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Transcriptional analysis of differential immune responses to *Sarcoptes scabiei* infestation in a porcine model

By

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Abstract

Scabies is one of the most prevalent skin diseases affecting over 100 million people globally. The prevailing knowledge of the disease processes and host immune response mechanisms is limited, and to identify novel vaccine and drug targets, a better understanding of the host-parasite relationship is essential. The objective of this study was to perform gene expression analysis of the skin immune response to infestation with *Sarcoptes scabiei* to gain a better understanding of the immune mechanisms and signaling pathways involved.

A 16-week animal trial employing experimentally *S. scabiei* infected (n=12) and control pigs (n=6) was conducted. Scoring for clinical phenotype, monitoring of mite infestation, skin biopsies and blood samples were collected at different time points of disease progression (at baseline week 0, and at weeks 1, 2, 4 and 8 post-infestation). Transcriptomic analysis was undertaken using RNA extracted from the skin biopsies (n=60) from infested pigs with crusted scabies (n=4), ordinary scabies (n=4) and non-infested controls (n=4) at each time point. Microarrays were performed with the Agilent Porcine Gene Expression Microarray platform and differential gene expression analysed.

A large number (>1000) of significantly differentially expressed genes were identified at each time point. The analysis revealed numerous genes with roles in allergy and inflammation, including pro-inflammatory cytokines and chemokines involved in immune cell activation and recruitment. In addition, the analysis demonstrated expression of transcripts of various cell surface markers, acute phase proteins, transcription factors, and signaling molecules. Key signaling pathways involved with scabies mite infestation included, “Th1 and Th2 Activation Pathway”, “JAK-STAT Signaling Pathway”, “NF-κB Signaling Pathway”, “Immune Cell Trafficking”, “Communication between Innate and Adaptive Immune Cells”, “Acute Phase Response Signaling” and “Role of IL-17F in Allergic Inflammatory Airway Disease”. In

crusted scabies relative to ordinary scabies, we detected gene expression associated with pathophysiologic pathways implicated in psoriasis, atopic dermatitis, rheumatoid arthritis and Th17 (IL17A, ARG1) pronounced responses.

To validate the differential gene expression, qRT- PCR confirmation was carried out for selected genes. ELISA analysis was also performed to characterise acute phase response proteins (Haptoglobin, Transferrin, Serum amyloid A and α1 Acid glycoprotein) in serum collected from pigs at selected time points. The analysis demonstrated an increased trend in SAA and significantly higher AGP levels post mite infestation in crusted scabies.

In summary, transcriptional profiling revealed genes in the T helper lymphocyte (Th1), Th2 and Th17 signaling pathways, acute phase response signaling, and allergic inflammatory response are associated with scabies. For the first time, we have described key transcriptional changes associated with the development of ordinary scabies and significantly, the distinction between ordinary and crusted scabies. This work provides the basis for follow-up studies in clinical patients with crusted scabies and may provide new control strategies for this severely debilitating disease.

Declaration

I hereby declare that the work and thesis presented here, is the result of my own independent research, and all references to ideas and work of other researchers have been specifically acknowledged. I declare that neither any part nor the whole of this thesis has been previously accepted for any degree or diploma, and is not being concurrently submitted in candidature for any degree or diploma to any other university or institution. I hereby give consent for my work, now submitted as a thesis for the degree of Doctor of Philosophy of the University of the Sunshine Coast.

Sajad A Bhat

2 August 2017

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Last year I was fortunate enough to attend the XIX International Congress for Tropical Medicine and Malaria 2016 (ICTMM 2016) in Brisbane with the support of the Australian Society of Parasitology. This conference was a fantastic opportunity to meet and interact with students, researchers, experts in the fields of Parasitology, Immunology, etc, to get know about their work and to keep abreast with the current happenings in our field. In addition, it was platform to present my work on Scabies to the wider research community.

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Publications

Journal Articles:

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Abbreviations

AGP: Alpha-1-acid glycoprotein 1;

AIDS: Acquired Immunodeficiency Syndrome;

ANOVA: Analysis of variance;

ARG: Arginase;

AREG: Amphiregulin;

APO: Apolipoprotein;

C: Complement;

CCL: C-C Motif chemokine ligand;

CCR: C-C Motif chemokine receptor;

CD: Cluster of differentiation;

CS: Crusted scabies;

CSF: Colony stimulating factor;

CTACK: Cutaneous T-cell-attracting chemokine;

CXCL: Chemokine (C-X-C motif) ligand;

CXCR: Chemokine (C-X-C motif) receptor;

DC: Dendritic cell;

DCT: Dopachrome tautomerase;

DEGs: Differentially expressed genes;

ELISA: enzyme-linked immunosorbent assay;

FC: Fold change;

FOXP3: Forkhead Box P3;

γδ: Gamma delta;

GM-CSF: Granulocyte-macrophage colony-stimulating factor;

HLA/SLA: Human leukocyte antigen/swine leukocyte antigen

HP: Haptoglobin:

ICAM: Intercellular adhesion molecules;

iCOS: Inducible T-cell costimulator;

IFN: Interferon;

Ig: Immunoglobulin;

IL: Interleukin;

IPA: Ingenuity pathway analysis;

JAK: Janus kinase;

MAP: Mitogen-activated protein;

M-CSF: Macrophage colony-stimulating factor;

MYD88: Myeloid differentiation primary response 88;

MMP: Matrix metallopeptidase;

NCF: Neutrophil cytosolic factor;

NF-κB: Nuclear factor kappa B;

NK cell: Natural killer cell;

OS: Ordinary scabies;

OSM: Oncostatin M;

PBMCs: Peripheral blood mononuclear cells;

PCA: Principal component analysis;

RFX5: Regulatory factor X5;

SAA: Serum amyloid A;

S100: S100 calcium binding protein;

SELPLG: P-selectin glycoprotein ligand 1;

SMIPPs: scabies mite proteases;

SMSs: scabies mite serpins;

STAT: Signal transducer and activator of transcription;

SOCS: Suppressor of cytokine signaling;

TGF: Transforming growth factor;

TF: Transferrin;

Th: T helper;

TNF: Tumour necrosis factor;

TLR: Toll-like receptor;

Tregs: Regulatory T cells;

Wpi: Weeks post-infestation.

Chapter 1

Introduction

Scabies remains a poorly understood parasitic disease affecting humans and animals worldwide. In humans, the disease is endemic in impoverished communities of developing countries and is a major public health problem in Indigenous populations of developed nations (Hay et al., 2014; Romani et al., 2015a). In addition, scabies associated secondary bacterial infections and post-infective complications such as rheumatic heart disease and acute post-streptococcal glomerulonephritis cause substantial morbidity (Hoy et al., 2012; Lawrence et al., 2005). *Sarcoptes scabiei* infestation (sarcoptic mange) is also a major concern in domestic and livestock animals, as it has significant economic implications and is detrimental to animal welfare.

Scabies occurs due to infestation of skin by the mite *S. scabiei* (Mellanby, 1941) and commonly results in an intensely pruritic skin rash caused by the host's allergic and inflammatory responses to the mite (Walton, 2010). However, in some cases, a debilitating condition called Crusted scabies (CS) occurs, characterised by hyperproliferation of mites and development of hyperkeratotic skin crusts (Walton et al., 2010). The reasons why some humans or animals contract Ordinary scabies (OS) while others develop the highly distressing CS phenotype remains unknown.

An important hallmark of primary *S. scabiei* infestation is that the hosts do not show disease manifestations until weeks after infestation (Liu et al., 2014b; McCarthy et al., 2004; Rampton et al., 2013). *In vitro* studies have demonstrated that the mites suppress the early host responses by inhibiting the production of pro-inflammatory mediators/factors (Arlian et al., 2007; Arlian et al., 2003; Arlian et al., 2004b; Elder et al., 2006; Morgan et al., 2013), the detailed mechanisms of which are not clear yet.

It is clear from previous studies (Abd El-Aal et al., 2016; Liu et al., 2014b; Mounsey et al., 2015; Roberts et al., 2005; Walton et al., 2008; Walton et al., 2010) that the immune response in scabies is intricate with marked differences between ordinary and crusted phenotypes. Ordinary scabies shows a Th1 skewed response whereas, CS is characterised as an allergic Th2 and IL-17 type response with patients exhibiting increased IgE and peripheral eosinophilia (Walton, 2010; Walton et al., 2008; Walton et al., 2010). The reasons for these increased and distinct host responses are not clear yet. To gain information about these processes is essential to understand the factors driving these events and immunological progression in OS and CS.

The susceptibility to CS may be associated with immunosuppressive conditions (Einsiedel et al., 2014; Gregorini et al., 2012; Roberts et al., 2005) but CS has also been seen in cases with no identifiable risk factor or immune deficit (Roberts et al., 2005). The underlying mechanisms of CS disease development are not yet known. In addition, in experimental infestations in animals, inoculation with similar numbers and same variant of mites result in some OS while in others it progresses to CS (Liu et al., 2014b; Rampton et al., 2013). Immunity related genetic factors may be responsible for susceptibility to CS and these key regulators still need to be investigated.

Despite recent advances in understanding immunological aspects of the disease, the immune and inflammatory responses in scabies and the associated immunopathology still remain poorly characterised. Studies so far have been limited and mostly performed *in vitro* using cell cultures and mismatched host-parasite systems (Arlian et al., 2003; Arlian et al., 2004b; Arlian et al., 2006; Morgan et al., 2013) wherein it is very hard to capture interactions between different cell types and difficult to extrapolate from the results of such works due to host specificity of mites (Walton et al., 2004). Furthermore, the interpretation of results from human studies are confounded by patients often presenting at an advanced stage of disease,

with validity further affected by the difficulties in assessment of disease severity and associated co-morbidities. It is therefore essential to obtain insights into the processes underlying the host responses to the mites especially early in the infestation prior to appearance of disease manifestations, with *in vivo* prospective studies.

Historically scabies research in humans has been and still remains a challenging task due to limited access to clinical cases, parasite material and more importantly due to the logistical and ethical difficulties to undertake longitudinal prospective immune response studies in humans. In this regard, animal disease models are of great value and provide an alternative approach to study the immunological aspects of the mite infestation.

My PhD work was a part of larger experimental trial wherein we used a novel porcine model to investigate the immune and inflammatory events in the skin, and to fill gaps in the knowledge, with following aims

1. Understand the temporal progression of immune and inflammatory responses in scabies.
2. Understand the immune mechanisms leading to development of ordinary or crusted scabies.
3. To gain insights into the underlying susceptibility to CS.
4. Comparing transcriptional profiles of immune and inflammatory responses in scabies with other skin inflammatory diseases.

and objectives

1. To identify the key regulatory/specific immune factors predisposing the animals to CS.
2. To identify pathogenesis associated gene signatures and differentially regulated pathways.

3. To identify overlapping gene expression profiles and common pathogenic mechanisms.

Microarrays have been used to study gene expression profiles and identify immune response mechanisms in various chronic inflammatory skin diseases like Psoriasis, Sheep scab, Atopic Dermatitis, etc. (Burgess et al., 2010; Burgess et al., 2012; Choy et al., 2012; Coda et al., 2012) which are clinically similar in various aspects to scabies. In this work, transcriptomic analysis using porcine microarrays combined with network and pathway mapping approach (IPA) was carried out to gain insights about the signaling pathways and factors involved in scabies pathogenesis, and identify the early key events differentiating the host immune and inflammatory responses in OS and CS phenotypes. Statistical significance and differential gene expression was determined using Partek software program. The microarray results were validated by qPCR using selected transcripts with significant differences in expression patterns. Transcriptomic analysis will help to identify key genetic factors, distinct patterns of gene expression and the signaling pathways involved in distinct responses in OS and CS. It will also provide a better understanding of immune and inflammatory responses which will be helpful in identifying novel diagnostic targets and the development of new therapeutic approaches against scabies.

Another aspect of my PhD was to study acute phase proteins (APPs). Acute phase proteins have been used as efficient biomarkers of inflammation, infection and stress in various human and animal diseases (Dayer et al., 2007; Eckersall and Bell, 2010; Germolec et al., 2010; Pomorska-Mól et al., 2013; Pomorska-Mól et al., 2015). In scabies, exact diagnosis is very challenging due to the fact that the early stages of the disease are symptomless and also due to non-availability of specific diagnostic tests. In this work, acute phase proteins were studied/investigated to characterise acute phase response and understand the use of APPs as

biomarkers of inflammation and disease severity, and which in turn will facilitate effective management and treatment of scabies prior to specific disease outcomes.

Chapter 2

Literature review

2. 1. Introduction

Scabies is an infestation of the skin caused by the burrowing ectoparasitic mite called *Sarcoptes scabiei* variety *hominis* (Greek word ‘sarx’ means flesh; ‘koptein’ means to smite or to cut and the Latin word ‘scabere’ means to scratch) (Hicks and Elston, 2009). It was reported in 2010 that about 100 million of the global population is infected with scabies (Hay et al., 2014) and prevalence in different regions ranged from 0.2% to 71.4% (Romani et al., 2015a). Scabies has been found to be more prevalent in developing countries and has a high impact on the health and social life of indigenous populations in developed countries (Hay et al., 2014). In particular, countries of the Pacific and Latin American regions have a high burden of scabies and prevalence is substantially higher in children than in adolescents and adults (Hay et al., 2014; Romani et al., 2015a). The global burden of scabies is reflected by the disability-adjusted life years (DALYs), a measure of health loss due to a disease or injury. One of the leading causes of skin related DALYs in 2010 was scabies, with around 1.5 million DALYs attributable to scabies alone (Murray et al., 2012). In addition to this direct burden, scabies is also linked to secondary complications such as rheumatic heart disease (RHD) and acute post-streptococcal glomerulonephritis (APSGN) (Hoy et al., 2012). These secondary complications if left untreated can lead to the development of serious downstream systemic and life-threatening conditions (Engelman et al., 2013).

People with scabies suffer from intense itching mediated through allergic and inflammatory reactions mounted by the host against the mite and its products. A wide range of clinical features, from mild to severely destructive, occurs in scabies but despite the significant

worldwide impact of the disease, the immune and inflammatory responses associated with the different clinical manifestations remain poorly characterized.

2. 2. Biology of the mite *S. scabiei*

The mite *S. scabiei* belongs to the phylum Arthropoda, family Sarcoptidae within the class of Arachnida. The mite is hardly visible to the naked eye but can be observed easily with a dissecting microscope. The mite is opaque, creamy white in colour with brown legs and mouth parts. The mite is recognized by the characteristic oval, ventrally flattened, and dorsally convex tortoise-like body, stout dorsal setae, numerous cuticular spines, and transversely ridged cuticular striations. The male (200-300 µm long by 150-200 µm wide) is about two thirds the size of the female (300-500 µm long by 230-420 µm wide) (Arlian, 1989).

All life cycle stages can penetrate intact epidermis and post transmission female mites dissolve the stratum corneum of the host epidermis (Arlian et al., 1984) with proteolytic secretions and burrow through to feed in the deeper moist layers of the epidermis. Female mites live for 4–6 weeks and produce 2–4 eggs per day, which are deposited in the burrowed tunnel (Mellanby, 1985). Larvae hatch 2–4 days after the eggs have been laid, and adult mites develop 10–14 days later (Arlian, 1989; Mellanby, 1985).

2. 3. Epidemiology and prevalence of scabies

Scabies is prevalent worldwide and it occurs both epidemically and endemically. Recent examples include an outbreak in the British Army where 100 Coldstream Guardsmen protecting the Queen contracted scabies (http://www.dailymail.co.uk/news/article-2305038/Queens-Guards-invaded--scabies-outbreak-Military-exercises-Germany-blamed-dozens-hit-skin-disease.html).

