentage of T cells was detected by flow cytometry in either PBMC or spleens. Alternatively, IDR-1002 may interact directly with T cells rendering them less responsive or anergic or inducing TR as has been demonstrated with other HDPs [224, 226]. This explanation could be addressed by examining the expression of cell surface marker CTLA and looking for increased production of IL-10.

In summary we have observed HDPs moderate immune responses depending on the context in which they are present. Notably, HDPs demonstrate a dual nature whereby they can enhance immune responses or suppress them. Examples of immune response enhancement by HDPs arises mostly from studies describing the role of HDPs as adjuvants in vaccine formulations [151-153, 156, 157, 230]. In contrast to these effects, HDPs also play an anti-inflammatory role. For example, treatment of human MoDCs with low concentrations of α-defensins1-3 (0.25– 1 μg/ml) enhanced immune responses (increased MoDC expression of costimulatory molecules, cytokine production and T cell

proliferation) whereas stimulation with high concentrations of α-defensins 1-3 (10-20 μg/ml) suppressed immune responses with decreased T cell proliferation [159]. In this study, the importance of HDP concentration as a regulating factor was highlighted. Di Nardo et al. (2007), demonstrated that the intradermal injection of the mouse cathelicidin CRAMP reduced swelling and cell recruitment in response to the application of 2,4-dinitrofluorobenzene [231]. Since DCs can link the innate and adaptive immune system, Di Nardo et al. (2007) examined the effects of CRAMP on TLR induced maturation of DCs. DCs were treated with CRAMP, then washed and exposed to LPS, DCs did not mature and IL-6 production was inhibited. Treatment with cathelicidin in human MoDCs, LPS induced production of IL-6, IL-8, IL-10 and TNF-α were reduced [231]. Also related to modulating LPS induced effects, hBD-3 downregulated TNF-α production in response to the TLR4 agonist LPS in human and mouse macrophages [232]. Semple et al. (2010), also demonstrated that Balb/c mice exposed to LPS in the presence of hBD-3 had lower serum TNF-α concentrations [232]. In human PBMC, LL-37 enhanced IL-1β induced IL-6 and IL-8 production but suppressed IL-6 production when the immune mediators IFN-y, IL-4 or IL-12 were present [233]. LL-37 also inhibited human DC activation by TLR ligands, including LPS, lipoteichoic acid and flagellin [234]. These studies described the immunosuppressant role of HDPs and also the importance of the local microenvironment. For example a change in one modulator can switch the role of LL-37 from being pro-inflammatory to anti-inflammatory or the exposure to a HDP prior to encounter LPS can modify cellular responses. The immunosuppressant role of HDPs may be key in resolving inflammation and preventing tissue damage. This dual nature of HDPs can be explored by administering them in the presence of different modulators to determine whether or not it is possible to orchestrate pro- or anti- inflammatory conditions.

In this study, our work began by examining the effects of HDPs on DC functions. However no strong effects on immune functioning were observed and further studies involved looking at PBMC and eventually examining *in vivo* effects in whole animals. Given the complex interplay between the tissue environment, cell cooperation and individual cells [235], HDP behavior is more completely depicted when it is examined in

this context (Figure 7-1). HDPs can be released from epithelial cells or from various other leukocytes and can recruit other immune cells. Subsequently HDPs in concert with other immunomodulators impact on the functioning of immune cells such as DCs, which then link the innate and adaptive immune systems.

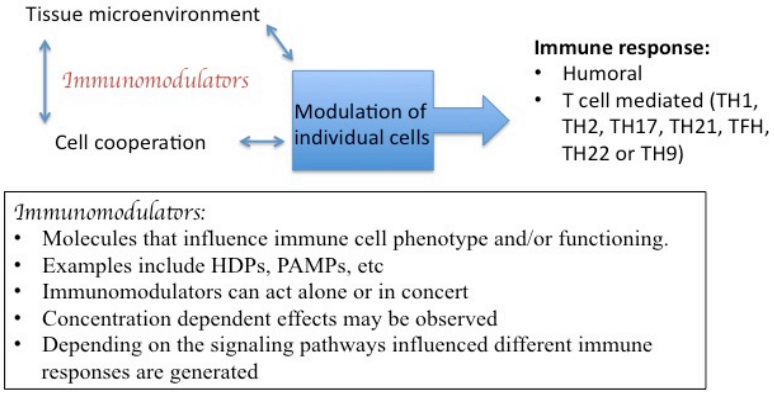

Figure 7-1 HDPs as innate immune system modulators

HDPs are immunomodulators that can act at various levels by recruiting immune cells, upregulating or downregulating cytokine production, modulating responses to TLR ligands and maturing DCs. The modulation of cells such as DCs by HDPs, depends on the local microenvironment created by tissue epithelial cells and stromal cells as well as by factors released by other cells.

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Chapter 8 Overall Conclusion

In conclusion, we have successfully isolated and characterized both BDCs and MoDCs. Regarding the immunomodulatory effect of HDPs and IDRs, we have demonstrated a lack of effect with IDRs HH2, HH17 and HH18 on the MoDC and BDC populations that we generated. We did however demonstrate that the IDR-1002, and various HDPs induced IL-8 secretion in PBMC and recruited PBMC. This *in vitro* production of IL-8 did not translate into neutrophil recruitment following the intradermal administration of these peptides into the pig dermis. Lastly, we demonstrated that the IDR-1002 decreased lymphocyte proliferation and therefore may represent a strategy for immunosuppression.

8.1 Future work

There are several directions that the work from this thesis can take. Of particular interest is developing an understanding for the mechanism of action of IDR-1002 in decreasing lymphocyte proliferation. It is possible that this peptide induces anergy as was demonstrated with the BMAP proteins [224]. It would be noteworthy to determine if this immunosuppressive state could be overcome by the administration of exogenous IL-2 in LPRs. Also of interest would be to examine which immune cells are recruited to the injection site via immunohistochemistry, using specific markers for DCs, macrophages and lymphocytes. One could also study the cytokine milieu at the injection site possibly by taking biopsies for RT-qPCR analysis of cytokine gene expression. Another interesting aspect that could be addressed, is the effect of using different routes of administration for example, subcutaneous or intramuscular administration as compared to intradermal. When IDR-1002 is administrated subcutaneously with PTd, antibody titres were increased and protection was observed [214]. Differences between these studies and ours include the antigen tested and the route of administration. Studies comparing the subcutaneous administration of KLH *versus* PTd, as an antigen in combination with IDR-

1002, would allow for a better understanding of the role played by each antigen as well as the immunization route. Another final matter to examine is that of synergy between CpG ODN and IDRs. In combination, IDRs demonstrate protective effects in vaccination studies. An understanding of which signaling pathways are activated by IDRs and CpG ODN would allow for a greater understanding of how these adjuvants work.

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Appendix A: Effects of synthetic host defense peptides on porcine dendritic cells

The immunomodulatory effect of the synthetic HDPs, HH2, HH17 and HH18 was examined in porcine MoDCs and BDCs. The effects of these HH peptides on cell surface marker expression via flow cytometry, cytokine production via ELISA, changes in gene expression in cytokines, chemokines and co-stimulatory molecules, and uptake ability via endocytic assay were studied.

A. Dose Response Assessment

To determine peptide safety, assays for cytotoxicity and for caspase activity were performed in porcine PBMC using kits (Promega) as per the manufacturers instructions. Peptide concentrations ranging up to 100 μg/ml were used at 1, 5 and 24 hours for the cytotoxicity assay (Figure 1A-C) and 5 hours for the caspase assay (Figure 2). We demonstrated that at these concentrations HH2, HH17 and HH18 were not cytotoxic nor did they induce apoptosis.

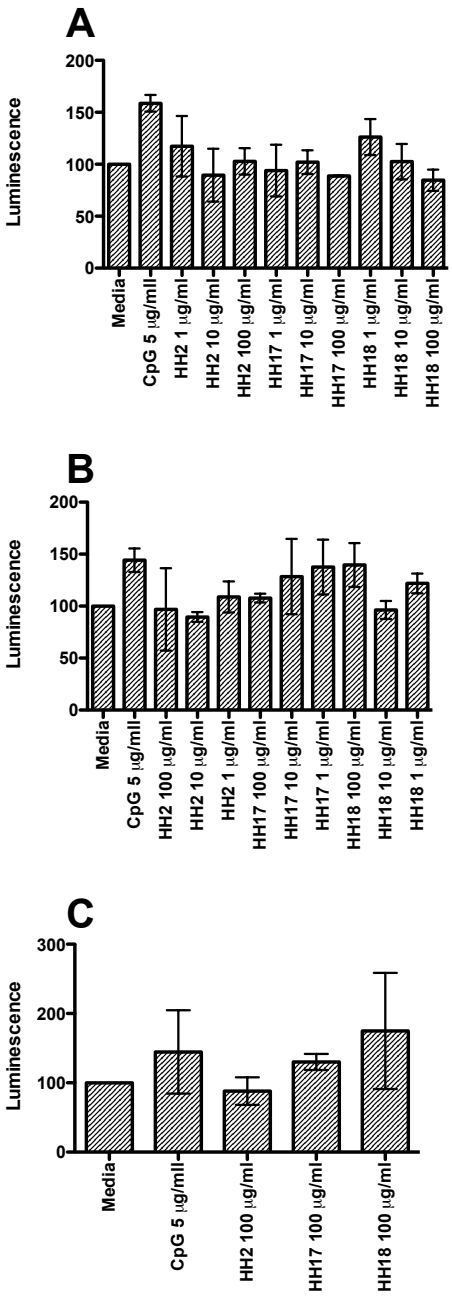


Figure 1. Cytotoxicity assay in PBMC.

PBMC were rested overnight and incubated with CpG ODN (5 μg/ml), HH2, HH17 and HH18 at respectively at the following respective concentrations: 100, 10 and 1 μg/ml, for 1 hour (A), 5 hours (B) and 24 hours (C) and then assayed by luminescence for cytotoxicity. Results are expressed as mean ± SEM (n=2).

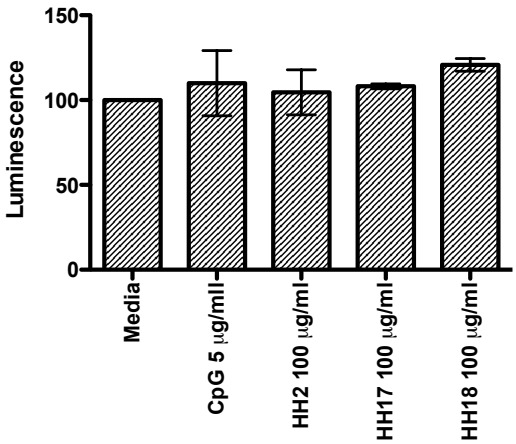
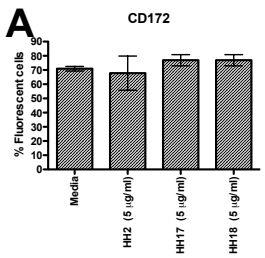
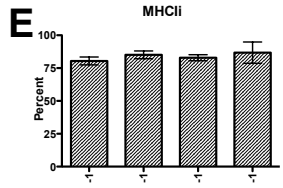


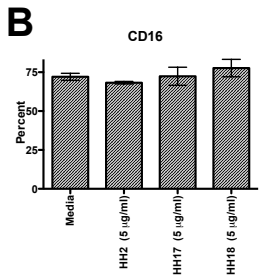
Figure 2. Caspase activity in PBMC following a 5 hour incubation with treatments. PBMC were rested overnight and incubated with CpG ODN (5 μg/ml), Peptide 1, 2, and 3 at respectively at the following respective concentrations: 100, 10 and 1 μg/ml, for 5 hours and then assayed by luminescence for caspase activity. Results are expressed as mean ± SEM (n=2).

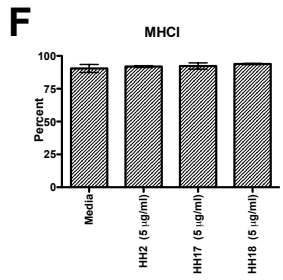
B. Cell surface marker expression

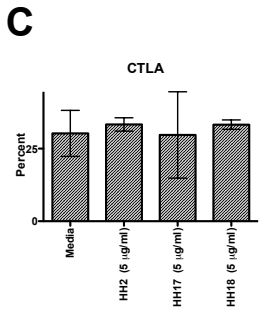
MoDCs were stimulated with HH2, HH17 and HH18 using a low dose of 5 μg/ml (n=2) for 8 hours. This peptide dose was chosen based on studies that used HDPs at similar concentrations [144]. MoDCs were stimulated with HH2, HH17 and HH18 for 8 hours and analyzed by flow cytometry for the percent of cells positive for CD172a, MHCII, CD16, MHCI, CTLA, CD1 and CD14 expression as well as for their mean fluorescence intensity (Figure 3). No changes in either of these parameters were observed. No differences were present for the percent of positive cells following peptide stimulation.

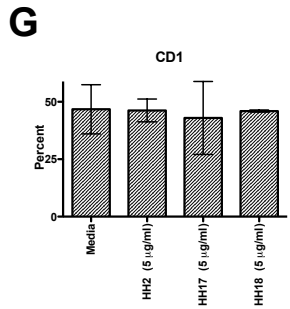

A
CD172
% Fluorescent cells
Media
HH2 (5 µg/ml)
HH17 (5 µg/ml)
HH18 (5 µg/ml)

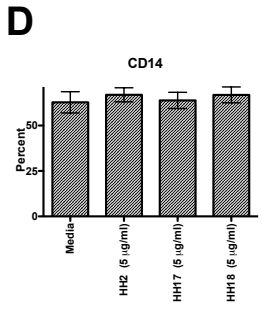

E
MHCIi
Percent


B
CD16
Percent
Media
HH2 (5 µg/ml)
HH17 (5 µg/ml)
HH18 (5 µg/ml)


F
MHCI
Percent
Media
HH2 (5 µg/ml)
HH17 (5 µg/ml)
HH18 (5 µg/ml)


C
CTLA
Percent
Media
HH2 (5 µg/ml)
HH17 (5 µg/ml)
HH18 (5 µg/ml)


G
CD1
Percent
Media
HH2 (5 µg/ml)
HH17 (5 µg/ml)
HH18 (5 µg/ml)


D
CD14
Percent
Media
HH2 (5 µg/ml)
HH17 (5 µg/ml)
HH18 (5 µg/ml)

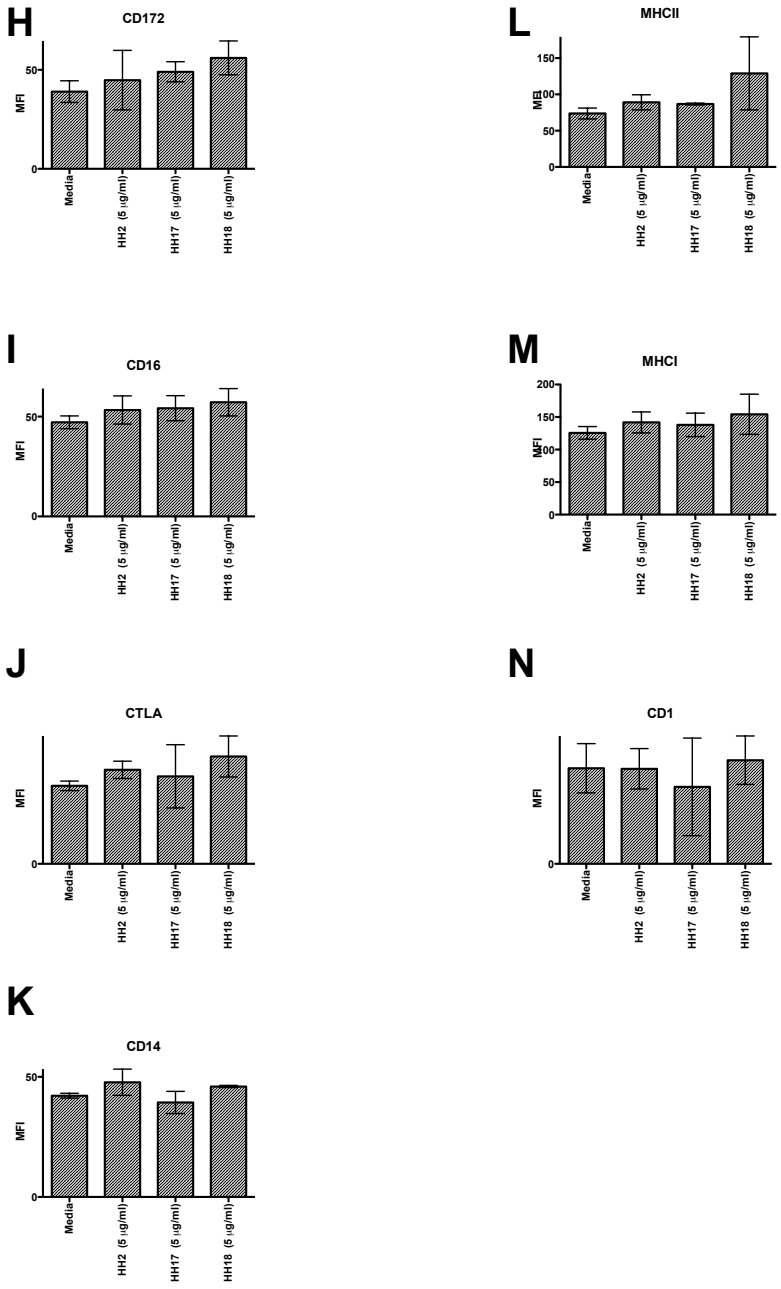


Figure 3. Cell surface marker expression in MoDCs. MoDCs were generated by isolating $CD14^{+}$ cells by MACs and then cultured in the presence of IL-4 and GM-CSF. They were assessed for the expression of their cell surface markers at day 6 (n=2) by flow cytometric analysis. Data are expressed as a mean ± SEM % of positive cells (A-G) and mean fluorescent intensity (H-K).