The Global Burden of Disease (GBD) Study 2010 estimated that about 100.6 million are infected with scabies worldwide and scabies occurs in the top 50 most prevalent skin diseases globally (Hay et al., 2014). According to the systematic review (Romani et al., 2015a) of prevalence studies of scabies, after accumulating data from 48 prevalence studies that reported on the epidemiology of scabies between 1985 and 2014, prevalence of scabies was reported in the range between 0·2% and 71·4%. It is notable that particularly high scabies prevalence was seen in the Pacific and Latin American regions. In general population, the highest prevalence of scabies was reported in Papua New Guinea 71% followed by Panama 32% and Fiji 32% (Bockarie et al., 2000; Haar et al., 2014; Taplin et al., 1991a).

Scabies prevalence was substantially higher in children than in adolescents and adults (Romani et al., 2015a). The highest scabies prevalence described in children was recorded in Panama, with scabies detected in 78% of children younger than 2 years and in 60% of those aged between 2 years and 6 years (Taplin et al., 1991a). In remote Aboriginal communities in Northern Territory of Australia clinic attendances for skin conditions have shown that 63% children by 1 year of age had presented with scabies (Clucas et al., 2008) and in Fiji, prevalence of 44% was reported in children aged 5-9 years (Romani et al., 2015b).

Mange caused by *S. scabiei* is responsible for epizootic disease in populations of many domestic and wild mammals worldwide, including dogs (*S. scabiei* var. *canis*), foxes, dingos, cats, pigs (*S. scabiei* var. *suis*), horses, camels, black bears, monkeys, boars, wombats and koalas, and great apes (Pence and Ueckermann, 2002). The mange epizootic diseases in livestock animals such as pigs is significant economically and worldwide estimation of between 50% and 95% of pig herds are reportedly infected with *S. scabiei* (Cargill et al., 1997).

2. 4. Clinical manifestations of scabies

Although a range of clinical presentations are apparent in scabies, for the purpose of this review we consider the two most commonly reported manifestations - ordinary scabies (OS, also known as classical or typical scabies) and crusted scabies (CS, also known as Norwegian scabies, or scabies crustosa).

Ordinary scabies is the common form of scabies with a mite burden estimated to be less than 15 mites per person (Mellanby, 1941). The main clinical signs include burrows, erythematous papules, and an allergic type skin reaction with intense, generalised pruritus. Occasionally, patients are asymptomatic (Wendel and Rompalo, 2002). Onset of the symptoms in a host with no previous infestation is delayed and occurs at 4 to 6 weeks post infestation (McCarthy et al., 2004). The primary papules may develop into secondary scabies lesions: excoriations and eczematisations. Patients usually show primary and secondary lesions existing together at the same time. Due to severe itching patients scratch the skin, opening up the lesion and making them susceptible to secondary bacterial infection.

Crusted scabies is relatively rare and an extreme manifestation with thousands of mites present which are the same variant as those causing OS (Walton et al., 1997). Due to the high number of mites present, CS is highly contagious as evidenced by nosocomial outbreaks of OS from index cases of CS (Mounsey et al., 2016). Clinically, CS is a hyperkeratotic skin disease with thick and scaly crusts containing large numbers of mites. In CS patients, the infectivity persists for longer because of the difficulty in eradicating mites from heavily crusted skin. Mite reinfestation frequently occurs in the same individual and it is extremely debilitating and can cause permanent skin disfiguration. CS patients may show deep fissuring of the crusts with pathogenic microbes gaining entry through these skin breaches and leading to serious secondary infections, frequently with the typical skin pathogens *Staphylococcus*

aureus and *Streptococcus pyogenes*. Generalized lymphadenopathy due to secondary sepsis is common carrying high mortality rate if left untreated (Currie and Carapetis, 2000; Hulbert and Larsen, 1992; McCarthy et al., 2004).

It is generally believed that immunosuppression and immunomodulation might be predisposing factors associated with CS. CS has been shown in immunocompromised patients such as those with human immunodeficiency virus (HIV) infection (Hulbert and Larsen, 1992), human T-lymphocytic virus 1 (HTLV-1) infection (Einsiedel et al., 2014; Roberts et al., 2005) and in patients undergoing organ transplantation (Youshock and Glazer, 1981). In addition, CS has been diagnosed in individuals with leprosy (Roberts et al., 2005) and developmental disability, including Down's syndrome, although the specific mechanisms linking these immune defects to crusted scabies have not yet been explored. Importantly, CS has also been detected in patients with no recognised immunodeficiency as evidenced in Aboriginal Australians (Gogna et al., 1985; Roberts et al., 2005). From these reports, it appears that the susceptibility of this cohort to CS may be due to a specific immune deficit, the nature of which is yet to be defined.

2. 5. Scabies and secondary bacterial infections

Scabies apart from the direct pathology it causes, is also responsible for secondary complications. The breaks in skin epidermis due to burrowing of scabies mites, the host scratching due to the pruritic rash and subsequent excoriations serve as an entry point for pathogenic bacteria and promote secondary skin infections (Heukelbach and Feldmeier, 2006). In tropical regions, scabies is frequently associated with pyoderma primarily caused by secondary bacterial infection of the scabietic lesions, in particular by *Streptococcus pyogenes* (group A streptococci, GAS) and *S. aureus* (Currie and Carapetis, 2000; Feldmeier et al., 2005). In the remote Aboriginal communities of northern Australia, 70% of children

were reported to have scabies and pyoderma before reaching two years of age (Clucas et al., 2008; Hay et al., 2012; Whitehall et al., 2013).

These secondary complications associated with scabies are of important significance because if left untreated they can lead to the development of serious downstream systemic and life-threatening diseases in the host (Engelman et al., 2013). An estimated 18 million individuals are affected globally by GAS associated diseases, such as rheumatic heart disease (RHD), acute post-streptococcal glomerulonephritis (APSGN) and severe invasive diseases causing over 0.5 million deaths per year (Carapetis et al., 2005).

Substantial evidence indicates that streptococcal pyoderma in turn contributes to the development of APSGN. For example, Streeton et al. (2008) in a survey of Aboriginal children in northern Australia reported symptomatic APSGN to be present in 10% of their scabies subjects. Hoy et al. (2012) have also shown that all APSGN cases in Australian aboriginal children were associated with group A streptococcal skin infections, and they were often related to scabies. APSGN has been shown to act as a strong risk factor for chronic kidney disease (CKD) later in life (Chung et al., 2014; Hoy et al., 2012) and an additional 24% of the study population demonstrated microscopic haematuria, suggesting that asymptomatic renal damage can also occur (Streeton et al., 2008). CKD is a progressive condition which may lead to chronic renal failure (Chen et al., 2009) and is also associated with a significantly increased risk of hospital admission, morbidity and death due to cardiovascular disease (Coresh et al., 2007). This association of scabies with APSGN is supported by intervention studies designed to treat scabies where it has been reported a substantial reduction in pyoderma even without use of antibiotics or other pyoderma-specific interventions (Carapetis et al., 1997; Currie and Carapetis, 2000; Lawrence et al., 2005; Taplin et al., 1991b).

Furthermore, it has been suggested that high burden of GAS on the skin of scabietic population of Australian Aboriginals communities contributes to the exceptionally high incidence of acute rheumatic fever and rheumatic heart disease (RF/RHD) recorded in these communities (Currie and Brewster, 2002; McDonald et al., 2004). In these communities RF/RHD prevalence has steadily risen to almost 2% in 2008 (Parnaby and Carapetis, 2010), translating to the highest incidences reported globally.

Additionally, the drug resistant bacterial strains can further complicate the secondary infections and more recently, methicillin resistant *S. aureus* (MRSA) has been reported in 64% of children presented with scabies in a regional hospital of North West Queensland (Whitehall et al., 2013).

The epidemiological evidence available so far supports the fact that secondary bacterial infections are closely associated with scabies, and mechanical disruption of the upper epidermal layers by the mites and host scratching considered as obvious prerequisites. However, the contribution of molecular interactions between host, parasites and bacteria is still poorly understood. It is highly important to consider the role of the complement system when analysing the scabies – pyoderma connection.

2. 6. Skin immune responses

The skin acts a barrier and defends the body against a host of toxins, pathogenic organisms, physical and chemical insults. The surveillance of this large and exposed organ by immune sentinels and effector cells is a huge but very important task. To this end human skin does appear to have reserves of immune sentinels and effector cells including tissue-resident T cells, macrophages, and Dendritic cells (DCs) (Boyman et al., 2007). Regulated immune responses are highly essential as improper response may result in infections or tumours and hyper response is associated with chronic inflammation and autoimmunity.

The epidermis and the dermis are the two main layers of the skin. The epidermis being the outer layer, is divided into four strata - the stratum basale, the stratum spinosum, the stratum granulosum and the outermost layer, the stratum corneum and is vital in maintaining the barrier function of the skin (as reviewed in Nestle et al., 2009). This compartment of the skin contains specialized resident immunocytes called Langerhans cells (LCs) and also maintains reserves of rare T cells, mainly $CD8^+$ cytotoxic T cells (Krueger and Stingl, 1989).

Keratinocytes are the major cell type of the epidermis, constituting more than 90% of epidermal cells which act as sensors of danger through the expression of molecular patterns called toll like receptors (TLRs) and alert systems such as the inflammasome (molecular platform composed of multiple proteins) responsible for the maturation of potent pro-inflammatory cytokines interleukin (IL) 1β and also IL-18 (Martinon et al., 2009). Keratinocytes upon expressing TLRs can lead to a predominant Th1 biased immune response and to the production of type I interferons (IFNs) and thereby promote skin immune responses (Miller and Modlin, 2007). Upon inflammasome activation, key pro- inflammatory cytokines IL-1β and IL-18 (Martinon et al., 2009) are secreted which in turn result in the activation of tissue-resident immune cells that initiate and propagate an inflammatory response such as in allergic contact dermatitis reactions (Watanabe et al., 2007).

Langerhans cells along with other DCs are highly specialized in sensing danger and presenting antigens to tissue-resident macrophages. These macrophages specialize in phagocytosis (Blander and Medzhitov, 2006; Filgueira et al., 1996) and help in clearing potentially dangerous substances and they also mediate tissue remodelling. Macrophages are generally phagocytic in nature (Blander and Medzhitov, 2006; Filgueira et al., 1996) and also these cells are able to act as effective antigen-presenting cells for $CD8^+$ T cells (Pozzi et al., 2005). Macrophages of the skin are usually not freely moving entities but these cells in times of inflammatory response move to nearby lymph nodes (van Furth et al., 1985) wherein they

secrete pro-inflammatory cytokines and immune mediators thereby take part in pro-inflammatory responses.

Langerhans cells regenerate in epidermis (Merad et al., 2002) and are among the first DCs to come into contact with microbial antigens and process these along with lipid antigens for presentation to effector T cells (Hunger et al., 2004). Also, it has been shown that human LCs are responsible for the differentiation of Th 2 cells which they carry out on preferential basis. In addition, these cells also can prime and cross-prime naive $CD8^+$ T cells (Klechevsky et al., 2008).

The skin dermis which is the layer beneath epidermis has been shown to contain numerous special immune cells, including dermal DCs and plasmacytoid DCs (pDCs) cells (Lenz et al., 1993; Nestle et al., 1993), and among the T cell subsets it contains $CD4^+$ T helper 1 (Th1), Th2 and Th17, gamma delta (γδ) T and natural killer T (NKT) cells. The dermal immune cell repertoire also contains macrophages, mast cells and fibroblasts. The dermal immune system is highly essential and participates actively in most of the chronic inflammatory skin disorders including psoriasis and atopic dermatitis (Nickoloff, 1993). This vast number of cells enter and leave the dermis via lymphatic and vascular conduits (Nickoloff, 1993). Dermal DCs are antigen-presenting cells as well and their main function is to process antigenic material and present it on the cell surface to the T cells. They act as messengers between the innate and the adaptive immune systems. Upon activation DCs traffic to lymph nodes and there they interact with T and B cells to orchestrate the adaptive immune response (Kissenpfennig et al., 2005). These activated dermal DCs take part in the inflammatory response by secreting cytokines and chemokines generating a network of these molecules which in certain circumstances helps in eliminating the pathogenic agents but in many settings it is responsible for the pathology (Guttman-Yassky et al., 2007). Dermal DCs that

produce both tumor necrosis factor (TNF) α and inducible nitric oxide synthase (iNOS) have been proposed to have a major role in psoriasis (Lowes et al., 2005).

Plasmacytoid DCs are hardly ever present in the healthy skin but they have been shown to play a role in the pathogenesis of systemic lupus erythematosus and psoriasis (Blanco et al., 2001; Nestle et al., 2005). Boyman et al. (2007) propose that upon activation, pDCs trigger an innate immune response in psoriasis which is followed on by the activation of myeloid DCs and then adaptive immune responses are mounted.

T cells residing in the normal healthy skin are twice the number found in the blood and exceed more than 2×10^{10} cells (Clark et al., 2006). Various distinct types of T cells reside in the skin epidermis predominantly memory $CD8^+$ T cells which frequently exist in nearby vicinity along with LCs (Bos et al., 1987; Foster et al., 1990). On the other hand it has been suggested by Bos and Kapsenberg (1993) that $CD4^+$ and $CD8^+$ T cells are present in dermis of the skin in equal numbers. T helper (Th) 1, Th2 and Th17 cells are the three main types of $CD4^+$ T cells that have been found in the skin during various inflammatory and infectious diseases (Abbas et al., 1996; Harrington et al., 2006; Sallusto and Lanzavecchia, 2009). For instance, in response to intracellular pathogens Th1 cells secrete interferon (IFN) γ and lymphotoxin and activate macrophages which eliminate these pathogens by phagocytosis. Previously it was thought that Th1 cell responses were responsible for the pathogenesis in autoimmunity and immune-mediated disease, such as psoriasis (Di Cesare et al., 2009), whereas Th2 cell biased immune responses were held responsible in allergic disease pathology, such as in asthma and atopic dermatitis (Di Cesare et al., 2008). Th17 cells have been shown to be vital in the first line defence against various fungal and bacterial infections (Weaver et al., 2007). However recently, Th17 cells have been implicated in the pathogenesis of psoriasis (Di Cesare et al., 2009), atopic dermatitis (Di Cesare et al., 2008), and mycobacterial disease (de Beaucoudrey et al., 2008; Milner et al., 2008), and their improper

functioning could also be responsible for chronic mucocutaneous candidiasis (Eyerich et al., 2008).