C. Cytokine production via ELISA

Preliminary data on cytokine production using the HH peptides at a low dose were suggestive that a higher peptide dose should be used. Similar findings were made by our collaborators therefore we decided to use a higher dose for future experiments (133 μg/ml). Subsequently, protein levels of IL-6, IL-8, IL-10, IL-12 and TNF-α were examined by ELISA following a 24-hour stimulation with HH2, HH17, HH18 and LPS. No changes were seen for IL-10 production (data not shown). TNF-α production was only seen following stimulation by LPS (data not shown). Results for IL-6, IL-8 and IL-12 for both MoDCs and BDCs are shown in Figure 4. With the exception of LPS, the only other remarkable finding was the ability of HH2 to induce high levels of IL-8 in MoDCs.

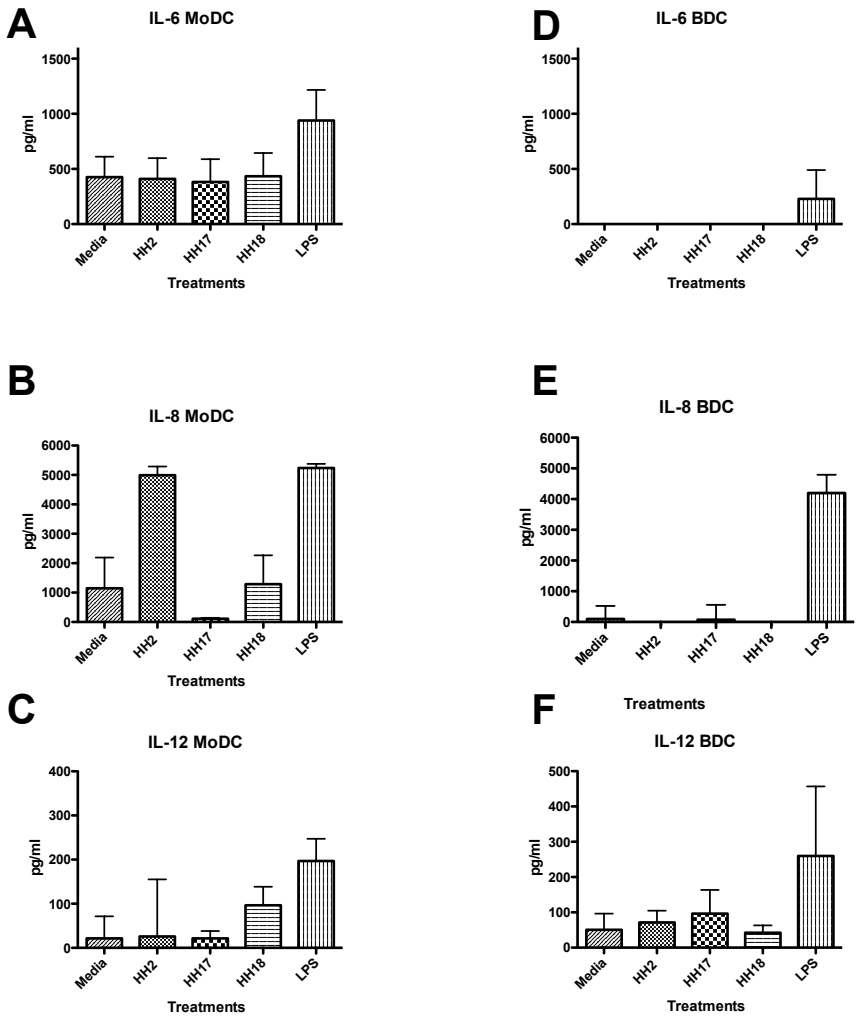


Figure 4. IL-6, IL-8 and IL-12 cytokine production by ELISA in MoDCs and BDCs. MoDCs (Figure 4A-C) at day5 and BDCs (Figure 4D-F) that were isolated and rested for 4 hours were cultured with HH2 (133 μg/ml), HH17 (133 μg/ml), HH18 (133 μg/ml) or LPS (100 ng/ml) for 24 hours. Supernatant was assayed by ELISA for IL-6, IL-8 and IL-12 protein. Results are expressed as median concentration ± range (n=4 pigs).

D. Gene expression studies on cytokines, chemokines and co-stimulatory molecules

Changes in gene expression were examined following an 8 hour stimulation with HH2, HH17, HH18 and LPS. Results are presented in Table 1 for both MoDCs and BDCs as an average fold change ± SEM (n=4). Only fold changes of more than 2 were considerable significant. Using this criterion HH2 increased the expression of IL-12p40 and IL-17 in

MoDC and of IFN-γ in BDCs. Neither HH17 nor HH18 resulted in changes in gene expression. LPS treatment resulted in an increase in CCL3, IL-6, IL-8, IL-12p40, IL-13, IL-17, TNF-α, CCR7, CCL2 and CCL20 expression.

Table 1. Changes in the relative fold change of gene expression in MoDC and BDC following an 8 hour stimulation with peptides and LPS. Change in DC gene expression of cytokine, chemokines chemokine receptors and co-stimulatory molecules following peptide and LPS stimulation. MoDC at day 5 were cultured with HH2 (133 μg/ml), HH17 (133 μg/ml), HH18 (133 μg/ml) and LPS (100 ng/ml) for 8 hours. Cells were collected in Trizol and then analyzed by RT-PCR for changes in gene expression of IL-6, IL-8, IL-12p35, IL-12p40, IL-13, IL-17, TNF-α, IFN- α, IFN-γ, TGF-β, NκFBp65, CCR5, CCR6, CCR7, CCL2 (MCP-1), CCL3 (MIP-1α), CCL4 (MIP-1β), CCL20 (MIP-3α), CD80, CD86 and MHCII (n=4 pigs).

		HH2 (133 μg/ml)	**HH17 (133 μg/ml)**	**HH18 (133 μg/ml)**	**LPS (100 ng/ml)**
BDC interleukins	IL-8	2.5 ±1.3	1.1±0.1	1.0±0.4	1.0±0.4
	TNF-α	2.5±1.3	1.1±0.1	1.0±0.4	1.0±0.4
	IFN-α	1.1±0.4	0.9±0.3	2.1±0.8	0.3±0.1
	IFN-γ	**6.7±4.1**	0.4±0.1	0.5±0.2	**8.1±4.7**
BDC chemokines/ chemokine receptors	CCR5	0.4±0.1	1.9±0.2	1.2±0.3	0.7±0.1
	CCR6	2.5±0.8	0.7±0.2	1.1±0.2	1.4±0.6
	CCR7	1.5±0.5	0.7±0.1	1.6±0.6	1.4±0.6
	CCL2	0.3±0.1	1.8±0.6	6.5±5.4	11.1±7.2
	CCL3	1.2±0.4	1.4±0.2	1.3±0.3	**21.3±13.3**
	CCL20	1.0±0.4	0.8±0.5	1.9±1.0	12.6±12.0
BDC markers	CD80	1.3±0.3	1.0±0.0	0.8±0.5	3.5±1.6
	CD86	1.1±0.4	1.4±0.4	0.5±0.1	3.6±1.4
MoDC interleukins	IL-6	18.0±17.7	1.5±0.6	1.0±0.7	**33.8±17.1**
	IL-8	17.6±17.0	1.3±0.5	0.4±0.2	**34.6±17.7**
	IL-12p35	1.4±0.9	1.2±0.4	1.0±0.1	2.4±0.8
	IL-12p40	**6.3±3.7**	0.6±0.2	0.7±0.2	**13.1±6.9**
	IL-13	6.4±6.2	2.8±1.8	1.0±0.3	**44.9±30.5**
	IL-17	**8.5±4.1**	0.9±0.3	0.5±0.3	**24.9±19.9**
	TNF-α	4.5±4.0	1.3±0.7	0.7±0.4	**12.6±5.8**
	IFN-α	2.0±0.6	1.5±0.5	2.0±0.4	0.3±0.3
	IFN-γ	1.7±0.8	0.5±0.1	1.4±0.2	5.4±4.3
	TGF-β	1.2±0.5	1.5±0.3	1.0±0.2	0.8±0.4
	NFKBp65	1.8±0.4	0.9±0.3	1.1±0.2	1.1±0.2

MoDC chemokines/ chemokine receptors	CCR5	2.5±1.2	1.1±0.2	1.5±0.3	0.7±0.3	
	CCR6	1.7±0.6	1.0±0.2	1.4±0.7	1.9±0.2	
	CCR7	4.1±2.9	0.8±0.2	0.9±0.2	**4.0±1.0**	
	CCL2	2.3±1.5	0.6±0.2	0.9±0.2	**4.8±2.2**	
	CCL3	-1.3±1.7	2.6±1.3	0.8±0.5	-3.5±1.2	
	CCL4	-1.1±1.7	2.5±1.1	1.1±0.7	-3.1±2.1	
	CCL20	9.2±8.8	2.3±1.1	0.3±0.1	**40.7±28.4**	
MoDC markers	CD80	1.9±0.9	1.1±0.3	0.8±0.2	2.9±1.3	
	CD86	1.0±0.1	1.0±0.3	2.6±1.1		
	MHCII	1.2±0.3	1.4±0.2	1.2±0.1	0.6±0.1	

E. Endocytosis

The ability of DCs to sample antigen is key in their functioning as antigen presenting cells. DCs sample antigen by various mechanisms including endocytosis. The goal of this experiment was to determine if HDPs are able to effect DC endocytosis via the uptake of FITC-dextran. Endocytosis assays for the HH peptides were performed in MoDCs (Figure 5 A-C) and BDCs (Figure 5 D-F). No changes were observed in either DC subset examined.

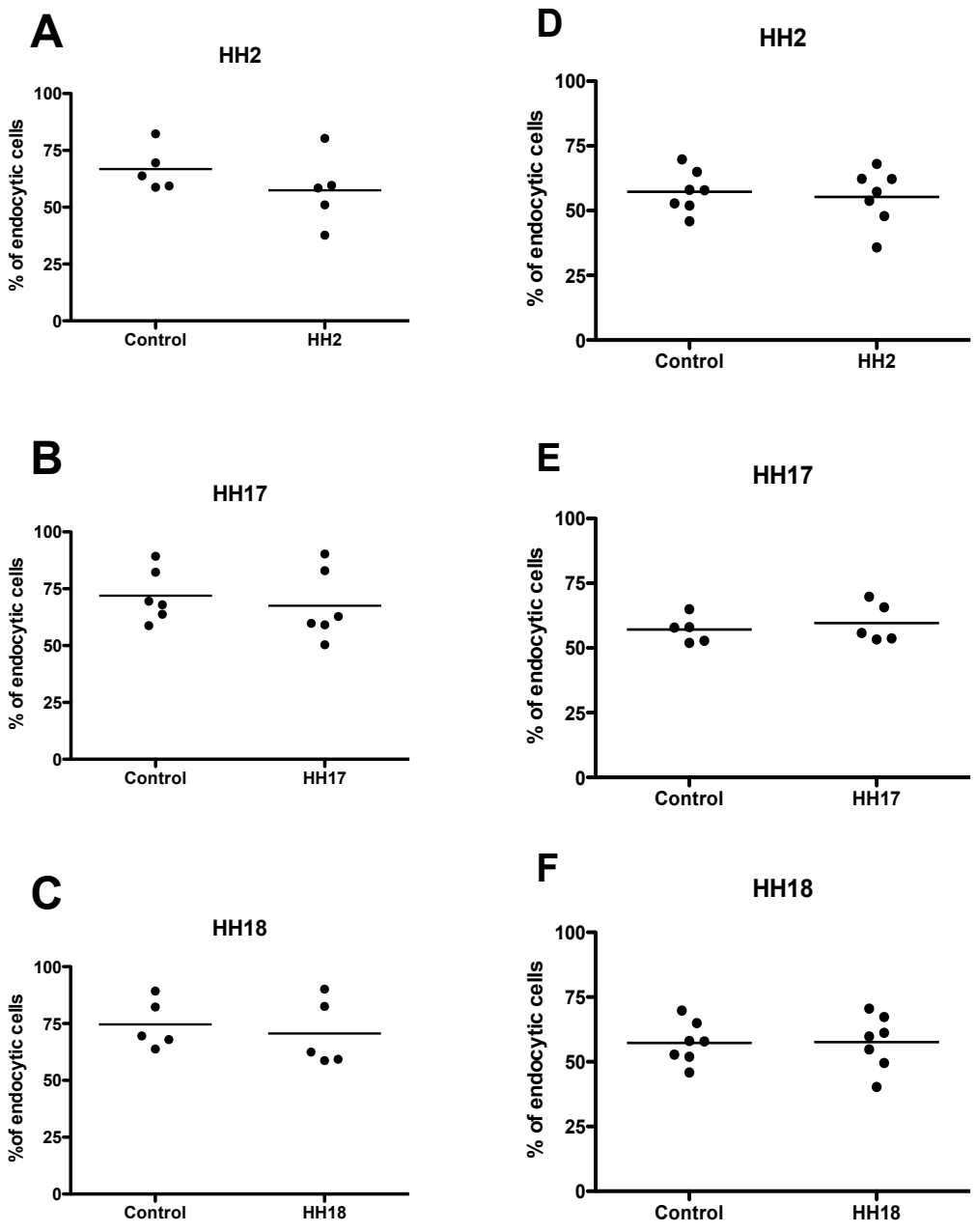


Figure 5. Endocytic activity MoDCs and BDCs following stimulation with HH peptides. Uptake ability of MoDCs (Figure 5A-C) and BDCs (Figure D-F) following stimulation with HH2 (133 μg/ml), HH17 (133 μg/ml) and HH18 (133 μg/ml) was assayed by examining the percentage of cells staining positive for FITC-dextran. Results are demonstrated as the mean of positively staining DCs (n=6 animals).

F. Summary

We examined the effects of stimulating porcine MoDCs and BDCs with HH2, HH17 and HH18. We did not see any significant changes in the parameters that we examined i.e. cell surface marker expression, cytokine production, chemokine production, co-stimulatory molecule expression and endocytic ability. For this reason we expanded our study to include other peptides some of which are naturally-occurring. We also moved from screening DCs to examining effects in PBMC. Reasons for which we did not see any effects are discussed in Chapter 4.5.

Appendix B: Recruitment and activation of dendritic cells by innate immune stimulators (as published in Facci et al., 2008; Recent Developments in Immunology)[45]

Abstract

The innate immune response to infection is characterized by the fast appearance of effector molecules within minutes after breaching of the mucosal lining or the skin. Effector molecules such as cytokines, chemokines and host defense peptides are subsequently secreted to recruit a variety of immune cells and to activate the cells upon arrival at the site of infection. Amongst the cells being recruited dendritic cells are critical immune cells, which play a major role in linking innate and acquired immunity, thereby ensuring that both acquired immune responses and immune memory are induced. Here, we review the early stages of an innate immune response and the recruitment, activation and maturation of these cells by innate immune molecules. Furthermore, the potential of using some of these innate immune modulators to modulate the immune response to vaccination is discussed.

Dendritic cells

1. Introduction

Innate immune responses are triggered in response to the recognition of pathogen associated molecular pattern (PAMPs) via pattern recognition receptors (PRRs). Typically, within minutes after breaching of the mucosal or dermal lining innate effector molecules that mediate the recruitment and activation of various types of immune cells to the site of infection are released. In fact, it is this first interaction between the pathogen and the host's innate immune system that sets the stage for the subsequent immune response. Among the cells recruited are dendritic cells (DCs). These cells are efficient antigen-presenting cells (APCs) found in peripheral tissues as well as in primary and

secondary lymphoid organs [3, 236]. DCs play a central role in the induction and regulation of innate and adaptive immune responses, and represent an important link between innate and acquired immunity [237]. Their strong capacities for acquiring, processing, retaining, and presenting multiple peptides explain their superior role as APCs. For example, compared to macrophages lung DCs home more efficiently to T cell zones in secondary lymphoid organs [238]. Different subtypes of DCs have been described at steady state in mice, pigs, and humans. The two major subtypes are the type-1 interferon-producing plasmacytoid DCs and the conventional DCs that are found in both non lymphoid and lymphoid tissues [162]. Conventional DCs can be further divided into subsets according to their tissue-specific localization, such as interstitial tissue DCs, lung DCs and mucosal tissue-associated DCs [162]. Depending on their maturation status, immature DCs are present in the periphery whilst in secondary lymphoid organs both mature and immature DCs are present.