Among the other T cells in the skin, gamma delta (γδ) T cells (Hayday and Tigelaar, 2003) and natural killer (NK) T cells (Kronenberg, 2005) constitute the majority of unconventional or innate-like T cells. γδ T cells constitute about 2-9% of skin dermis and 1-10% of epidermis. γδ T cells secrete cytokine IL-17 which has been implicated in the pathogenesis of psoriasis and other autoimmune inflammatory diseases (Fitch et al., 2007). γδ T cells constitute 3–5% of all lymphoid cells found in the secondary lymphoid tissues and the blood. These cells are the first immune cells found in the foetus and provide immunity to newborns prior to activation of the adaptive immune system (Sinkora et al., 2005). γδ T cells are T cells with a distinctive T cell receptor (TCR) on their surfaces than the αβ T cells (CD 4^+ and CD 8^+ T cells) and this TCR does not engage MHC-antigen complexes in the same manner as αβ T cells (Crowley et al., 1997). γδ T cells are generally less common than αβ T cells. They are likely to originate from foetal γδ thymocytes. γδ T cells are preferentially localized in barrier tissues and have been found in substantial numbers in the peripheral mucosal tissues such as the dermal layer of the skin (Price et al., 2012). While αβ T cells require antigen-specific priming and an inflammatory environment in order to develop (Weaver et al., 2007) and function on the other hand γδ T cells are different in that they do not seem to require antigen processing but are triggered through the engagement of pathogen pattern recognition receptors (PRRs) and/or by inflammatory cytokine receptors (Hamada et al., 2008; Kapsenberg, 2009; Lockhart et al., 2006). γδ T cells have been shown to secrete IL 17 and this is promoted through cytokine signaling, in particular by IL-23 in combination with IL-1β or IL-18. The inflammatory cytokine IL-17 plays a critical role in immunity to infection (Cho et al., 2010; Hamada et al., 2008; Lockhart et al., 2006; Peng et al., 2008) and is involved in

the inflammatory pathology associated with certain autoimmune diseases, such as psoriasis and rheumatoid arthritis (Cai et al., 2011).

2. 7. Allergic/inflammatory responses

Allergy is one of the four forms of hypersensitivity and is formally called type I (or immediate) hypersensitivity. Allergic reactions are distinctive because of excessive activation of eosinophils, mast cells and basophils by immunoglobulin (Ig) E. The membrane bound IgE interacts with an allergen causing the release of chemicals by basophils and mast cells leading to an allergic reaction or inflammatory response which can range from uncomfortable to fatal (Kay, 2000). Substances that cause allergic reactions are called "allergens" and examples include pollens, dust and scabies mite products, mold, insect bites, animal proteins, foods, and even drugs (Schwartz, 2011). Allergies can cause a variety of symptoms such as an immediate cutaneous weal and flare reaction, runny nose, sneezing, wheezing, itching, rashes, swelling, or asthma (Kay, 2000).

Allergic diseases are classified into two types – atopic and non-atopic (Greek ‘atopos’ means ‘out of place’). In atopic disorders, IgE is produced that mediates the disease like in allergic rhinitis, asthma and atopic eczema.

On the other hand, in non-atopic individuals, IgE-independent mechanisms are responsible for the pathogenesis of type IV (or delayed) hypersensitivity like in contact dermatitis and hypersensitivity pneumonitis and hence considered as non-atopic, allergic disease (Kay, 2000). Delayed hypersensitivity reactions are inflammatory responses that do not involve antibodies but rather is a type of cell-mediated response (T cells and monocytes/ macrophages). Type IV hypersensitivity — cell-mediated reactions are a common response to many intracellular and to large or complex pathogens including mycobacteria, fungi, and certain parasites, and it also occurs in transplant rejection and tumour immunity. These

reactions appear 48-72 hours after antigen exposure as compared to immediate hypersensitivity response which generally appears within 12 minutes of an antigen challenge, hence the term delayed hypersensitivity is used (Janeway et al., 2001a).

Type IV cell mediated hypersensitivity reactions occur in two effector mechanisms (1) delayed-type mediated by $CD4^+$ cells and (2) direct cytotoxicity mediated by $CD8^+$ cells. $CD4^+$ Th 1 cells recognize complexes of antigen: MHC II major histocompatibility complex on the surface of antigen-presenting cells like macrophages and release inflammatory cytokines, such as IL 2 and IFN-γ and induce the further release of other Th1 cytokines, thus mediating the immune response. On the other hand, in direct cytotoxicity, recognition of a cell-surface antigen by the MHC class I receptor on a $CD8^+$ cell triggers the T-cell to release perforin and granzyme which cause apoptosis of the target cell (Janeway et al., 2001a).

Immune response towards various allergens (such as those derived from pollen, house dust, mites and cats) by non-atopic adults and children is usually low; they produce allergen-specific IgG1 and IgG4 antibodies (Kemeny et al., 1989) and *in-vitro* their T cells respond to the allergen with a modest degree of proliferation and production of IFN-γ which is typical of Th1 cells (Ebner et al., 1995; Romagnani, 1991; Till et al., 1997).

In contrast, atopic individuals mount an exaggerated allergen-specific IgE response; with elevated serum IgE levels and show positive skin tests to extracts of various common allergens. It has been shown that T cells from the peripheral blood respond to allergens *in vitro* by producing cytokines of the Th2-type, i.e. IL-4, IL-5 and IL-13 (Ebner et al., 1995; Romagnani, 1991; Till et al., 1997), rather than cytokines of the Th1-type (IFN-γ and IL-2). There are many exceptions to this rule e.g., T cells from atopic subjects have been found to produce a mixed (Th1 and Th2 type) cytokine pattern when challenged *in vitro* by an allergen from house dust mite (Byron et al., 1994; Wierenga et al., 1991). Also in scabies, a mix of

Th1 and Th2 cytokines with increased total IgE, allergen-specific IgE production and eosinophilia has been documented (Abd El-Aal et al., 2016; Walton et al., 2010).

Nevertheless, the immuno-pathological hallmark of allergic diseases is the infiltration of affected tissue by cells with a Th2-type cytokine profile (Durham et al., 1992; Kay et al., 1991; Robinson et al., 1992), elevated total IgE, allergen specific IgE secretion and eosinophilia resulting in allergic reaction or inflammatory response.

2. 8. Immune response in scabies

In animals, *S. scabiei* infestation (sarcoptic mange) results in inflammatory and adaptive immune responses occurring relatively late in the infection (4-6 weeks after initial contact with mite), in contrast to related Psoroptic mange where inflammatory responses are seen almost immediately after mite infestation. Given the parasite's long co-evolution with its hosts, it is believed scabies mites have developed the capability of modulating various aspects of the host immune responses resulting in the delayed onset of symptoms (Cote et al., 2013; Morgan et al., 2013). The rash and itch associated with scabies shows features of both type I (immediate) and type IV (delayed) hypersensitivity reactions. The initial inflammatory response, as reviewed by Walton (2010), towards the mite and its products consists of LCs and eosinophils with smaller number of monocytes, macrophages and mast cells.

2. 8. 1. Innate immune responses

2. 8. 1. 1. Complement system

The complement system is an essential and a far-reaching component of innate immunity and is the first line of defence against invading pathogens. It consists of almost 40 plasma and membrane associated proteins and together this complex network represents one of the major effector mechanisms of the innate immune system (Ricklin et al., 2010). Complement

proteins have been documented in host defence against blood feeding ticks (Wikel, 1979) and also in immune response to other pathogens (Zipfel et al., 2007). Studies analyzing skin biopsies and circulating serum from scabies patients have revealed presence of complement components C3 and C4 (Roberts et al., 2005; Walton et al., 2008) suggesting both local and systemic sources of complement during infection. Complement fragments C3a and C4a act on specific receptors causing local inflammatory responses. In addition, C3a and C5a can activate mast cells to release mediators such as histamine and TNF-α that contribute to the inflammatory response (Janeway et al., 2001b). The observation of these components in skin biopsies of CS patients (Roberts et al., 2005) indicate an activated complement system which may be participating in the early inflammatory responses in scabies. Somewhat counterintuitively, low circulating C3, C4, or both have been reported in CS patients (Roberts et al., 2005), suggesting some potential defect with complement function in CS, or possibly due to massive overload of mites and bacteria the system is unable to maintain production. Furthermore, there is evidence of scabies mite inactivated protease paralogues (SMIPPs) and serpins (SMSs) inhibiting complement activation and promoting bacterial growth *in-vitro*, presumably protecting mites from complement mediated destruction (Mika et al., 2012b; Swe and Fischer, 2014). As suggested (Holt and Fischer, 2013) production of such inhibitory molecules might be a way to evade host defence and also by promoting bacterial growth might provide further mechanisms contributing in disease pathogenesis. While these mechanisms still need to be defined *in-vivo*, a recent study in a porcine model demonstrated the influence of scabies infestation on skin microbiota, with the microbial population changing from commensal to more pathogenic *Staphylococcal* species (Swe et al., 2014b). Such studies are beginning to provide biological insights into the close association between scabies and bacterial skin infection.

2. 8. 1. 2. Innate immune cells

The various innate effector cells detected in response to *S. scabiei* mites in OS and CS include eosinophils, mast cells, basophils, neutrophils, DCs and macrophages (Table 1. 1).

Table 1. 1. Immune response in scabies

	Ordinary Scabies (OS)	Crusted Scabies (CS)
Skin cellular responses	Mostly $CD4^+$ T cells, eosinophils and macrophages (Walton et al., 2008).	Mostly $CD8^+$ T cells, increased $\gamma\delta^+$ T cells, eosinophils and few macrophages (Liu et al., 2014b; Roberts et al., 2005; Walton et al., 2008; Walton et al., 2010)
Blood cell responses	T and B cells and T-cell subsets within normal ranges.	T and B cells and T-cell subsets within normal ranges. Increased $\gamma\delta^+$ T cells, eosinophilia (Liu et al., 2014b; Walton et al., 2008)
Th1/Th2 responses	Th1 mediated with increased production of Th 1 cytokines IFN-γ, IL-2 and TNF-α (Abd El-Aal et al., 2016; Mounsey et al., 2015; Walton et al., 2010). Increased production of IL-10 (Abd El-Aal et al., 2016).	Th2 mediated with increased production of Th2 cytokines IL-4, IL-5 and IL-13 (Mounsey et al., 2015; Roberts et al., 2005; Walton et al., 2010). Increased production of Th17 cytokines IL-17, IL -23 (Liu et al., 2014b; Mounsey et al., 2015). Decreased production of IL-10 (Walton et al., 2008; Walton et al., 2010).
Systemic Ig responses	Variable reports of elevated levels of total IgG, IgE, IgA and IgM. Increased levels of scabies-specific IgE, IgG and IgA (Walton et al., 2008; Walton et al., 2010).	Increased levels of total IgG, IgG1, IgG3, IgG4, IgE and IgA. Elevated levels of scabies specific IgG4, IgE and IgA (Walton et al., 2008; Walton et al., 2010).

Eosinophils are produced in high numbers in allergic inflammation and helminth infections, and tissue eosinophilia is often found at inflammatory sites associated with these diseases (Prussin and Metcalfe, 2006). Histological examination of 25 skin biopsies of scabies infection has shown the presence of dermal eosinophils in 22 patients with 68% of these showing numerous eosinophils and 20% of cases showing few eosinophils (Elwood et al., 2015). In CS, skin biopsy sections from two patients have shown large numbers of eosinophils in the dermis (Walton et al., 2008) and 58% of a cohort of CS patients were reported to have peripheral eosinophilia (Roberts et al., 2005). In *Psoroptes ovis* infested

sheep and cattle, lesional histology studies also show an eosinophil dominated immunoinflammatory infiltrate (Sarre et al., 2015; van den Broek et al., 2000). In addition, eosinophil infiltrations have been detected in the skin dermis of red foxes infested with *S. scabiei* (Little et al., 1998). This eosinophil detection is consistent with the high expression of Th2 representative cytokines IL-4, IL-5 and IL-13 in CS (Walton et al., 2010). Eosinophils have been shown to express Th2 specific cytokines. IL-5 is involved in the attraction, activation and maturation of eosinophils and its production may be an autonomous mechanism for promoting the recruitment and survival of these granulocytes (Hamelmann and Gelfand, 2001; Prussin and Metcalfe, 2006). The presence of eosinophils in CS and their ability to express Th2 profile cytokines (Voehringer et al., 2006) suggests that these granulocytes may themselves modulate or sustain the local Th2 inflammatory responses (Cadman and Lawrence, 2010; Jacobsen et al., 2007) in scabies. Eosinophils may also regulate Th1 inflammatory response. Eosinophils have been shown to produce IL-12 and IFN-γ (Lamkhioued et al., 1996), and express several TLRs (e.g., TLR 7) (Nagase et al., 2003) which are part of innate immunity and responsible for Th1 biased responses. Furthermore, it is also suggested that eosinophil expression of IL-10 and transforming growth factor (TGF) β may suppress local inflammatory responses by modulating the activities and development of regulatory T cells (Tregs). Alternatively cytokine IL-2 is highly important in the development and survival of Treg cells (Boyman and Sprent, 2012) and eosinophil expression of IL-2 can result in the expansion of these T lymphocytes. In addition, eosinophil production of IL-10 and TGF-β (Lamkhioued et al., 1996; Ohno et al., 1996) may alter the local character of the Th2/Th1 responses by preventing the differentiation of naïve T lymphocytes to either the Th1 or Th2 phenotype (Jacobsen et al., 2007). By producing indoleamine 2, 3,-dioxygenase eosinophils may also drive Th1/Th2 imbalance (Jacobsen et al., 2007). Eosinophils are key players in defence against helminthic parasites but also

contribute to tissue dysfunction and damage in allergic disease. However, the function and relative importance of eosinophils in the immune and inflammatory responses of both ordinary and crusted scabies is still undetermined.

Mast cells and basophils share morphological and functional similarities and are essential components in IgE mediated allergic diseases and the immune response to parasitic infections. Mast cells and basophils have been detected in skin lesions of scabies patients (Amer et al., 1995; Ito et al., 2011), and in sheep with psoroptic mange (van den Broek et al., 2000). In pigs, immunohistochemistry of skin lesions has revealed increased mast cells numbers in CS while their number remained steady over the course of infestation in OS (Mounsey et al., 2015). A recent histological analysis of skin lesions of 86 Red Foxes with sarcoptic mange have shown numerous mast cells (Nimmervoll et al., 2013) and mast cells have also been detected in the dermis of free-living wombats with severe sarcoptic mange compared to normal wombats (Skerratt, 2003). Upon activation, mast cells and basophils rapidly produce TNF-α, IL-6, Th2 cytokines IL-4, IL-5 and IL-13, which are the main molecules responsible for the allergic Th2-type inflammation (Prussin and Metcalfe, 2006; Schroeder, 2011). The mechanisms for the infiltration of mast cells and basophils into the blood and skin remains to be addressed to elucidate their role and importance in scabies inflammatory and allergic responses.

Macrophages, neutrophils, and DCs are immune effector cells involved in phagocytosis, antigen presentation and differentiation of T cells. These cells are associated with pro-inflammatory and allergic responses, parasitic infections and possibly humoral responses. IL-4, IL-13, TNF and IFN-γ play a role in alternative macrophage activation (Brombacher, 2000) and these cytokines have been reported in immune response to scabies (Abd El-Aal et al., 2016; Mounsey et al., 2015; Walton et al., 2008; Walton et al., 2010). Macrophages, although very few in number, have been detected in skin of patients with scabies (Walton et

al., 2008) and cellular infiltrates of skin lesions in dogs infested with scabies mites (Arlian et al., 1997; Stemmer et al., 1996). Low numbers of macrophages may be due to the production of immune modulating molecules secreted by the scabies mites. It has been suggested that early in the infestation mites inhibit the ability of macrophages to migrate to the site of inflammation allowing the mites to grow and establish (Cote et al., 2013).

Neutrophils are an essential part of the innate immune system. They drive the initiation of inflammation and are implicated as mediators of tissue-destructive events in various inflammatory diseases as previously reviewed (Barrett and Austen, 2009; Hahn et al., 2016). In a recent histopathological study of human scabietic infections, 25 skin biopsies obtained from scabies patients showed the presence of dermal neutrophils in 52 % of cases (Elwood et al., 2015). In a more recent review, histological findings of skin lesions in 44 cases of bullous scabies (a rare form of scabies, which may develop concurrently with, or after, the occurrence of regular scabietic lesions) revealed neutrophils as the predominant inflammatory cell infiltrates (Luo et al., 2016). Neutrophils have also been detected in inflammatory infiltrates in the skin of common wombats, sheep and red foxes infected with *S. scabiei* (Dagleish et al., 2007; Little et al., 1998; Skerratt, 2003). In an *in-vitro* study using human whole blood, with *S. aureus*, the recombinant *S. scabiei* mite protein SMSB4 was found to suppress bacterial killing by inhibiting opsonisation and phagocytosis by neutrophils (Swe and Fischer, 2014).

Dendritic cells are among the first skin antigen presenting cells to come into contact with antigens, migrate to draining lymph nodes and process the antigens for presentation to effector T cells which results in T cell differentiation and activation. These cells are responsible for pathologies in infections, inflammatory disorders and have also been implicated in modulating the balance between immunity and peripheral tolerance (Hunger et al., 2004; Loser and Beissert, 2007). Histological analysis of the scabietic lesions of dogs have revealed infiltration of DCs in the skin epidermis (Stemmer et al., 1996) and DCs

derived from human peripheral blood mononuclear cells (PBMCs) have been shown to secrete pro-inflammatory cytokines upon stimulation with scabies mite extract (Arlian et al., 2004b). This engagement of DCs, neutrophils and macrophages in scabies warrants further investigations into their function, role and importance in immune and inflammatory responses in scabies mite infestations.

2. 8. 2. Humoral immune responses

Scabies infestation is known to elicit robust antibody-mediated immune responses, especially in crusted scabies which is associated with extremely high levels of antigen specific IgG and IgE (Table 1. 1). However, the timing of these responses, and their relative importance in establishing protective immunity remains poorly understood.

2. 8. 2. 1. IgM

IgM is the first antibody to appear in response to antigen exposure and hence is traditionally considered the first line of the humoral immune response. In a recent study, ELISA analysis of serum in OS patients showed IgM antibodies that bound to scabies antigens in 74% of cases, although a canine antigen mite extract (*S. scabiei* var. *canis*) was used, and high cross reactivity between antigens of scabies and house dust mites was shown which somewhat confounds the interpretation of this finding (Arlian et al., 2015). Nevertheless, these results suggest that IgM may be useful in detecting serum IgM to scabies antigens. IgM is the first antibody class to be produced and may allow earlier detection of scabies. However, given its low affinity for antigens and the cross-reactivity between house dust and scabies mite proteins the utility of IgM for serodiagnosis of scabies should be further investigated.

2. 8. 2. 2. IgA

Secretory IgA is usually more abundant in mucosal regions than in serum and plays a critical role in immune function in the mucous membranes. In OS, it is not clear whether scabies associated IgA secretion is increased or decreased as compared to non-infected individuals or CS, as studies have reported contradictory results (Arlian et al., 1994a; Kenawi et al., 1993; Morsy et al., 1993). Elevated levels of circulatory IgA were documented in 64% of study patients with CS (Roberts et al., 2005). In addition, Walton et al. (Walton et al., 2010) showed increased IgA binding to a recombinant scabies mite antigen in OS and CS patients compared to controls. In porcine studies, increased IgA serum levels in mange positive pigs was reported to whole mite antigen extract, with significant levels detected at week 10 in the infection and positively correlated with severity of infestation (Rampton et al., 2013).

2. 8. 2. 3. IgG

In animals, studies have demonstrated elevated serum levels of total IgG as compared to the controls in *S. scabiei* var. *canis* infested rabbits and dogs (Arlian and Morgan, 2000; Arlian et al., 1996a; Arlian et al., 1994a; Morgan and Arlian, 1994). Serum studies involving whole mite extracts of *S. scabiei* var. *suis* and recombinant antigen Sars s 14.3 demonstrated increased IgG, IgG1 and IgG2 responses in mange infected pigs from weeks 6 -12 post mite infestation (Rampton et al., 2013). *S. scabiei* var. *ovis* primary infestation in sheep resulted in significant increases in the serum levels of specific IgG (Rodríguez-Cadenas et al., 2010). In the same study, secondary challenge in sheep induced a decreased IgG response in comparison to those observed during the primary infestation (Rodríguez-Cadenas et al., 2010). In goats with sarcoptic mange, analysis of antibody profiles has revealed a strong serum IgG response in primary infestation and repeated mite experimental challenges (Sarasa et al., 2010; Tarigan, 2004). In contrast, goats vaccinated with specific *S. scabiei* mite

antigens showed high levels of scabies-specific IgG in the serum but this vaccination failed to provide protection against infestation despite the presence of elevated levels of IgG (Tarigan, 2004). Further studies in dogs, where the IgG titres were inversely proportional to protection, also suggested that IgG antibodies conferred limited protection to sarcoptic mange in dogs (Bornstein and Zakrisson, 1993), with similar conclusions in rabbits (Arlian et al., 1994a; Casais et al., 2014). These results also suggest that cell-mediated immune responses may be providing immune protection. In humans, mite infestations result in circulatory IgG responses in both OS and CS (Arlian et al., 2004a; Dougall et al., 2005; Walton et al., 2008; Walton et al., 2010) with CS patients showing stronger IgG responses compared to OS (Arlian et al., 2004a). Increased serum levels of total IgG were reported in 56 of 58 of cases with CS (Roberts et al., 2005). On the other hand, only 27% of patients with OS showed circulatory IgG response directed at scabies mite antigens although *S. scabiei* var. *canis* mite extract was used which might limit sensitivity (Arlian et al., 2015). IgG subclass serology investigations have revealed elevated levels of total and antigen specific IgG1, IgG3 and IgG4 in CS patients compared to non-infected controls (Dougall et al., 2005; Walton et al., 2008; Walton et al., 2010). Serum IgG4 levels are similarly elevated in chronic helminth and other parasite infections and also rise during allergy desensitization therapy, after repeated exposure to low doses of allergen (Aalberse et al., 2009). The reason behind these elevated levels of total IgG and IgG isotypes especially in CS is unknown and may be due to high antigenic load imparted by mite hyper-infestation. Increases in total IgG could also result from concomitant bacterial infections (Walton et al., 2010). Increased expression of IgG4 is likely due to the production of IL-4 and IL-13 in CS (Roberts et al., 2005; Walton et al., 2010) as these cytokines are known to drive antibody class switching and induce expression of IgG4 (Collins and Jackson, 2013).

2. 8. 2. 4. IgE

IgE is important in the host defence against a variety of parasites and along with mast cells, basophils, and eosinophils constitutes an essential element in allergic and parasitic inflammation. In humans, earlier studies have shown that scabies results in an increased production of circulating IgE antibodies but with highly divergent results (Falk, 1980; Hancock and Ward, 1974; Morsy et al., 1993; Roberts et al., 2005). In recent studies, increased total IgE levels have been observed in OS patients (Abd El-Aal et al., 2016; Walton et al., 2008). In a recent study, ELISA analysis revealed only 2% of 91 cases with OS had circulating IgE antibodies that bound to *S. scabiei* var *canis* antigens (Arlian et al., 2015). Conversely, OS patients from Australia had increased IgE antibodies specific to recombinant scabies antigens compared to naïve controls (Walton et al., 2010). In another similar study, IgE binding to recombinant scabies mite antigen (Sar s 14) for OS was higher compared to controls, with a 100% diagnostic sensitivity and 94% specificity (Jayaraj et al., 2011). Furthermore, IgE binding to another scabies mite recombinant antigen was observed in patients with OS from Pakistan, and the ELISA used for detection showed high (over 90 %) sensitivity and specificity (Naz S, personal communication). In comparison, in CS dramatic increases in total IgE levels have been consistently reported (Arlian et al., 2004a; Walton et al., 2008) with one study showing 96% of 56 cases had elevated total IgE levels (Roberts et al., 2005). Immunoassay studies using plasma from subjects with CS showed increased specific IgE response to recombinant scabies mite molecules (Jayaraj et al., 2011; Walton et al., 2010). Similar to IgG responses, as suggested by Roberts et al. (2005), these results of increased IgE response in CS individuals are expected given the high amount of antigenic material/stimuli provided by the large number of mites. In the earlier studies reporting variable IgE changes, specific IgE responses to scabies mite antigens were not determined. In addition, altered serum IgE levels could be attributed to the different techniques and antigenic

compositions of whole mite extracts used. For example, antigenic similarities exist between scabies and house dust mites, and IgE antibody cross-reactivity has been demonstrated (Falk, 1981; Walton et al., 2015). Hence studies using whole mite extracts may reflect a component of cross-reactive IgE binding. In contrast, the recent ELISA studies using various scabies mite specific recombinant antigens show high specificity and sensitivity in IgE detection in both OS and CS phenotypes from different populations/demographics indicating their potential for serodiagnosis of scabies.

In animals, *S. scabiei* var. *canis* infestation in rabbits and dogs resulted in elevated serum levels of specific IgE (Arlian and Morgan, 2000; Arlian et al., 1996a; Arlian et al., 1994a; Morgan and Arlian, 1994). In sheep, primary *S. scabiei var. ovis* infestation resulted in significant increases in the serum levels of specific IgE and in secondary challenge a higher IgE response was observed than during the primary infestation (Rodríguez-Cadenas et al., 2010). In goats with sarcoptic mange, studies have demonstrated a strong IgE response in primary infestation and repeated mite experimental challenges (Tarigan, 2004). IgE responses in pigs to *S. scabiei* mite infestation have not been explored previously due to non-availability of commercial porcine specific IgE antibody.

While the above studies provide some insights into the humoral immune responses of scabies, a major limitation is the absence of robust prospective studies of human infestation. Such studies are difficult to perform due to the delayed appearance of symptoms and ethical considerations. Moreover, sensitivity is decreased when using non var. *hominis* mite antigen extracts, but obtaining sufficient amounts of scabies mites from human patients for research purposes to generate such extracts is logistically difficult, although recombinant antigens have been utilised (Rampton et al., 2013; Walton et al., 2010). To further understand the humoral response in scabies it remains important to investigate the timing of onset of each Ig response; Ig profiles early in the infestation; after treatment and in reinfection; in both

humans, and in suitable animal models; and the relationship between different antibody responses and their role in immunity. This information would be helpful for the development of improved diagnostic tools which would facilitate improved treatment and control of scabies at both the individual and community level.

In summary, in CS significantly higher levels of total and scabies scabies-specific IgE and IgG antibody responses have been observed in comparison to OS where weaker and more varied responses are documented. From these studies, it also appears that differences may exist between the immune responses of humans and other animals to scabies, between primary and secondary infestations. These responses might also be affected by the sex of the host (Sarasa et al., 2010), type of infestation in humans (ordinary versus crusted) and the validity of the antigen used for diagnosis. From these studies, it also appears that in some individuals/animals ineffective/dysregulated immune responses result in reduced acquired immunity to mite infestation (Little et al., 1998; Sarasa et al., 2010). In addition, immunomodulation exerted by the mites appears to affect the immune response to infestation (Cote et al., 2013; Morgan and Arlian, 2010; Morgan et al., 2013; Mullins et al., 2009). This might explain why some animals/individuals fail to develop resistance to reinfection by *S. scabiei* and remained fully susceptible to recurrence of sarcoptic mange/scabies (Little et al., 1998). Also, this dysregulated/ineffective immune response early in the infestation in some individuals/animals might be playing a role in disease susceptibility especially to CS phenotype and the effects of these factors still need to be fully explored.

Elevated IgE and IgG responses have also been observed in parasitic infections such as in schistosomiasis and lymphatic filariasis (Hagan, 1993; Hagan et al., 1991; Nyindo et al., 1999) conferring protective immunity. However, the increased IgE and IgG scabies-specific antibody responses seen in CS seems not to be effective at clearing the scabies parasite, as shown by high rates of re-infestation (Roberts et al., 2005) despite these increased antibody

levels. It is suggested (Roberts et al., 2005) that these increased serum levels of non-protective IgE and IgG in CS might be related to an inappropriate Th2 biased immune response but the reasons for this remain unknown.

2. 8. 3. Cell-mediated immune responses

2. 8. 3. 1. T cell infiltrates in S. scabiei infested skin

T cell infiltrates to scabies mite infestation (Table 1. 1) of the skin have been demonstrated in humans (Walton et al., 2008), pigs (Liu et al., 2014b) and dogs (Arlian et al., 1997; Stemmer et al., 1996). T cells are the main players in cell-mediated immune responses and cluster of differentiation (CD) 4^+ T cells have been demonstrated as the most prevalent T lymphocytes in inflammatory skin lesions in OS (Table 1. 1) in humans (Falk and Eide, 1981; Falk and Matre, 1982) pigs and dogs (Arlian et al., 1997; Liu et al., 2014b). This corresponds to inflammatory cells in the skin lesions from patients with atopic dermatitis where a significantly greater number of infiltrating $CD4^+$ lymphocytes compared with $CD8^+$ subtypes is reported with $CD4^+/CD8^+$ ratios similar to peripheral blood levels (Akdis et al., 1999).
In contrast, immunohistology and flow cytometry studies using biopsies from CS skin lesions of humans and pigs have revealed increased number of infiltrating $CD8^+$ T cells (Table 1. 1) compared with minimal or no $CD4^+$ cells in the skin dermis (Liu et al., 2014b; Walton et al., 2008). The number of T and B lymphocytes and T-cell subsets in the blood of CS patients have been reported within normal ranges (Roberts et al., 2005; Walton et al., 2008). This presence of a greater number of $CD8^+$ T cells in the skin than in the blood suggests a selective movement of $CD8^+$ T cells. It is further hypothesised (Walton et al., 2008; Walton et al., 2010) that these $CD8^+$ T lymphocytes might be the cause of keratinocytes apoptosis leading to epidermal hyper proliferation. This has also been observed in psoriasis patients with marked levels of $CD8^+$ T cells in the skin epidermis and dermis (Bovenschen et al.,

2005). The apoptotic keratinocytes may secrete cytokines which could exacerbate the inflammatory response by targeting resident skin cells causing further tissue damage. Therefore, these skin homing cytotoxic T cells may be responsible for the imbalanced inflammatory response and may contribute to the failure of the skin immune system to induce an effective response resulting in uncontrolled growth of the parasite. The precise role, importance and function of $CD8^+$ T cells in the pathogenesis of CS needs to be investigated. Additionally, $CD4^+$ T cells in the skin may be essential in the immune response to scabies conferring protection as it has been seen that acquired immunodeficiency syndrome (AIDS) patients often develop CS if infected with scabies mites (Fuchs et al., 2007; Orkin, 1993).

Recently our group reported that PBMC $\gamma\delta^+$ T cells increased in mange infected pigs relative to controls from as early as one week post mite infestation with these increases sustained throughout the infestation (Liu et al., 2014b). Similarly, strong PBMC proliferation of $\gamma\delta^+$ T cells was reported in cattle infested with *P. ovis* (Sarre et al., 2015). These peripheral blood responses mirrored cutaneous responses, with skin cell infiltrates in the lesions of CS pigs showing significantly higher $\gamma\delta^+$ T cell numbers than those with OS (Liu et al., 2014b). These elevated numbers of $\gamma\delta^+$ T cells suggest their role in the disease pathogenesis as it has been demonstrated that IL-17 secretion by $\gamma\delta^+$ T cells plays a critical role in the pathogenesis of psoriasis (Cai et al., 2011).

2. 8. 3. 2. Cytokine profiles in ordinary and crusted scabies

Cytokines, chemokines, and other mediators secreted by $CD4^+$ (Th1, Th2, Th17 and Tregs) and $CD8^+$ T cells along with other effector cells orchestrate the immune and inflammatory responses to scabies mite or its products (Table 1. 2). These cells and their secreted molecules have been associated with specific immune responses and have been implicated in various inflammatory skin and infectious diseases.