DCs are regulators of the innate and adaptive immune system that can be found in almost every organ in the body. Their recruitment and activation is mediated by a variety of innate immune molecules including chemokines, TLR ligands and host defense peptides. The presence of DCs at the mucosal surfaces and spleen allows them to continuously sample antigens in their surrounding environment by macropinocytosis, receptor-mediated endocytosis and phagocytosis [239, 240]. Resting DCs display a low surface expression of class II MHC and co-stimulatory molecules such as CD40, CD80 or CD86. In response to microbial and endogenous stimuli, these resting DCs undergo a complex maturation process that leads to changes in antigen capture and presentation, migration, expression of co-stimulatory molecules and production of T cell polarizing cytokines. Some of these stimuli are presented in the following sections.

2. Recruitment of dendritic cells

A large number of molecules are involved in the homing and recruitment of DCs including integrins, adhesins, lipid mediators, host defense peptides, cytokines and chemokines [241, 242] Here, we focus on the role of chemokines and host defense peptides as effector molecules of the innate immune system.

The role of chemokines in DC recruitment

Chemokines, a family of structurally similar peptides, are involved in the regulation of inflammation, leukocyte trafficking, and immune cell differentiation [243, 244]. These molecules are highly basic proteins of 70-125 amino acids, with molecular masses ranging from 6 to 14 kDa. They exert their functions by binding specific seven-transmembrane G-protein-coupled receptors located on the cell surface [243, 244]. Most chemokines contain at least four cysteines that form two disulfide bonds, one between the first and third and one between the second and fourth cysteine residues [243, 244]. According to the relative position of the cysteine residues, chemokines can be subdivided into four families: CXC- (α-chemokines), CC- (β-chemokines), C- (γ-chemokines), and CX_3C- chemokines (δ-chemokines) [243, 244]. Different chemokines are involved in the various stages of DC migration.

DCs originate mainly from hematopoietic stem cell precursors that are released into the bloodstream and account for approximately 1% of all peripheral blood mononuclear cells (Figure 1) [44, 241, 245]. However, some DCs are of lymphoid origin [162]. The egress of DCs from the bone marrow into the bloodstream is tightly controlled by various integrin/addressin and chemokine/chemokine receptor interactions, the most important being CXCR4/CXCL12 [241, 246-249]. CXCL12, secreted by stromal cells, controls the egress of DC precursors even in the absence of inflammatory stimuli [250, 251]. This chemokine interacts with CXCR4, expressed on monocyte and DC precursors, inducing an up-regulation of cell surface integrins that retain DC precursors in the bone marrow [252, 253]. Thus, the immune system controls the release of precursors by modifying CXCL12 expression. Another receptor, CX3CR1, is involved in the recruitment of DCs during homeostasis and under inflammatory conditions [254-256]. Interestingly, other innate immune molecules such as host defense peptides also attract dendritic cells [257] (Figure 1).

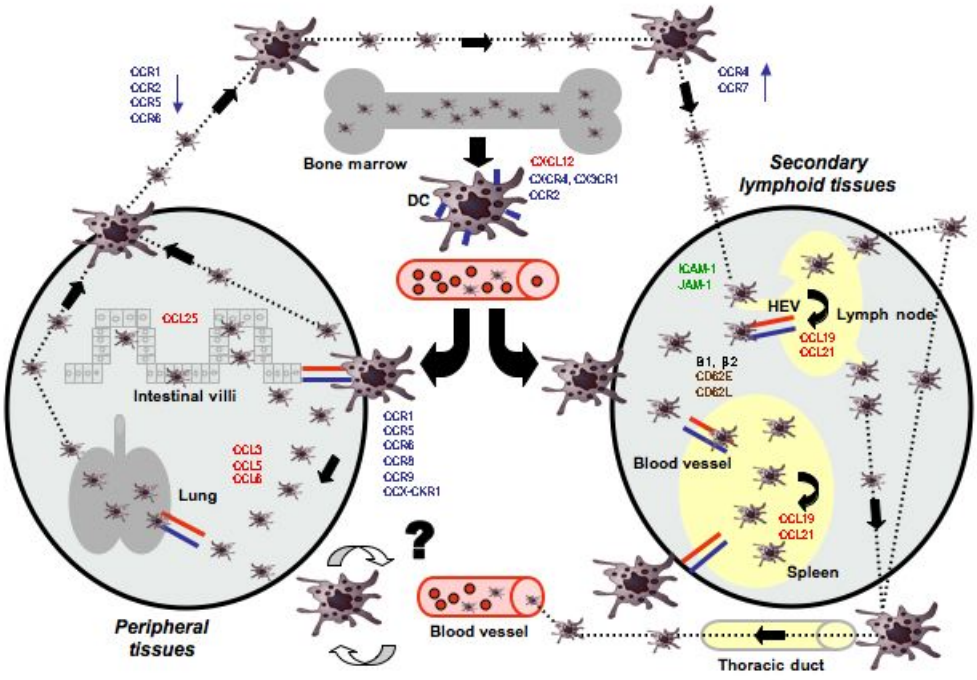
DCs reach the spleen via the blood at a very low frequency, and then migrate to the marginal zone where they develop into conventional populations of immature DCs

(Figure 1) [44, 254, 258, 259]. Once matured and activated, DCs leave the marginal zone towards the T cell zone in the white pulp. This migration is mediated by an upregulation of CCR7, which enables the DCs to respond to the chemokines CCL19 and CCL21 [260-262]. While some DCs colonize lymph nodes via the blood [7, 263, 264], their main route of entry is via the afferent lymph [7, 263, 264]. Interestingly, this migration is substantially increased after stimulation with microbial stimuli and inflammatory cytokines [241, 259, 265, 266]. This stimulation leads to the downregulation of CCR1, CCR2, CCR5 and CCR6 and upregulation of CCR4 and CCR7, eventually enabling DCs to migrate to secondary lymphoid tissues in response to CCL19 and CCL21 [241, 259, 267, 268]. Secondly, it also reduces their responsiveness to inflammatory chemokines (e.g., CCL3, CCL5 and CCL20). DC entry into lymphatic vessels is mediated by adhesion molecules such as ICAM-1 or JAM-1 [241, 269]. From lymphatic vessels DCs reach the subcapsular sinus of the regional lymph node in a CCL21-dependent manner [241, 269]. Upon entry into the sinus, DCs are further directed towards the paracortex by CCL21 and CCL19 [241, 269]. Although their entry into the lymph node via the afferent lymph is the best described route of entry, DCs can also enter the lymph node via high endothelial venules (HEV) [7, 15, 21, 270]. Among the different DCs subsets, plasmacytoid DCs, $CCR2^{high}$ monocytes and conventional DCs including their precursors have been described to use this route [7, 15, 21, 270]. Non-inflamed lymph nodes support adhesion but not transmigration of plasmacytoid DCs, whereas inflamed lymph nodes allow strong migration of DCs across the HEV. This occurs using CD62L and CD62E during attachment and rolling, and involves β1 and β2 integrins for endothelial attachment [7, 21]. CCR5 as well as ChemR23 in humans also appears to mediate the recruitment of DCs across HEV [7, 21, 271].

DCs enter the periphery either directly, or via precursors such as monocytes (Figure 1). However during inflammation, mononuclear phagocytes and DCs are recruited [44, 263, 272, 273]. This recruitment is controlled by various chemokines and matrix metalloproteinases [274]. For example, CCL3 interacts with the receptors CCR1 and CCR5. Within the chemokine network, several functional redundancies exist, as a single chemokine can bind to more than one receptor, and a single receptor can be bound

by various chemokines (CCL3, CCL5 and CCL6 for CCR1 and CCR5; CCL25 for CCR9 and CCX-CKR1) [241]. Recently, CCX-CKR1, which is exclusively expressed by stromal cells, has been implicated in the regulation of homeostatic $CD11c^{+}$ $MHCII^{high}$ DC migration by controlling the availability of chemokines into the extracellular space [275]. This finding adds another level of complexity in the understanding of leukocyte homeostatic migration [275]. Plasmacytoid DCs can enter normal tissues at very low levels in humans and mice in the presence or absence of inflammatory stimuli [162, 241, 276] most likely via chemokines and their cognate receptors.

Figure 1: Schematic representation of dendritic cells (DC) migration pathways. Chemokines, chemokine receptors, selectins, integrins and addressins are in red, blue, brown, black and green, respectively.