Table 1. 2. Effect of scabies mites or mite extracts on key cytokines and molecules from cultured cells *in-vitro* and *in-vivo*.

Cell Type(s)	Cytokines Up Regulated (↑)	Cytokines Down Regulated (↓)	References
A. Cultured cells treated with mite extracts			
Human skin equivalents (HSE)	CTACK, IL 1α, IL 1β, IL 1Rα, IL-6, IL-8, IL-23A, GM-CSF, M-CSF	Not reported	(Morgan and Arlian, 2010; Morgan et al., 2013)
Human dermal microvascular endothelial cells (HMVEC-D)	ICAM-1	IL-6, IL-8, VCAM-1	(Elder et al., 2006; Elder et al., 2009)
Human keratinocytes, fibroblasts	IL-6, CTACK, TGF α, CXCL1, G-CSF	IL-8, GM-CSF	(Arlian et al., 2003) (Mullins et al., 2009)
Dendritic cells	TNF-α	IL-6, IL-8	(Arlian et al., 2004b)
B. In-vivo studies - Human			
PBMCs	IL-10, IFN-γ, IL-6, IL-8, TNF-α, IL 1β, IL-4, IL-5, IL-13	IL-10	(Dagleish et al., 2007; Mounsey et al., 2015; Walton et al., 2010)
Serum	IL-10, TNF-α, IFN-γ	IL-6	(Nimmervoll et al., 2013)
Skin biopsies (crusted scabies)	IL-1β, TGF-β	IFN-γ, IL-10?	(Zipfel et al., 2007)
C. In-vivo studies - other animals			
Porcine PBMCs	IL-17, IFN-γ	Not reported	(Liu et al., 2014b)
Spleen (from exposed/vaccinated mice)	G-CSF, IL-2, IL-13	ICAM-1, ICAM-2, L-selectin, M-CSF, TNF α, TGF β	(Arlian et al., 2007)

Canine PBMCs	IL-4, IL-5, TGF-β	TNF-α	(Singh et al., 2014)
Pig skin biopsies	IL-13, IL-17, IL-23, IL-4, IL-2, TGF-β	Not reported	(Mounsey et al., 2015)

Scabies mite infestation skews the Th1/Th2 immune response (Lalli et al., 2004). It is suggested that the host immune response to OS is a Th1 cell-mediated protective response. Th1-biased immune responses are dominated by $CD4^+$ and $CD8^+$ T cells secreting the cytokines IFN-γ, TNF-α and IL-2 (Kidd, 2003; Romagnani, 2000). Studies have shown strong IFN-γ and TNF-α proliferative responses in PBMCs to scabies mite antigens (Walton et al., 2010) and studies have shown the presence of these cytokines in the serum of OS patients (Abd El-Aal et al., 2016). Th2 cells secrete IL-4, IL-5 and IL-13, and mediate humoral immunity by upregulating antibody production to fight extracellular parasites. Th2 cells are also dominant effector cells in the pathogenesis of IgE-mediated hypersensitivity in asthma and other allergic inflammatory diseases. PBMCs isolated from CS patients secreted increased levels of Th2 cytokines IL-4, IL-5 and IL-13, and decreased secretion of the Th1 cytokine IFN-γ as compared to OS upon stimulation with scabies mite antigens (Walton et al., 2010), similar to those seen with Der p 1 and HDM allergy (Thomas et al., 2004). Transcriptional analysis of skin biopsies from pigs with CS and cattle susceptible to severe psoroptic mange similarly revealed increased expression of IL-4, IL-5 and IL-13 (Mounsey et al., 2015; Sarre et al., 2015). IL-4 and IL-13 play important roles in class switching of B cells and induce co-expression of IgE and IgG4 (Coffman et al., 1986; Punnonen and de Vries, 1994), and thus the presence of these cytokines in CS is in alignment with the extremely high levels of IgG4 and IgE observed (Roberts et al., 2005). This strong humoral response in severe CS is not surprising given the antigenic load imparted by the hyper-proliferation of

scabies mite. What is not understood is whether the Th2 skewed response occurs as a cause of, or in response to scabies mite infestation, as porcine studies have demonstrated up-regulation of these cytokines relatively early in infestation before mite numbers reach extremely high levels (Mounsey et al., 2015).

IL-17 is a potent proinflammatory cytokine, commonly recognised to be secreted by Th 17 cells but it is also secreted by other cell types such as $\gamma\delta^+$ and $CD8^+$ T cells. Th 17 cell generation and IL-17 secretion is promoted through cytokine signaling, in particular by IL-6, TGF-β, IL-23 and IL-1β or IL-18 (McGeachy and Cua, 2008). Studies have demonstrated increased TGF-β, IL-23 and IL-1β expression in immune response to scabies mite infestation (Morgan et al., 2013; Mounsey et al., 2015), indicating that there are immune signals available in the local skin environment which may foster the generation of IL-17 secreting T cells. Furthermore, our group has shown increased IL-17 and IL-23 production in T cells isolated from skin lesions of pigs with CS (Liu et al., 2014b; Mounsey et al., 2015) and increased expression of IL-17 have also been documented in the skin biopsies of cattle breeds susceptible to psoroptic mange but not in those breeds resistant to *Psoroptes* infestation (Sarre et al., 2015). IL-17 secretion further exacerbates the ongoing inflammatory responses by inducing expression of TNF-α, IL-1β and IL-6 in epithelial cells as well as keratinocytes and fibroblasts (Fouser et al., 2008). Th 17 cells and the cytokine IL-17 play a critical role in the inflammatory pathology associated with skin diseases, such as psoriasis and atopic dermatitis (Di Cesare et al., 2008; Di Cesare et al., 2009) and parasitic *Leishmania* major and *Schistosoma japonicum* infections (Chen et al., 2013a; Gonzalez-Lombana et al., 2013).

TGF-β and IL-10 secreted by Tregs suppress pathological inflammatory responses (Miyara and Sakaguchi, 2011). It is suggested that Tregs may play a role in the control or development of scabies (Liu et al., 2014b). In CS, it has been demonstrated that IL-10 secretion in both PBMCs and lesional skin is significantly reduced compared to OS and

control cases (Walton et al., 2008; Walton et al., 2010). In agreement with this, Abd El-Aal et al. (Abd El-Aal et al., 2016) reported a negative correlation between IL-10 secretion and severity of lesions in ordinary scabies. It has been postulated that the activity of Tregs and IL-10 secretion (Nimmervoll et al., 2013) in OS cases may contribute to inhibition of inflammatory and immune reactions to the parasite which may partially explain the 4 - 6 week incubation period in a primary infestation of *S. scabiei*. It has been demonstrated in PBMCs that scabies mite extract can induce IL-10 expression and by extrapolation, influence Tregs activity (Arlian et al., 2006). The delay in symptoms may also be in part due to the well-known ability of IL-10 in inhibiting synthesis of the proinflammatory cytokines TNF-α, IFN-γ an IL-2 (Moore et al., 2001; O'Garra et al., 2004). It is suggested that this reduced IL-10 expression may cause expansion of IL-17-secreting T cells resulting in a Treg/Th17 dysfunctional immune response. Such a hypothesis is supported by a recent murine study of mucocutaneous leishmaniosis, where blocking of IL- 10R resulted in increased IL-17 responses and more severe skin pathology (Gonzalez-Lombana et al., 2013).

2. 9. Treatment

To control scabies transmission and prevent re-infestation, treatment in all patients and close contacts is highly important. Unfortunately, we do not have an ideal treatment against scabies yet. Physicians prescribe topical agents in first-line treatment to scabies patients. Permethrin cream 5% is probably the most effective with low toxicity in mammals and is standard treatment in USA, UK and Australia (Strong and Johnstone, 2007). Other topical treatments in use are benzyl benzoate (25%), monosulfiram (25%), malathion (0.5%), crotamiton (10%), and sulphur in petrolatum (2–10%) (Heukelbach and Feldmeier, 2006; Mounsey and McCarthy, 2013). Oral ivermectin is generally given to recurrent, difficult-to-treat cases, or for patients with CS (Currie and McCarthy, 2010).

Over the years increased use of currently available chemotherapeutics topical permethrin cream 5% and oral ivermectin have resulted in the development of resistance by scabies mites against these treatments (Currie et al., 2004; Mounsey et al., 2009; Walton et al., 2000). Availability of effective treatments being limited, and also given the recent reports of evolving drug resistance to ivermectin and permethrin, asks for attention concerning the control of scabies in times ahead particularly in patients with crusted scabies and also in regions with continual treatment programs being carried out. One solution lies in comprehensively studying fundamental aspects of mite biology, host- parasite interactions, pathogenesis and host immune responses which might facilitate in finding new targets for chemotherapeutic, immunological intervention or vaccine development.

2. 10. Conclusions, recent developments and future directions

In summary, our current understanding indicates that the immune responses to scabies are complex, with distinct profiles between the different clinical manifestations (summarised in Figure 1. 1, Table 1. 1). Crusted scabies shows a picture of increased $CD8^+$ and $\gamma\delta^+$ T cell infiltration (Liu et al., 2014b; Walton et al., 2008), increased production of IgE (Roberts et al., 2005; Walton et al., 2008; Walton et al., 2010), elevated secretion of Th2 cytokines IL4, IL-5 and IL-13, decreased IL-10 production (Walton et al., 2010) and increased production of Th17 cytokines IL-17 and IL-23 (Liu et al., 2014b) suggesting a mix of non-protective allergic Th2 and IL-17 responses contributing in disease pathogenesis. However, the underlying mechanisms of these elevated responses in CS is not yet known and the knowledge about these events is important in understanding the development and immunological progression in CS.

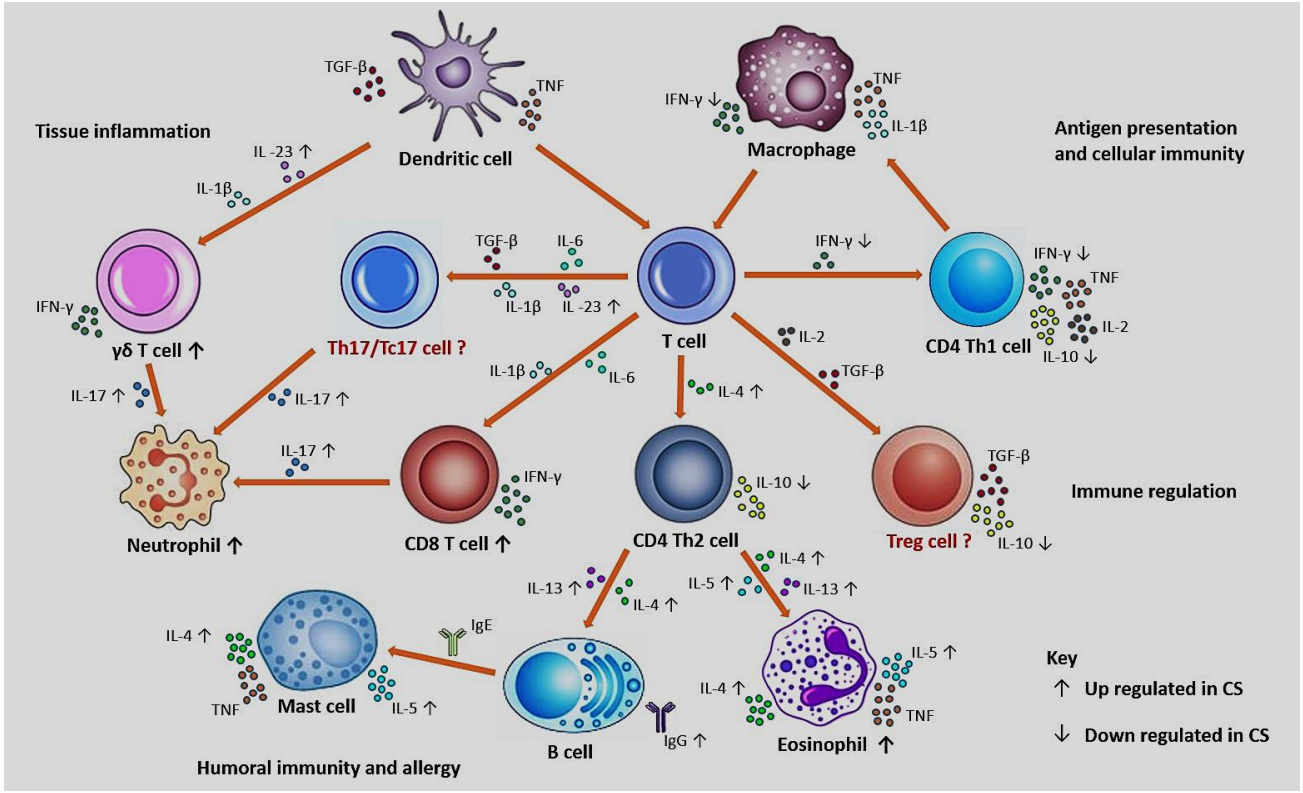

Figure 1. 1 Current knowledge on immune mechanisms in scabies: The figure shows possible mechanisms of immune responses to scabies mite infestation. Keratinocytes, langerhans cells, and macrophages in the skin respond to mite antigens, secreting proinflammatory cytokines such as TNF-α, IFN-γ, TGF-β, IL-1β and IL-23. This leads to the differentiation and recruitment of $CD8^+$ and $CD4^+$ Th1 and Th2 cells into the skin. Secreted cytokine milieus of IL-6, TGF-β and IL-23 promote the differentiation of Th17 or Tc17 cells and IL-17 production. IL-23 and IL-1β also have firmly established roles in promoting IL-17 production by γδ T cells, and their increased expression observed in crusted scabies (CS) may act in an amplification loop for IL-17 production, promoting inflammation and aggravating immune pathology. TGF-β and IL-2 induce Tregs. IL-10 and TGF-β production by Tregs may contribute to the delayed inflammatory response in scabies and suppress pathological inflammation in ordinary scabies (OS), regulating innate and adaptive responses.
Immune responses to OS appear Th1 oriented as evidenced by strong IFN-γ secretion in response to mite antigens. Increased expression of the Th2 cytokines IL-4 and IL-3 in CS leads to immunoglobulin switching in B cells resulting in secretion of large amounts of IgE and IgG. IL-5 activates and promotes the maturation of eosinophils at the site of infestation, sustaining the local Th2 inflammatory responses. IgE through its high affinity receptor (FcεRI) activates mast cells. These cells produce inflammatory mediators such as TNF, histamine, leukotrienes, IL-4, IL-5 and IL-13, supporting their contribution to allergic inflammation in CS.

Cells highlighted in “red” and with “?” are not yet defined in scabies.

Although CS is often associated with general immunosuppressive conditions such as HIV, HTLV-1 infections, or patients undergoing organ transplantation, some people with no recognised immunodeficiency still develop CS (Roberts et al., 2005). In addition, studies with pigs have shown that some pigs develop OS while others exhibit CS phenotype after infestation following inoculation with a similar number of mites (Liu et al., 2014b; Rampton et al., 2013). This indicates that these individuals/animals may have an immunity related genetic predisposition increasing susceptibility to CS. These genetic changes may not directly cause CS but may play a role in its development. Detailed gene expression studies would be beneficial for identifying such genetic changes, particularly if conducted early in infestation, prior to the development of high mite burdens and severe clinical pathology with confounding co-morbidities.

One of the characteristic features of a primary *S. scabiei* infestation is that clinical signs of cutaneous inflammation and pruritus do not appear until weeks after infestation. Studies show that *S. scabiei* may be inhibiting the early immune responses by down regulating the expression of proinflammatory mediators and cytokines (Arlian et al., 2007; Arlian et al., 2003; Arlian et al., 2004b; Elder et al., 2006; Morgan et al., 2013). However, these studies were mostly *in-vitro*, utilising mite extracts and cultured cells or skin equivalents.

To gain valuable insights into the mechanisms behind the immune and inflammatory responses driving disease outcomes in scabies, it is highly imperative to look into the immune responses upstream of those seen at clinical presentation, with *in-vivo* prospective studies. As access to patients with scabies is very limited and carrying out a study of infection in humans can be logistically and ethically a difficult process, thus animal models provide an alternative to investigate the immunopathological mechanisms of scabies development. In addition to studies undertaken with experimental infestation in dogs (Arlian et al., 1997; Stemmer et al., 1996) and rabbits (Morgan and Arlian, 1994), a model involving pigs has been recently

utilised (Mounsey et al., 2010). Pigs are a natural host to *S. scabiei* var. *suis* and develop clinical manifestations resembling both CS and OS, making this model preferable for comparative immunology studies (Morsy et al., 1989).

This porcine model has already allowed more detailed study of scabies immunology than previously possible and importantly validated critical findings from human crusted scabies where interpretation was limited by small sample numbers. Further studies have also begun to shed some light into the effect of scabies mite infestation on the skin microbiota, and helping to unravel the complexities between *S. scabiei* and concomitant bacterial infection (Swe et al., 2014a). Recent genome sequencing (Mofiz et al., 2016; Rider et al., 2015) and proteomic analysis (Arlian et al., 2016; Morgan et al., 2016) of *S. scabiei* promises to provide further insights in understanding the biology of the mite, molecular basis of host specificity, host-parasite interactions, parasitic adaptations and immune evasion. The knowledge of host immune responses and genetic changes in scabies is essential and may aid in the development of novel therapeutics, diagnostic and disease control, as well as allow the early discrimination of ordinary scabies from the severe form of the disease.

In conclusion, development of immunodiagnostics, vaccines, and immunotherapeutics represents a promising long term strategy to control scabies in affected communities globally. A comprehensive understanding of the immune events in the skin and peripheral blood occurring during scabies may provide multiple points at which immunological interventions may intersect the infection and target the responses away from pathology to immunity.

Chapter 3

Materials and methods

3. 1. Ethics statement/approval

The study was approved by the Animal Ethics Committees of the University of the Sunshine Coast (Approval number AN/A/13/71), the QIMR Berghofer Medical Research Institute (Approval number P1266) and the Queensland Department of Agriculture, Forestry and Fisheries (Approval number SA/2013/02/416). All animals were handled in strict accordance with good animal practice as defined by the Australian code of practice for the care and use of animals for scientific purposes.

3. 2. Porcine *in vivo* trial

This work was a subset of a larger experimental trial undertaken at the Queensland Agricultural Science Precinct (QASP), University of Queensland, Gatton QLD, Australia from March-June 2013 (Liu et al., 2014b). Trial design was developed in consultation with the Queensland Department of Agriculture, Forestry and Fisheries (DAFF).

For this study, eighteen 3-week-old piglets (*Sus scrofa*) of the large white breed – a common meat producing breed in Australia were investigated. These pigs had been randomly allocated to mite infested group (n = 12, designated as B1-B12) or non - infested control groups (n = 6). In the infested group, ears of pigs were infected/inoculated with approximately 200 mites and in the non-infested group (controls) no mite infection was carried out. The infested and non-infested pigs were housed separately in identical rooms to avoid accidental disease transmission. The rooms were at a constant temperature of 24 °C and provided with 12 hour/dark photo period. These experimental ear infections/inoculations were carried out with

Sarcoptes scabiei var. *suis* mites obtained from our existing mange pig model. The experimental infestations and housing protocols were as described previously (Liu et al., 2014b; Mounsey et al., 2010). The duration of the trial was 16 weeks.

3. 3. Mange/Disease monitoring

Pigs were monitored on weekly basis for disease progression. As described previously (Liu et al., 2014b; Mounsey et al., 2010), the severity of the skin lesions was scored on a scale from 1–8 (where 1 = mild papular rash, 2–4 = papular rash of increasing intensity, accompanied by exudates and increasing inflammation, > 4 = development of hyperkeratotic lesions of increasing area, 8 = severe hyperkeratosis with development external to ears).

3. 4. Sample collection

3. 4. 1. Skin punch biopsies

During the animal trial, for this study skin samples for RNA extraction from all mite infested (n=12) and non-infested control (n=6) animals were collected using sterile disposable 3.5 mm biopsy punches (McFarlane Medical, Surrey Hills, Australia) at various time points between week 0 and 8. The average weight of skin biopsies was 10 milligrams. Biopsies were stored in 1 ml of RNA later (Qiagen, Victoria Australia), transported on ice and stored at - 80˚C. Biopsies from animals in the infested group were taken directly from the lesional areas (centre of the lesion) in the ear where scabies lesions were apparent and from the same area of the ear from animals in the non-infested control group.

3. 4. 2. Blood samples

Blood was collected from all mite infested and non-infested control animals at several time points between week 0 and 8 during the trial. Approximately 10mL of blood was collected

from the cephalic vein or anterior vena cava into Lithium Heparin CPT Tubes (BD Biosciences, Australia) or Serum Tubes (ThermoFisher Scientific, Australia). Plasma was prepared by centrifuging whole blood at 1000 g for 15 min at temperature. Plasma was then aliquoted and stored at −80 °C until use.

3. 5. Total RNA extraction

For microarray analysis, total RNA was extracted from skin biopsies from infested (OS and CS, n = 8) and non-infested (C, n = 4) animals at time points week zero (pre-infestation), one, two, four and eight weeks post-infestation (wpi), giving a total of 60 biopsy samples. Skin biopsies in RNA Later were thawed on ice, removed from RNA later and cut into smaller pieces using a sterile scalpel blade. The skin biopsy pieces were then homogenised with 1mL of TRIzol reagent (Life Technologies, Victoria, Australia) using a Minilys Homogeniser with CK 28 ceramic beads (Bertin Instruments, Bretonneux, France). Phase separation with TRIzol was undertaken according to the manufacturers' instructions. The aqueous phase was column purified using the DirectZol RNA MiniPrep kit (Zymo Research, Integrated Sciences, New South Wales, Australia) as per the manufacturer's protocol and on-column DNAse digestion was carried out using PureLink TM DNase (Life Technologies). RNA was eluted in 50 μL nuclease free dH_20 and stored at -80°C.

RNA sample integrity was assessed on a 2100 Bioanalyzer System (Agilent Technologies, Inc., California, USA), an RNA Integrity Number (RIN) was obtained for each sample and RNA concentration was quantified using a ND-2000 NanoDrop spectrophotometer (Thermo Scientific, Delaware, USA). RNA samples with a RIN >7.0 were considered to be of acceptable quality (Schroeder et al., 2006).

3. 6. Transcriptomic analyses using microarrays

Transcriptomic analysis was performed using the A-GEOD-16571 Agilent Porcine Gene Expression Microarray V2 4x 44K platform containing 43,803 probe sets from *Sus scrofa*. The One-Colour Microarray Based Gene-Expression workflow using Low Input Quick Amp Labelling and RNA Spike-In Kit (Agilent) was used to amplify and process the total RNA samples, following the manufacturer's recommended protocols. As recommended 50ng total RNA was used as starting material for each animal at each time point for the generation of fluorescently labelled (Cy3 dye) complementary RNA (cRNA), using T7 RNA polymerase. The RNA samples were then converted to cyanine-3 (Cy3) labelled fluorescent cRNA using Low Input Quick Amp Labelling Kit (Agilent) according to manufacturer's protocol. The quality and quantity of cRNA samples was assessed by NanoDrop ND-2000 spectrophotometer. cRNA levels of 1.65 µg and specific activity of 6 pmol Cy3 per µg cRNA were recommended. The recommended amount of 1.65 µg of each cRNA samples were processed at the Ramaciotti Centre for Genomics, (Sydney NSW) following the manufacturer's protocol and hybridisations were performed at 65 °C (Microarray Incubator, Agilent) for 17 hours. Samples from all animals in a group (from either infected or non-infected) at each time point were hybridised onto the arrays on a single slide to limit technical variation. In summary, two slides were used for infested group and one slide for non-infested group at each time point with a total of 15 arrays. After hybridisation, scanning for Cy3 dye intensity (Microarray Scanner, Agilent) was carried out as per the manufacturer's recommended protocols. Feature Extraction software version 10.7.3 (Agilent) was used to extract data signals from the probe features on the arrays. Quality control analysis by reviewing the control information from all arrays was carried out to ensure quality and consistency of sample labelling and array hybridisations and was found to be consistent with the manufacturer's (Agilent) recommendations. Feature extraction data were then imported

into Partek Genomics Suite Version 6.5 (Partek) as text files for further downstream filtering, statistical and differential gene expression analysis. In Partek, to explore the preliminary data, box and whisker visualizations, histogram plot and principal component analysis (PCA) were carried out to assess the distribution profile of the dataset, identify outliers, sample to sample variation and to assess relationships between samples. Quantile Normalisation (Bolstad et al., 2003) of the raw expression data was carried out to normalize the distribution of probe fluorescence intensities among different arrays and the data were log transformed.

3. 7. Statistical assessment of differential gene expression

Differential gene expression was determined using a two way-analysis of variance (ANOVA) with a Fisher's Least Significant Difference (LSD) post-hoc test. For analysis 1 (described in chapter 4) infested (CS and OS, n = 8) were compared to non-infested (C, n = 4) samples at one, two, four and eight weeks post-infestation. For analysis 2 (described in chapter 5), crusted scabies (CS, n = 4) were compared to ordinary scabies (OS, n = 4) samples at zero (pre-infestation), one, two, four and eight weeks post-infestation. Multiple test correction was performed to generate the gene lists using the False Discovery Rate (FDR) (Benjamini and Hochberg, 1995) procedure with FDR corrected p-value threshold of ≤ 0.05 and a fold change (FC) threshold of $\geq \pm 2.0$.

3. 8. Gene annotation

The probes which were not annotated on the Agilent Array annotation file were annotated by individual BLAST analysis of the EST sequences represented by each probe to *Sus scrofa* cDNA using genome assembly Sus_scrofa.scrofa10.2.cdna.all.fa.gz (Ensembl) in CLC (Genomics Workbench 8.5.1, Qiagen). For filtering stringent parameters of greater than 40 base pair (on average the base pair length of the probes on the array was 60) match, $\geqslant$ 95%

identity and E value of 1.00E-5 were applied. The pig cDNA was then annotated to coding sequences (CDS) of *Homo sapiens* cDNAs using genome assembly GRCh38.cds.all (Ensembl) in CLC. Here as well, for filtering stringent parameters of greater than 200 base pair match, above 60% identity (as we aligned against the coding regions) and E value of 1.00E-5 were applied. Gene symbol (HUGO Gene Nomenclature Committee, HGNC) level annotation was used and the relevant homologous human gene symbol was used where the porcine annotation was not available.

3. 9. Comparative analysis using Venn diagrams

Venn diagrams are widely used for comparing multiple datasets to help identify candidate genes – shared or different between the datasets. Statistically significant ($p \leq 0.05$) differentially expressed genes (DEGs) with FC $\geq \pm 2.0$ from Partek analysis were input into the Venny (Version 2.1, BioinfoGP Service) software program available at http://bioinfogp.cnb.csic.es/tools/venny/index.html to generate Venn diagrams. A comparative data analysis of DEGs from comparison groups – CS vs C and OS vs C was performed to depict similar/shared and unique sets of genes in CS and OS.

3. 10. Pathway and gene network analysis

It is important to note here that a large number of genes were represented by multiple probes (ranging from 3 probes to over 30 probes) in the porcine array we used. To identify significant pathways, in our datasets, the similar expression (- - or + +) of multiple probes of the same gene was averaged while the probes of the same gene with dissimilar expression (- +) were excluded from the analyses. The annotated gene lists were uploaded as the input data set into Ingenuity Pathway Analysis (IPA) (Ingenuity Systems, Qiagen) program and a network/pathway based analysis was carried out, representing all differentially expressed

genes (DEGs) from across the time period of infestation. Each gene identifier was mapped to its corresponding gene object in Ingenuity's Knowledge Base. Gene networks, which are graphical visualisations of the molecular relationships between the genes, were created based on their connectivity with the genes in the input data and these networks are supported by a published reference. According to how relevant they are to the genes in the dataset each network was assigned a Z-score and a right tailed Fisher's exact test was used to calculate a p-value for each network. Canonical pathway analysis was performed to identify the biological pathways associated with the molecules in the input dataset. The significance values (p-value) of the association of genes from the dataset with each canonical pathway were calculated using a right tailed Fisher's exact text. The functions of the differentially expressed genes were confirmed from IPA, GeneCards (http://www.genecards.org/) and Uniprot (http://www.uniprot.org/) online databases.

3. 11. qRT-PCR validation

Quantitative real-time PCR (qRT-PCR) was used to validate the microarray and verify differential gene expression, 9 genes were selected from the final list of genes with an FDR corrected p-value of ≤ 0.05 and FC of $\geq \pm 2$. One microgram of total RNA from each porcine skin biopsy was, prepared as described above, reverse transcribed into complementary DNA (cDNA) using the QuantiTect Reverse Transcription Kit (Qiagen) following the manufacturer's recommended protocol. Primers (Table 3. 1) were either as previously described (Levast et al., 2010; Mounsey et al., 2015; Petrov et al., 2014) or newly designed using Primer-BLAST (Primer3 and BLAST) (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Hypoxanthine phosphoribosyl transferase 1 (HPRT1) was used as a housekeeping gene (Mounsey et al., 2015; Nygard et al., 2007).

Table 3. 1. Primer sequences and amplicon details for porcine qRT-PCR.

Gene	Primer Sequence (5'-3')	Amplicon Size (bp*)	GenBank Accession	Primer Source
HPRT1	F: GCAGCCCCAGCGTCGTGATT R: CGAGCAAGCCGTTCAGTCCTGT	142	NM_001032376.2	(Mounsey et al., 2015)
GLO1	F: GGATGCTCGGTTGTTCCTGT R: GGAAGACGACTGAGCCGATT	145	XM_001927957.3	
IL17	F: GACCACAGCGTGAGAGTTGA R: CCGGGTGATGTTGTAATCCCA	111	XM_001924366.3	
FOXP3	F: GGTGCAGTCTCTGGAACAAC R: GGTGCCAGTGGCTACAATAC	148	NM_001128438.1	(Levast et al., 2010)
IFNγ	F: CCAGGCCATTCAAAGGAGCATGGA R: GGCTTTGCGCTGGATCTGCAGA	140	NM_213948.1	(Mounsey et al., 2015)
IL1β	F: GTGCTGGCTGGCCCACA R: GAACACCACTTCTCTCTTCA	71	NM_214055	(Petrov et al., 2014)
NLRP3	F: TCTGTGAGGGACTGTTGCAC R: CGCAGGCTCTGGTTAGAAGT	121	NM_001256770.1	
TGFβ	F: CACGGCATGAACCGGCCCTT R: TGTAGAGCTGCCGCACGCAG	148	NM_214015.1	(Mounsey et al., 2015)
CD274	F: ACCAGTTCCCAGAGAGAGGA R: CACATATGGTTCTGGGATGACC	150	NM_001025221.1	
TNF	F: GCCCCCAGAAGGAAGAGTTTC R: TCCCTCGGCTTTGACATTGG	128	NM_214022.1	

*bp = base pair.