The role of host defense peptides in DC recruitment

Host defense peptides are cationic peptides that can be grouped into defensins and cathelicidins based on the respective presence of β-sheets and α-helices [191]. These two families differ in their disulfide content and peptide length allowing them to have different functions. Being positively charged, host defense peptides are able to form pores

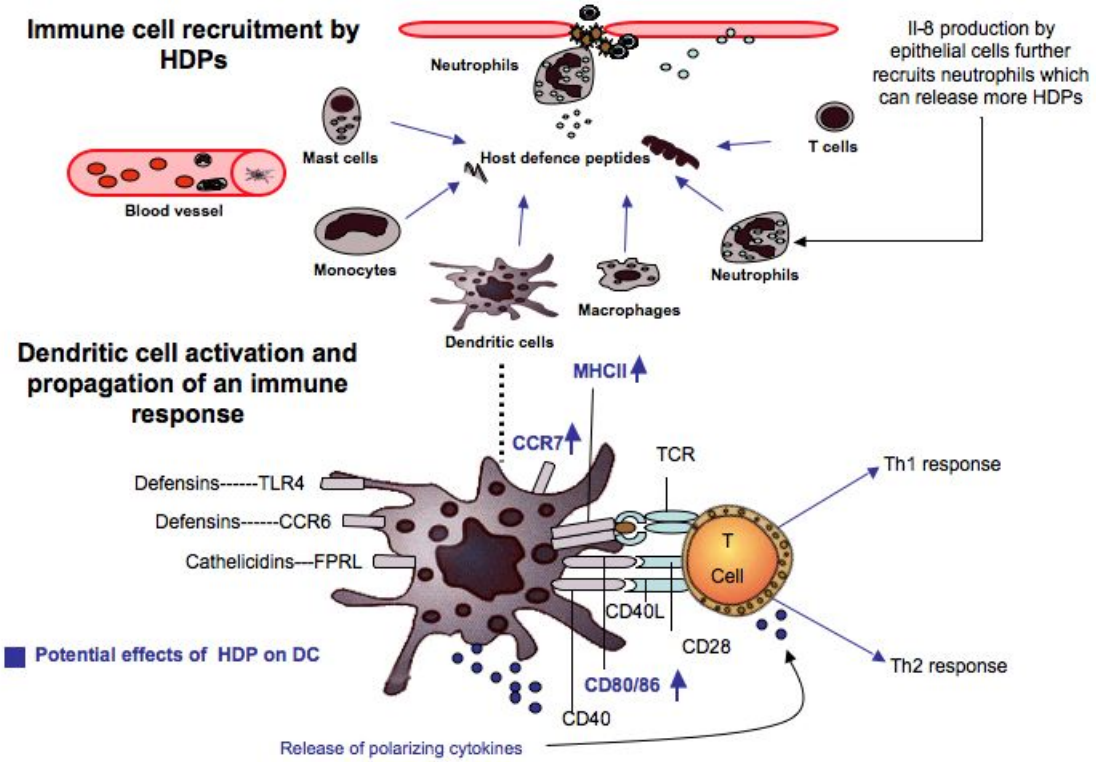
in bacterial membranes thereby being antimicrobial [193]. In addition to this property, host defense peptides have been demonstrated to be immunomodulatory by recruiting immune cells to sites of pathogen entry (Figure 2). Neutrophils are amongst the first cells to arrive and possess various granules that contain antimicrobial [277] and immunomodulatory factors [215] such as host defense peptides. The release or activity of these peptides is regulated by the activation state of the neutrophil and by enzymes that cleave the propeptide form of the peptide into its mature form. For example, neutrophil-derived elastase cleaves Bac7 into its mature form [109], whilst the cleavage of LL-37 from HCAP18 occurs upon exocytosis and consequent cleavage via proteinase 3 [110]. Interestingly, whilst the immature form of Bac7 is chemotactic [134] and does not have any antimicrobial activity, its mature form is antimicrobial against *Klebsiella pneumoniae* and *Salmonella typhimurium* [109]. This example demonstrates the importance of peptide maturity in determining the peptide's function and ability to recruit immune cells. The ability of peptides to chemoattract other immune cells serves as means to propagate and amplify immune signals.

Host defense peptides from various species have been demonstrated to recruit immune cells (Figure 1). For example, hCAP, the propeptide of the human cathelicidin LL-37 recruited human monocytes [135], rat mast cells [143], human neutrophils [135-137], human DCs [136] and human T cells [135, 138]. The mouse cathelicidin CRAMP was chemotactic for human monocytes, neutrophils, macrophages, and mouse peripheral blood leukocytes [149]. The human beta defensins 1-4 were chemotactic for human macrophages [139], mast cells [139], immature DC [278], $CD4^+/CD45RA^+$ naive and $CD8^+$, but not $CD4^+/CD45RO^+$ memory T cells [141, 278]. Murine β-defensins 2 and 3 were able to recruit murine immature DC [145, 155].

Many of the receptors used by host defense peptides for recruitment remain unknown. Whereas some defensins have been shown to recruit immature DCs via CCR6 [145, 146], both the human and mouse cathelicidins LL-37 and CRAMP act mostly via FPRL (formyl peptide receptor like) [135, 147-149]. Different cells possess distinct sets of receptors thereby adding specificity to the recruitment process. For example DCs migrate

in response to fMLP (representative of formyl peptides of bacterial origin), C5a, and the CC-chemokines CCL7, CCL3 and CCL5, but not to the CXC-chemokines IL-8 and CXCL10 and the CC-chemokines CCL2 and CCL8 [147, 279] whereas neutrophils migrate in response to cytokines such as IL-8. In pigs, we observed that after 24-hour stimulation of monocyte-derived DCs with a Bactenecin peptide derivative increased protein levels of IL-8. Theoretically given that IL-8 recruits neutrophils and that neutrophils release host defense peptides, this peptide may have the potential to indirectly recruit more DCs. Furthermore, following an 8-hour stimulation of monocyte-derived DCs with the same peptide, increases in gene expression of IL-12p40 and IL-17 were observed. These changes in gene expression indicate the potential of host defense peptides to activate DCs and to skew the immune response towards a particular type of immune response.

Figure 2: The role of host defence peptides in cell recruitment and propagation of an immune response



3. **Activation of dendritic cells**

Activation of DCs is partially induced by the recognition of pathogens via PRRs (Figure 3). The three main families of PRRs comprise Nod-like receptors (NLRs), C-type lectin receptors (CLRs) and Toll-like receptors (TLRs). Nod-like receptors function as cytosolic sensors for bacterial products and endogenous danger signals. This family includes innate receptors like nucleotide-binding oligomerization domain (nod)1 and nod2, which activate NF-κB, and other receptors, such as NALPs, which can activate caspase-1 leading to the processing of pro-IL-1β and pro-IL-18 into their active forms [280]. The CLR family recognizes various bacterial and fungal carbohydrate moieties such as D-mannose, L-fucose and *N*-acetylglucosamine present on the surface of pathogens. Their major function is to internalize antigens for further processing and presentation by DCs. This family includes DC-SIGN (dendritic cell-specific intercellular adhesion molecule 3 grabbing nonintegrin), dectin-1 and the mannose receptor [281].

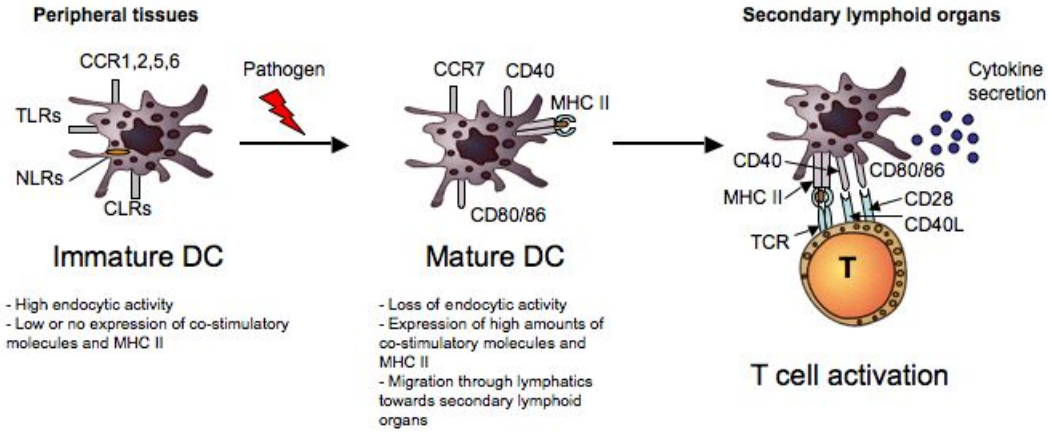
The TLR family includes 10 different receptors in humans (TLR1-10) and 12 in mice (TLR1-9 and 11-13) [282]. Members of the TLR family recognize a wide range of bacterial, viral and parasitic molecular patterns. These TLRs are either expressed on the cell surface or within endosomes. Amongst the cell surface expressed TLRs, TLR5 is triggered by flagellin from both Gram-positive and Gram-negative bacteria [283]. TLR4 detects lipopolysaccharide (LPS) and TLR2 recognizes a variety of microbial components such as peptidoglycans and lipoteichoic acid (LTA) from Gram-positive bacteria, lipoarabinomannans from mycobacteria and zymosan from fungi [284]. TLR2 also forms heterodimers with TLR1 or TLR6. The TLR1/2 heterodimer recognizes triacyllipopeptides from gram-negative bacteria whereas the TLR2/6 heterodimer is triggered by diacyllipopeptides expressed by mycoplasma [285]. Amongst the TLRs expressed in the cytoplasm, TLR3 recognizes double-stranded RNA, produced by most viruses, [286], whereas TLR9 recognizes unmethylated 2'-deoxyribo(cytidine-phosphate-guanosine)(CpG) DNA motifs present in bacterial DNA [287]. Synthesized oligodeoxynucletides with unmethylated CpG dinucleotides (CpG ODN) can mimic the immunostimulatory activity of bacterial DNA through TLR9 activation. Depending on the number of CpG motifs present, the spacing of the CpG motifs, the flanking sequences

and the backbone of the ODN, three classes of ODN can be distinguished, namely A-class ODN, B-class ODN and C-class ODN. These three classes have different stimulatory activities with the C-class ODN combining intermediate effects of both the A-class and the B-class ODN [29]. TLR7 and TLR8 specifically recognize single-stranded RNA from pathogens such as RNA viruses. They are highly homologous to TLR9 and are involved in viral recognition [288]. Certain synthetic anti-viral imidazoquinolines were also demonstrated to stimulate TLR7- and TLR8-dependent signaling [289].

Interestingly, the pattern of expression of TLRs by DCs differs by both subset and species. In humans, conventional DCs express TLR2, TLR3, TLR4, TLR5, TLR6 and TLR8 whereas plasmacytoid DCs express TLR7 and TLR9. In mice, both conventional and plasmacytoid DCs express TLR9. Depending on the TLR and the DC subpopulation different types of immune responses are elicited. For example, in conventional DCs, triggering of TLR4 or TLR5 induces phosphorylation of p38 and JNK1/2 kinases, which stimulate Th1 responses via IL-12 production. In contrast, the TLR2 agonists Pam3Cys and *Schistosoma* egg antigens stimulate phosphorylation of ERK1/2, which results in the stabilization of the transcription factor c-Fos (a suppressor of IL-12) and polarization of the immune response towards a Th2 type [290]. TLR3 engagement leads to IRF-3 activation via the adapter protein TRIF and IFN-β production, which induces IRF-7 in an autocrine fashion and subsequent production of IFN-α [291]. In plasmacytoid DCs, triggering of TLR7 or TLR9 by their natural ligand (single stranded RNA and bacterial DNA respectively) or by synthetic ligands (imidazoquinolines and CpG ODN respectively) induces the production of large amounts of IFN-α through a signaling cascade that rapidly activates IRF-7 [292], making those cells the most efficient type I interferon producers. Stimulation of TLR7 and TLR8 also induces the production of Th1-like and pro-inflammatory cytokines and chemokines other than type I IFN including IFN-γ, CXCL10, IL-12, IL-6, TNF-α [37]. Signalling via TLR9 induces upregulation of costimulatory molecules, resistance to apoptosis and secretion of Th1 promoting chemokines and cytokines such as type III IFNs, CCL3, CCL4, CXCL10 and other IFN-inducible genes [29]. Human pDCs do not produce significant IL-12p70 whereas mouse

pDCs produce measurable amounts of IL-12 in response to these TLR ligands, even in smaller amounts compared to the conventional DC subset [293].

Figure 3: Maturation of DCs and induction of adaptive immunity



CCR: C-Chemokine Receptor, TLR: Toll-like receptor, CLR: C-type lectine receptor, NLR: Nod-like receptor, MHC II: Type II Major Histocompatibility Complex, TCR: T cell receptor

4. Maturation of dendritic cells

Under steady-state conditions, DCs remain in an immature state and do not differentiate into functional initiators of immunity. Without inflammation or infection, DCs continuously migrate from the periphery to the lymph nodes. DCs migrating during homeostasis are considered quiescent, semi-mature or not fully mature. In contrast to mature DCs, homeostatic DCs do not express the full set of stimulatory signals that are required for T cell activation. They present self-antigens or non-immunogenic proteins leading to T cell deletion, anergy or differentiation into regulatory cells [294]. This is an important immunological process designed to purge the peripheral T cell repertoire of autoreactive T cells that have escaped thymic depletion and potentially could give rise to autoimmunity.

As discussed previously, immature DCs express a wide repertoire of PRRs that specifically recognize highly conserved PAMPs. During an infection or tissue injury resting DCs will be exposed to pathogens and mediators of inflammation that can act as "danger signals" to alert these cells [295]. The triggering of these receptors by microbial products combined with the presence of host-derived inflammatory molecules such as TNF-α, IL-1, IL-6, type I interferons, CD40/CD40 ligand interaction, or molecules released by damaged host tissues will lead to the activation and full maturation of DCs. This maturation process is characterized by a loss of endocytic and phagocytic capacities and an increase in the surface expression of co-stimulatory molecules such as CD80, CD86 and CD40 [296, 297]. The mature DCs also change their pattern of chemokine receptor expression from the expression of receptors recognizing chemokines expressed in the peripheral tissues (CCR1, CCR2, CCR5 and CCR6) towards the expression of CCR7. This receptor recognizes CCL19 and CCL21; two chemokines constitutively expressed in the T-cell zones of secondary lymphoid organs, thus allowing the migration of mature DCs in the lymph nodes to present antigens to and activate naïve T cells [268, 298, 299]. Depending on the nature of the maturation stimulus, the subset of DCs, and the local environment, DCs are able to prime naïve T cells and induce their clonal expansion and differentiation into Th1, Th2 or Th17 cells, which can be distinguished on the basis of their cytokine production [300, 301].

Plasmacytoid DCs can be activated and undergo maturation, which enhances their potential for antigen presentation. Similar to conventional DCs, plasmacytoid DC maturation includes the upregulation of MHCII and co-stimulatory molecules CD80, CD86 and CD40. However plasmacytoid DCs present antigens less efficiently since their uptake, processing and loading of antigen onto MHC molecules is not as effective as that of conventional DCs; and even fully matured, they express less MHC class II and co-stimulatory molecules than conventional DCs. However, plasmacytoid DCs can efficiently induce proliferation of previously experienced T cell clones [41, 302, 303].

Other molecules such as host defense peptides can also modulate DC maturation. For example, in the presence of LL-37 immature DCs increase their expression of HLA-

DR and CD86 [158], become more endocytic and secrete Th1 cytokines [136]. When monocyte-derived DCs were stimulated with LPS, lipoteichoic acid and flagellin they produced IL-6, IL-12p70 and TNF-alpha and increased their surface expression of HLA-DR, CD80, CD83, CD86 and the chemokine receptor CCR7. Interestingly, when these stimulants were combined with LL-37 the surface marker expression of the above was decreased. Furthermore when DC were stimulated with LL-37 and LPS and then co-cultured with naïve T cells the production of IL-2 and IFN-y was decreased [234]. This data demonstrates the extent to which HDPs can alter immune responses.

All stimuli involved in the activation and maturation of DCs can cooperate or synergize in the induction of particular aspects of DC maturation, especially cytokine production. For example, the secretion of large amounts of IL-12p70 by DCs requires synergizing stimuli such as IFN-γ, which acts as a conditioner by inducing the upregulation of TLR expression [304, 305], triggering of TLRs by PAMPs acting as specifying inducers [306, 307], and CD40L interaction that boosts the IL-12p70 production already triggered by microbial stimuli [305, 308]. Synergy between innate immune receptors has been demonstrated following microbial infections. For example, during infection by *Mycobacterium sp.* or *Trypanosoma sp.*, TLR2 and TLR9 synergize to induce IL-12p40 production and a Th1 response [309, 310]. Another example is the synergy between Nod receptors and TLR3, TLR4 or TLR9 in the induction of IL-12p70 production [311]. Host defense peptides also contribute to the activation of DCs and deficiencies in host defense peptide expression can contribute to disease pathogenesis [118]. However in contrast, in psoriasis LL-37 appears to activate plasmacytoid DC to respond to self DNA and trigger TLR9 [312] a receptor, that is used to detect foreign DNA.