Optimal annealing temperatures for each primer set were tested with gradient PCRs using pig skin cDNA and the MyTaq TM Red Mix kit (Bioline) or AmpliTaq Gold® 360 Master Mix kit (Applied Biosystems) as per the manufacturer's instructions. To confirm the identity of the amplified target gene, the PCR products were purified using the QIAquick PCR Purification Kit (Qiagen) and sequenced at the Australian Genome Research Facility (Melbourne, Victoria, Australia). PCR products were cloned (pGEM-T, Promega) to generate plasmids for the selected genes as previously described (Mounsey et al., 2015). To check the amplification efficiency, plasmids containing the gene of interest were linearized using restriction endonucleases (New England BioLabs® Inc.) and serially diluted (using nuclease free dH_20). qRT-PCR reactions were carried out using the QuantiTect SYBR green PCR kit

(Qiagen) as previously described (Mounsey et al., 2015). Each qPCR assay was performed in 20μL reaction volumes containing 2x SYBR green master mix 10 μL, 1μL of each forward and reverse primer (10μM), 3μL diluted plasmid DNA and nuclease free dH2O. Reactions were cycled in a Rotor Gene 6000 real-time cycler (Qiagen, Hilden, Germany). The thermal cycling parameters included: initial denaturation at 95°C, 15 min, followed by 40 cycles of denaturation at 94°C, 15 s; annealing at 54 or 56°C, 30 s (40 cycles); 72°C, 30 s; with data acquisition at 76°C, 15 s. The melting curves were recorded from 65°C to 95°C at an increasing rate of 1°C/s as described previously (Mounsey et al., 2015) with minor modifications. Standard curves and melting temperatures for the selected genes and the endogenous control HPRT1 were prepared using the Rotor Gene software. PCR reaction efficiencies were calculated from the reaction slopes and were consistently $\geqslant$90% for all of the assays performed.

PCR amplifications for the selected genes in parallel with HPRT1 were performed using diluted cDNA (1:4 dilutions with nuclease free dH_2O) samples with the same qPCR assay, PCR kit and cycling parameters as above following the manufacturers' instructions. A no-RT control containing RNA as template was maintained to confirm that co-amplification of genomic DNA was not occurring. Each qPCR also included a no template control as negative control and a positive control containing cDNA (same cDNA used to generate the plasmids) as template. In each qPCR reaction duplicates of each sample and controls were maintained, and the reaction mixtures were the same as above. Relative quantification of gene expression levels was determined by normalising to the HPRT1 control using the Comparative Ct method with the formula $2^{-\Delta\Delta CT}$ (Schmittgen and Livak, 2008) and expressed as fold change.

3. 12. Acute phase protein analysis using ELISAs

For this analysis, we used/compared CS (n = 4), OS (n = 4) and C (n = 4) plasma samples at baseline (week 0 pre-infestation), 2 and 4 week post-infestation. These plasma samples were obtained from the same pigs used in microarray analysis. The concentration of Haptoglobin (HP), Serum amyloid A (SAA), Transferrin (TF) and Alpha-1-acid glycoprotein (AGP) was measured by using commercially-available ELISA kits (pig HP #AB205091 and AGP #AB205068 from Abcam, Australia; multispecies SAA #KAA0021 from Life Technologies Pty Ltd, Australia and TF #CSB-E13764P from Cusabio, China) in accordance with the manufacturer's instructions. Prior to assay optimal dilutions were determined using pooled plasma from three pigs with CS at week 8. For the final assay, all plasma samples were diluted in the provided buffer as per manufacturer's instructions (1:100 for TF; 1:500 for SAA; 1:10000 for HP and 1:20000 for AGP). Samples were tested in duplicates alongside standards and the absorbance was read at 450 nm using 630nm as a reference. The blank standard absorbance value was subtracted from all other sample and standard absorbance values. The absorbance values of replicates of all samples and standards were averaged. Standard curve was generated from the average absorbance values of standards using CurveExpert Basic (Version 1.4, CurveExpert Software) software program available from www.curveexpert.net. To determine the concentration of APPs in the plasma samples, the absorbance values of the samples were interpolated against the standard curve.

The acute phase protein concentrations were compared and tested for statistical significance in Graph Pad Prism (Version 7.0, Graph Pad Software Inc.) using repeated measures two-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test. Differences with $p \leq 0.05$ were considered as significant.

Chapter 4

Transcriptomic analysis of the host response to skin infestation with the ectoparasitic mite *Sarcoptes scabiei*.

4. 1. Introduction

In scabies, infestation of the host skin by the itch mite, *Sarcoptes scabiei* results in intense itching mediated through allergic and inflammatory reactions (Walton, 2010). The host responses to mite infested skin include infiltrates of inflammatory cells – eosinophils and neutrophils with smaller numbers of macrophages, basophils and mast cells (Elwood et al., 2015; Ito et al., 2011; Walton, 2010). The humoral immune response towards *S. scabiei* infestation results in increased circulating antibody production with both total and scabies-specific immunoglobulin (Ig) G and IgE being documented (Roberts et al., 2005; Walton et al., 2008). In primary mite infestation, hosts exhibit delayed inflammatory and adaptive immune responses with clinical signs appearing 4 to 6 weeks after the initial contact with the mite (Liu et al., 2014b; McCarthy et al., 2004; Rampton et al., 2013). This lag period can complicate accurate diagnosis and results in delayed treatment of the disease. Several studies have shown that the scabies mites may dampen the immune responses by inducing immunoregulatory cells, up-regulating anti-inflammatory cytokines, producing complement and chemotaxis inhibiting proteins and reducing expression of cell receptors (Arlian et al., 2007; Arlian et al., 2003; Arlian et al., 2004b; Elder et al., 2006; Mika et al., 2012a; Morgan et al., 2013; Swe and Fischer, 2014). However, these studies have mostly been performed *in-vitro*, utilising mite extracts and cultured cells or skin equivalents. The exact mechanism of this potential suppression of the immune response is not fully understood and may be an important step in mite colonization, and therefore critical in determining disease progression.

Despite the significant worldwide impact of the disease, immune and inflammatory responses in scabies remain poorly characterized (Walton, 2010). To gain valuable insights into the mechanisms and signaling pathways behind the immune responses driving disease outcomes in scabies, it is imperative to investigate the responses prior to clinical presentation, with *in-vivo* prospective studies. As access to patients with scabies is very limited and carrying out a study of infection in humans can be logistically and ethically a challenging process, animal models provide a useful alternative to investigate the immunopathological mechanisms of scabies development. In this study, differential gene expression profiles were investigated between scabietic and control skin samples from pigs to better understand disease pathology and to identify also disease associated gene signatures and differentially regulated pathways in immune and inflammatory responses to the mite at the local tissue level.

DNA microarray is a high dimensional gene expression technology which comprehensively surveys the transcriptome and allows the simultaneous comparison of thousands of messenger RNAs that may reveal the disease-specific pattern of tissue inflammatory and immune responses. Infestation of the skin with *S. scabiei* mites results in localised cutaneous inflammation, pruritus and skin lesions caused by the allergic and inflammatory responses. The clinical signs begin to manifest from 4-6 weeks post infestation. As such the focus of this analysis was on the first 8 weeks post-infestation to identify the genes and elucidate the signaling pathways involved in the early pre-clinical disease development. Porcine microarray combined with a network and pathway mapping approach was employed to identify the key signaling events and the factors involved in disease pathogenesis. This information will be useful in identifying novel diagnostic targets and may also aid in the discovery of new therapeutic treatments.

4. 2. Materials and methods

The materials and methods relating to the microarray data analysis are described in more detail in chapter 3.

4. 3. Results

4. 3. 1. Clinical progression of mange infection in pigs

Based on previous studies using the porcine model (Mounsey et al., 2010; Rampton et al., 2013) a range of clinical manifestations were expected in infested pigs. Some pigs were expected to develop hyperkeratotic mange with high mite burdens, similar to the crusted scabies (CS) phenotype in humans, whereas others would maintain infestation with few clinical signs and low mite burden, similar to ordinary scabies (OS). Pigs in the infested group began to exhibit clinical signs of lesion development from 4 weeks post-infestation. The non-infested pigs did not show skin lesions at any time during the trial. Based on clinical scores, by week 8 post-infestation, five infested pigs were classified as OS (score < 4) and seven pigs were classified as CS (score ≥ 4).

From the experimental infested group four pigs with CS (lesion score > 4 at more than one time point) and four with OS (lesion score < 4) (Figure 4. 1) were randomly selected for this analysis. Four pigs were also randomly selected from the non-infested control group (C). In total 12 pigs (8 infested and 4 non-infested) were used in this analysis.

4. 3. 2. Total RNA extraction from pig skin biopsy samples

Total RNA was successfully extracted from skin biopsy samples of all 12 animals – infested (n = 8) and non-infested (C, n = 4) at 0 pre-infestation, 1, 2, 4 and 8 weeks post-infestation (wpi), giving a total of 60 samples.

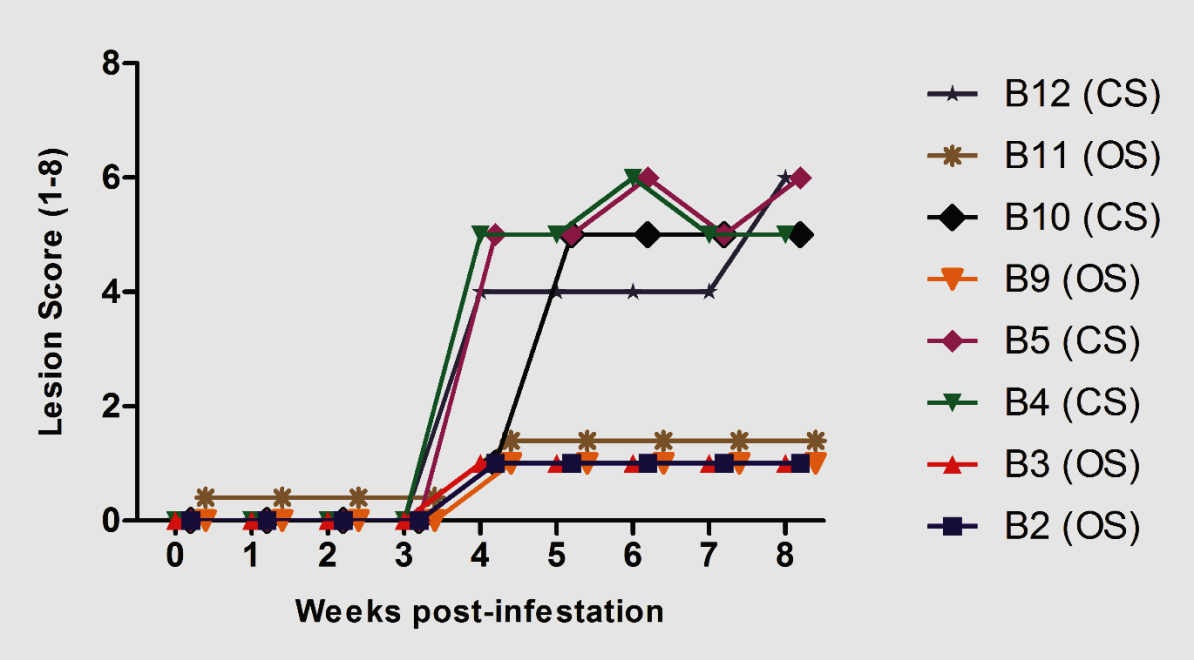

Figure 4. 1. Clinical score and lesion progression in mite infested pigs (n=8). Ear lesions were scored weekly. Score of 1 – 4: acute mange with generalised rash and papular lesions of increasing density, 4: development of increasing encrustment, 8: extensive encrustment spreading externally to the ears. Clinical progression of individual pigs with CS (n = 4, B4, B5, B10 and B12) and OS (n = 4, B2, B3, B9 and B11) phenotype.

The mean RNA integrity number (RIN) value of 7.75 and optical density (OD) A260/A280 ratio between 1.8 and 2.10 indicated high quality, purified RNA with minimal degradation. RNA yield of > 100 ng per biopsy was obtained. The RIN values, yield and OD A260/A280 ratios of total RNA samples are provided in appendix A (Supplementary Table 1).

The resulting complementary RNA (cRNA) samples showed yields between 1.80 μg and 5.60 μg (recommended >1.65 μg of cRNA), and specific activity between 6.45 pmol and 17.40 pmol (recommended >6 pmol Cy3 per μg cRNA). The cRNA samples showed average OD A260/A280 values of 2.10 (range 1.78 – 2.34 with 12 samples showing above 2.10) demonstrating high-quality cRNA. The yield, specific activity and OD A260/A280 ratios of cRNA samples generated from total RNA are provided in appendix B (Supplementary Table 2).

4. 3. 3. Microarray data processing and quality control

Following microarray (cRNA labelling, hybridization and feature extraction), to ensure quality and consistency of sample labelling and array hybridizations, quality control of all arrays was carried out prior to data analysis. The quality of array data was found to be consistent with the manufacturer's (Agilent) recommendations. Box and whisker visualizations and histogram plots from preliminary Partek analysis confirmed the data had comparable distributions and were of sufficient quality for further analysis. The box and whisker plot of the array data is provided in appendix C (Supplementary Figure 1). An initial principal component analysis (PCA) analysis revealed that the infested samples grouped together according to their clinical phenotype (CS and OS) and non-infested (C) samples grouped together at each time point of the study (appendix D, Supplementary Figure 2).

4. 3. 4. Determination of differentially expressed genes

To compare the differences in gene expression profiles between infested and non-infested animals' two-way analysis of variance (ANOVA) combined with a Fisher's Least Significant Difference (LSD) post-hoc test was performed on all 43,803 gene probes. Overall 17,938 probes were significantly differentially expressed between infested and non-infested animals over the eight-week time course of infestation. Of the probes in the gene lists, gene symbol level annotation was initially available for 25 - 30 %. Further gene annotation of the probes, which were not annotated/assigned a function on the manufacturers' array, was carried out and with another 40% of these probes annotation was available. In total, 60 - 65% annotation was available at the end. Fold changes of probes of the same gene showing similar expression (- - or + +) were averaged and probes of the same gene showing dissimilar expression (- +) were excluded from the final lists.

For this analysis, infested (n = 8) samples were compared with non-infested (C, n = 4) samples at time points 1, 2, 4 and 8 wpi, and 948, 1505, 482 and 1145 genes respectively were identified as being significantly (p-value ≤ 0.05) differentially expressed with a fold change (FC) of ≥ 2.0 (Table 4. 1).

The complete gene lists for the comparisons are provided on a separate USB drive due to their large size (appendix E, Additional File 1). The microarray data has been deposited in an online public genomics data repository of the European Bioinformatics Institute (ArrayExpress, https://www.ebi.ac.uk/arrayexpress/) with accession number E-MTAB-6433.

In this analysis, following mite infestation at 1wpi, 449 genes were up-regulated and 499 genes down-regulated. At week 2, a prominent trend for gene down-regulation in gene expression was observed. Week 4 showed the lowest number of genes being differentially

expressed. At 8wpi, a trend towards up-regulation was observed (740 genes being up-regulated versus 405 being down-regulated) (Table 4. 1).

Table 4. 1. Number of differentially expressed genes (DEGs) following infestation with *S. scabiei* in pigs. In Partek, gene expression analysis was carried out with a 2- way ANOVA combined with a Fisher's Least Significant Difference (LSD) post-hoc test across infested (CS and OS) (n=8) and non-infested (C) pigs (n=4) at time points 1, 2, 4 and 8 weeks post-infestation (wpi). Number of differentially up (↑) or down (↓) regulated genes in infested relative to non-infested pigs with a p-value of ≤ 0.05 and fold change of ≥ ± 2.0 at each time point are indicated.