5. Induction of acquired immunity

Once matured, DCs link innate and adaptive immunity by being able to drive Th1 or Th2 immune responses. The presence of immunomodulators, such as host defense

peptides or TLR ligands such as CpG ODN, is particularly important in determining the type of the immune response. DCs interact with naïve T cells by delivering different types of signals. The first signal is delivered through the T cell receptor (TCR) after its engagement with the peptide-MHC complex expressed by the DC. The second signal is a co-stimulatory signal and is mediated by signaling through CD28 when it engages CD80 or CD86 expressed by the DCs [313]. DCs also deliver a third signal to direct the T cell response towards a Th1, Th2, Th17 or regulatory T cell profile by producing different sets of cytokines.

Subsequent production of IL-12, IL-18 or IFN-α by DCs results in a bias of the $CD4^+$ T-cell priming towards a Th1 profile characterized by the production of high levels of IFN-γ and TGF-β, cytokines that are fundamental for defense against intracellular pathogens. DCs are also able to promote a Th2 cell response, characterized by the high production of IL-4, IL-5, IL-10 and IL-13, and induction of IgE antibodies. The induction of Th2 cell responses by DCs is still not completely understood, but the expression of members of the Jagged family of Notch ligands could play a role [314]. Lastly, production of TGF-β together with IL-6 by DCs can mediate the differentiation of Th17 T cells. These cells are able to produce high amounts of IL-17 and seem to be involved in autoimmune diseases as well as in general inflammation [315] [316].

Evidence suggests that host defense peptides are able to enhance and strengthen the nature of the immune response. For example when defensins were administered intraperitoneally with Keyhole Limpet Hemocyanin (KLH), higher KLH-specific antibody titers and KLH-specific proliferative responses were seen [150]. Ovalbumin-specific immune responses were enhanced after intranasal co-administration of ovalbumin and HNP1-3 in C57/Bl mice [317] and intraperitoneal injection of HNP1-3 and KLH of B-cell lymphoma idiotype Ag into mice enhanced the resistance to subsequent tumor challenge [150]. Fusion of beta-defensins mBD2 or mBD3 to a B–cell lymphoma epitope sFv38 induced stronger anti-tumor immune responses in mice [145, 318]. Therefore host defense peptides have the potential to strengthen immune responses. The presence or absence of these peptides during disease can be advantageous or

disadvantageous depending on the immune environment. Moreover, fusion of the murine β-defensin 2 with the gene encoding the human immunodeficiency virus-1 glycoprotein 120 (HIV gp120) resulted in specific mucosal, systemic, and CTL immune responses after immunization [145, 318].

Two major cell types, B cells and plasmacytoid DCs, express TLR9 and therefore can respond to CpG ODN stimulation. Stimulation of plasmacytoid DCs by CpG ODNs lead to secretion of type I IFN and TNFα. Those cytokines lead to the secondary activation of other immune cells such as Natural Killer cells, monocytes or neutrophils. The TLR9-stimulated plasmacytoid DCs are able to migrate to the T-cell zones of secondary lymphoid tissues, express greater amounts of costimulatory molecules, allowing them to activate naïve and memory T cells and cross-present antigens to $CD8^+$ T cells. As a consequence, CpG ODN promote strong Th1 $CD4^+$ and $CD8^+$ T cell responses [29]. With its capacity to switch immune responses towards a Th1 profile, CpG ODNs are good candidates for prophylactic or therapeutic treatment of infectious diseases as well as vaccine adjuvants.

6. Use of immunomodulators as future adjuvants

Several types of vaccines are currently being used in humans and animals. These include live attenuated-, inactivated-, subunit- and DNA-vaccines. Live attenuated vaccines are the most immunogenic and their use is common practice in animals. However, because of safety concerns, only very few live attenuated vaccines are licensed for use in humans, Subunit vaccines, consisting of recombinant protein subunits, synthetic peptides and DNA vaccines are safer but often less immunogenic [319]. Thus, adjuvants are required that can enhance the immunogenicity of these vaccines.

Adjuvants can improve vaccine efficacy by i.) optimizing the delivery of the vaccine, ii.) increasing depot effect iii.) improving recruitment and activation of immune cells to the site of vaccination and iv.) modulating the immune response towards either a

Th1- or Th2-type immune response. DCs are key cells that link innate and acquired immunity. Thus, adjuvants that can enhance the recruitment and activation of DCs represent very promising adjuvant candidates for future vaccines. Indeed, the potential of CpG ODN and host defense peptides to enhance both innate and acquired immune responses against a variety of infectious agents has already been demonstrated in a large body of studies. It is our belief that future vaccines will be dramatically improved with the introduction of these 'molecular adjuvants', which will not only enhance the kinetics and magnitude of the immune response but also modulate the quality of the response to ensure both long term memory and appropriate elimination of the pathogen.

Appendix C: Strategies to link innate and adaptive immunity when designing vaccine adjuvants (as published in Garlapati, Facci et al. 2009; Vet Immunol Immunopathol)[229]

Abstract

Adjuvants are important components of vaccine formulations. Their functions include the delivery of antigen, recruitment of specific immune cells to the site of immunization, activation of these cells to create an inflammatory microenvironment, and maturation of antigen-presenting cells for enhancement of antigen-uptake and -presentation in secondary lymphoid tissues. Adjuvants include a large family of molecules and substances, many of which were developed empirically and without knowledge of their specific mechanisms of action. The discovery of pattern recognition receptors including Toll-like-, nucleotide-binding oligomerization domain (NOD)- and mannose-receptors, has significantly advanced the field of adjuvant research. It is now clear that effective adjuvants link innate and adaptive immunity by signaling through a combination of pathogen recognition receptors (PRRs). Research in our lab is focused towards the development of novel adjuvants and immunomodulators that can be used to improve neonatal vaccines for humans and animals. Using a neonatal pig model for pertussis, we are currently analyzing the effectiveness of host defence peptides (HDPs), bacterial DNA and polyphosphazenes as vaccine adjuvants.

Appendix D: Biological roles of host defense peptides: lessons from transgenic animals and bioengineered tissues (as published in Dybvig, Facci et al. 2011; Cell Tissue Res)[320]

Abstract

Host defense peptides (HDPs) have long been recognized as microbicidal agents, but their roles as modulators of innate and adaptive immunity have only more recently been appreciated. The study of transgenic animal and tissue models has provided platforms to improve our understanding of the immune modulatory functions of HDPs. Here, the characterization of transgenic animals or tissue models that over-express and/or are deficient for specific HDPs is reviewed. We also attempt to reconcile this data with evidence from human studies monitoring HDP expression at constitutive levels and/or in conjunction with inflammation, infection models, or disease states. We have excluded activities ascribed to HDPs derived exclusively from *in vitro* experiments. An appreciation of the way that HDPs promote innate immunity or influence the adaptive immune response is necessary in order to exploit their therapeutic or adjuvant potential and to open new perspectives in understanding the basis of immunity. The potential applications for HDPs are discussed.

Appendix E: Differential activation and maturation of two porcine DC populations following TLR ligand stimulation (as published in Auray, Facci et al. 2010; Mol Immunol)[92]

Abstract

Dendritic cells (DCs) are at the interface of innate and adaptive immune responses. Once activated via triggering of their pattern recognition receptors (PRRs), they acquire a mature state and migrate to the lymph nodes where they activate T cells and direct the immune response. Compounds that trigger PRRs are potential vaccine adjuvants, hence in this study we stimulated two porcine DC populations, namely monocyte-derived DCs (MoDCs) and blood DCs (BDCs), with a broad range of toll-like receptors (TLRs) ligands and assessed the activation/maturation state of these porcine DCs. In order to determine if TLR ligands would have an effect on porcine DCs, we characterized the expression of TLRs and demonstrated that MoDCs and BDCs expressed the same set of TLRs but at different levels. Of the TLR ligands examined, lipopolysaccharide (LPS) and poly I:C were the most potent activators of MoDCs, inducing the up-regulation of co-stimulatory molecules CD80/86 and the chemokine receptor CCR7, and production of pro-inflammatory cytokines interleukin (IL)-6 and tumor necrosis factor (TNF)alpha. The most effective in inducing BDCs activation were LPS and class A CpG oligodeoxynucleotide (ODN), resulting in up-regulation of chemokine receptor (CCR)7 and down-regulation of CCR2 and CCR5, production of IL-12p40, and expression of a broad range of chemokines that were able to attract porcine immune cells.

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