Time	Partek analysis		
	Number of DEGs	**Up-regulated ↑**	**Down-regulated ↓**
1wpi	948	449	499
2wpi	1505	293	1212
4wpi	482	196	286
8wpi	1145	740	405

Interestingly, genes which were down-regulated earlier in the infestation at week 1 and/or 2 showed up-regulation towards week 4 and/or 8, and vice versa (Table 4.4). Several genes were only expressed (up-regulated) at 4wpi and their expression levels further elevated at 8wpi. At 8wpi, a large number of genes were expressed (up-regulated) as compared to other time points with several genes among them showing strong up-regulation. Table 4. 2 lists the top 10 differentially expressed genes (DEGs) at each time point with the highest fold-change and shows S100 calcium binding protein A7 (S100A7), Gamma-tubulin complex component 3 (TUBGCP3), Sodium/iodide cotransporter (SLC5A5), ARG (arginase) 1 and interleukin (IL) -8 as the most highly up-regulated genes, and Secretoglobin family 1A member 1 (SCGB1A1), Dopachrome tautomerase (DCT), Fucose mutarotase (FUOM) and Sad1 and UNC84 domain containing 5 (SUN5) the most down-regulated genes.

Table 4. 2. Top 10 high fold changing genes at each time point of the study following infestation with *S. scabiei* in pigs. In 2-way ANNOVA analysis, these genes showed $\geqslant \pm$ 2.0 fold change in expression with FDR corrected p-value of $\leqslant$ 0.05 in infested relative to non-infested (control) pigs. Wpi = weeks post-infestation.

Time	Genes	Gene Description	Fold change
1wpi	U2AF2	U2 small nuclear RNA auxiliary factor 2	7.92
	CNPY2	Canopy FGF signaling regulator 2	7.43
	CACNG1	Calcium voltage-gated channel auxiliary subunit gamma 1	7.34
	KCNE2	Potassium voltage-gated channel subfamily E regulatory subunit 2	7.32
	CD9	CD9 molecule	7.17
	DCT	Dopachrome tautomerase	-11.84
	RBM38	RNA binding motif protein 38	-7.24
	ADIPOQ	Adiponectin, C1q and collagen domain containing	-7.21
	DEPDC5	Dep domain containing 5	-7.09
	DNAJC2	DnaJ heat shock protein family (Hsp40) member c2	-7.05
2wpi	DLX3	Distal-less homeobox 3	25
	RAB34	RAB34, Member RAS oncogene family	12.21
	KAT7	Lysine acetyltransferase 7	9.83
	PNMT	Phenylethanolamine n-methyltransferase	9.23
	POLR3A	RNA polymerase III subunit A	8.82
	SALL4	Spalt like transcription factor 4	-6
	SUN5	Sad1 and UNC84 domain containing 5	-5.55
	ITGB1	Integrin subunit beta 1	-5.29
	TFAM	Transcription factor A, mitochondrial	-5.04
	HECW1	HECT, C2 and WW domain containing E3 ubiquitin protein ligase 1	-4.66
4wpi	GAL	Galanin and GMAP pre-propeptide	8.05
	ADSS	Adenylosuccinate synthase	8.04
	CGA	Glycoprotein hormones, alpha polypeptide	7.49
	BPIFB3	BPI fold containing family B member 3	6.97
	TDP1	Tyrosyl-DNA phosphodiesterase 1	6.97
	FUOM	Fucose mutarotase	-9.93
	RPL8	Ribosomal protein L8	-4.98
	ATAD1	ATPase family, AAA domain containing 1	-4.73
	KLRG1	Killer cell lectin like receptor G1	-4.73
	G6PC	Glucose-6-phosphatase catalytic subunit	-4.72
8wpi	S100A7	S100 calcium binding protein A7	132.92
	TUBGCP3	Tubulin gamma complex associated protein 3	70.69
	SLC5A5	Solute carrier family 5 member 5	57.58
	CXCL8/IL8	C-X-C motif chemokine ligand 8/ interleukin 8	54.19
	ARG1	Arginase 1	52.45

	SCGB1A1	Secretoglobin family 1A member 1	-32
	SUN5	Sad1 and UNC84 domain containing 5	-9.73
	NPFFR1	Neuropeptide FF receptor 1	-8.6
	GHITM	Growth hormone inducible transmembrane protein	-6.78
	WDFY2	WD repeat and FYVE domain containing 2	-6.14

4. 3. 5. Network/Pathway analysis of host response to *S. scabiei* infestation

To determine the biological function of the DEGs, and the molecular signaling pathways and gene networks involved in the host cutaneous response to mite infestation across the time course, the data were analysed with Ingenuity Pathway Analysis (IPA) program (Ingenuity Systems) (Table 4. 3).

Table 4. 3. Number of differentially expressed genes (DEGs) for pathway and network analysis in IPA. In IPA, pathways and networks associated with the DEGs (with a p-value of ≥ 0.05 and fold change of $\geq \pm 2.0$) were identified. The number of DEGs entering IPA are indicated. Wpi = weeks post-infestation.

Ingenuity Pathway Analysis			
Time	**Number of DEGs entering IPA**	**Number of DEGs mapped with IPA data base**	**Number of DEGs not mapped with IPA data base**
1wpi	948	917	31
2wpi	1505	1461	44
4wpi	482	465	17
8wpi	1145	1095	50

Preliminary analysis of the ANOVA results, pathways and gene networks generated in IPA revealed differential gene transcription of an array of cytokines – (proinflammatory and immunomodulatory), transcription factors, signaling molecules – (receptors and ligands), immune cell surface markers and chemokines (from IPA, GeneCards and Uniprot programs and databases) in response to mite infestation (Table 4. 4).

Table 4. 4. Immunity related genes in response to *S. scabiei* infestation in skin. Genes that differed significantly in expression (either up or down-regulated) levels upon mite infestation at 1, 2, 4 and 8 weeks post-infestation (wpi). Gene expression was carried out with a 2- way ANOVA combined with a Fisher's Least Significant Difference (LSD) post-hoc test in Partek. These genes had a FDR corrected p-value of ≤ 0.05 and fold change (FC) of ≥ ± 2.0 in infested relative to non-infested (control) pigs. In IPA, gene networks and signaling pathway analysis was carried out on DEGs from each time point. The gene function was verified with online databases IPA, UniProt and GeneCards. The notable genes with the fold changes associated with signaling pathways and gene networks in the processes of immune, inflammatory and allergic responses are indicated.

Genes	Gene Description	1wpi (FC)	2wpi (FC)	4wpi (FC)	8wpi (FC)
	Pro-inflammatory cytokines and receptors				
IFNγ	Interferon gamma	+2.26			
IL13Rα1	Interleukin 13 receptor subunit alpha 1		-2.18		
IL17F	Interleukin 17F			+2.74	+3.34
IL17RB	Interleukin 17 receptor B		-2.7		
IL1RL1	Interleukin 1 receptor Like 1				+2.14
IL1α	Interleukin 1 alpha				+2.61
IL1β	Interleukin 1 beta				+5.65
IL2	Interleukin 2				+3.07
IL20	Interleukin 20				+2.82
IL2Rγ	Interleukin 2 receptor subunit gamma				+2.76
IL4	Interleukin 4				+3.75
IL4R	Interleukin 4 receptor				+2.31
IL6	Interleukin 6				+4.75
IL8/CXCL8	Interleukin 8/C-X-C motif chemokine ligand 8				+54.19
IL8R/CXCR2	Interleukin 8 receptor/C-X-C motif chemokine receptor 2				+3.43

OSM	Oncostatin M	+3.31			
TNF	Tumour necrosis factor				+2.49
	Pathogen recognition receptors				
NLRP3	NLR family, pyrin domain containing 3		-2.03		+2.1
NOD2	Nucleotide-binding oligomerization domain containing 2				+3.57
TLR10	Toll-like receptor 10				+4.9
TLR4	Toll-like receptor 4		-2.12		
TLR5	Toll-like receptor 5		-2.31		
TLR6	Toll-like receptor 6		-2.04		
TLR9	Toll-like receptor 9				+2.01
	Cell surface receptors				
CD19	CD19 molecule	-2.53	-2.38		
CD274	CD274 molecule	-3.74	-2.59	-3.01	+2.93
CD36	CD36 molecule			-2	
CD3γ	CD3 gamma molecule		+3.02		+3.42
CD3δ	CD3 delta molecule				+5.86
CD3ε	CD3 epsilon molecule				+2.23
CD4	CD4 molecule				+2.47
CD40	CD40 molecule				+2.06
CD5L	CD5 antigen-like		-2.44		
CD70	CD70 molecule	-3.49	-2.78		

CD81	CD81 molecule		+2.25		
CD86	CD86 molecule				+2.53
CD8α	CD8 alpha molecule	-3.3	-2.07		
	Chemokine receptors and ligands				
CCL20/MIP3α	Chemokine (C-C motif) ligand 20/Macrophage inflammatory protein 3-alpha				+6.16
CCL22	C-C Motif Chemokine Ligand 22				+3.11
CCL27/CTACK	Chemokine (C-C motif) ligand 27/Cutaneous T-cell activating chemokine				+2.39
CCR3	C-C motif chemokine receptor 3	+2.92		-2.13	
CCR7	C-C motif chemokine receptor 7				+9.33
CXCL10	C-X-C motif chemokine ligand 10		-3.98		
CXCL13/BCA-1	C-X-C motif chemokine ligand 13/B cell-attracting chemokine 1		-4.22		
CXCR4	C-X-C motif chemokine receptor 4		+2.34		-2.97
ICAM3	Intercellular adhesion molecule 3	+3.11			+2.71
VCAM1	Vascular adhesion molecule 1		-2.25		
	Complement factors				
C1QA	Complement C1q A chain		-2.12		
C1R	Complement C1r		-2.28		
C1S	Complement C1s		-2.49		
C2	Complement C2		-2.14		
C3	Complement C3			+2.48	+6.45
C5	Complement C5		-2.93		-2.09

C5AR1	C5a anaphylatoxin chemotactic receptor 1		-2		
C6	Complement C6	+3.27	+2.68	-3.07	-2.3
C8A	Complement C8 alpha chain		-2		
	Immunomodulatory cytokines				
IL10	Interleukin 10				+2.06
IL27	Interleukin 27	+3.02			
TGFβ1	Transforming growth factor beta 1				+2.47
	Cell growth factors, cell signaling molecules and transcription factors				
CSF1/M-CSF	Colony stimulating factor 1 / Macrophage colony-stimulating factor				+2.38
CSF2/GM-CSF	Colony stimulating factor 2 / Granulocyte-macrophage colony-stimulating factor				+11.24
FOXP3	Forkhead Box P3	+3.87			+2.11
iCOS	Inducible T-cell costimulator				+5.39
JAK1	Janus kinase 1		-2.42		
JAK2	Janus kinase 2		-2.53		
JAK3	Janus kinase 3				+2.92
MYD88	Myeloid differentiation primary response 88		-2.45		+2.09
NFκB1	Nuclear factor kappa B subunit 1				+2.13
SOCS2	Suppressor of cytokine signaling 2		-2		
SOCS5	Suppressor of cytokine signaling 5		-2.17		
STAT1	Signal transducer and activator of transcription 1		-2.36		
STAT2	Signal transducer and activator of transcription 2		-2.01		

STAT4	Signal transducer and activator of transcription 4	-2.23		+2.14	+2.68
TNFSF13B	Tumour necrosis factor superfamily member 13b		-2.06		
TRAF3	TNF receptor associated factor 3		-3.15		
TYK2	Tyrosine kinase 2		+2.52		
VEGFA	Vascular endothelial growth factor A				+2.12
	Metallopeptidases				
MMP1	Matrix metallopeptidase 1 (Interstitial collagenase)				+24.48
MMP9	Matrix metallopeptidase 9 (Gelatinase B)				+10.22
MMP12	Matrix metallopeptidase 12 (Macrophage elastase)				+23.45
MMP13	Matrix metalloproteinase 13 (Collagenase 3)			+2.13	+2.23
	S100s				
S100A7	S100 calcium binding protein A7 (Psoriasin 1)				+132.92
S100A8	S100 calcium binding protein A8 (Calgranulin A)				+44.12
S100A9	S100 calcium binding protein A9 (Calgranulin B)				+50.78
S100A12	S100 calcium binding protein A12 (Calgranulin C)				+23.45
	Acute phase response proteins and signaling molecules				
AGP/ORM1	Alpha-1-acid glycoprotein 1/orosomucoid 1				-2.05
APOA1	Apolipoprotein A1	+3.64		-2.08	-3.17
APOA2	Apolipoprotein A2		+3.16	-2.27	
CP	Ceruloplasmin		-3.28		
IKBKE	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase epsilon	+2.75	+2.5		

IRAK1	Interleukin 1 receptor associated kinase 1	+2.01			
MAP2K1	Mitogen-activated protein kinase kinase 2		-3.94	+2.74	
PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha		-2.01		
PIK3CG	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit gamma	+2.03			+2.28
PIK3R3	Phosphoinositide-3-kinase regulatory subunit 3	+2.06			
PLG	Plasminogen	+3.8			+2.2
PTPN11	Protein tyrosine phosphatase, non-receptor type 11	+2.87			
SAA/SAA1	Serum amyloid A/Serum amyloid A1	-2.53	-2.16		
SERPINA3	Serpin family A member 3		2.12		
SERPIND1	Serpin family D member 1		2.39		
TF	Transferrin	+2.9			
	Miscellaneous – other important genes				
AREG	Amphiregulin		-3.73		
ARG1	Arginase 1				+52.45
ARG2	Arginase 2				+2.22
CASP1	Caspase 1		-2.14		
CTSB	Cathepsin B		-2.09		+2.53
DCT	Dopachrome tautomerase	-11.84	-4.51	2.64	
FCER1A	Fc fragment of IgE receptor Ia				+2.73
FCGR2B	Fc fragment of IgG receptor IIb	+2.17		-2	+2.97
GZMB	Granzyme B				+2.16

HLA-DMA/SLA-DMA	Major histocompatibility complex, class II, DM alpha		-2.16		
LTA	Lymphotoxin alpha		-2.08		+2.75
LY75	Lymphocyte antigen 75				+2.67
LY96	Lymphocyte antigen 96	+2.12	+2.93		-2.21
LYZ	Lysozyme				15.3
MAP2K1	Mitogen-activated protein kinase 1		-3.94	+2.74	
NCF2	Neutrophil cytosolic factor 2	+3.19			
NOS2	Nitric oxide synthase 2	+3.07			
RFX5	Regulatory factor X5		+4.21		
SERPINA1	Serpin family A member 1				-2.37
TGFβ3	Transforming growth factor beta 3	+2.72			-2.4
TNFSF10	Tumour necrosis factor superfamily member 10	+6.43			

In general, IPA analysis revealed that the genes differentially expressed following *S. scabiei* infestation were associated with various important biological functions such as, "Cellular Growth and Proliferation", "Cell Morphology", "Cell-mediated Immune Response", "Cell-To-Cell Signaling and Interaction", "Immune Cell Trafficking", "Inflammatory Response", "Immunological Disease" and "Inflammatory Disease. There were often many significant networks associated with the datasets at each time point, but we are only showing the top ranked networks based on how relevant they are to the DEGs in the dataset. The top 5 gene networks associated with each time point are provided in Table 4. 5. The top biological functions and the number of DEGs involved in each process are listed in Table 4. 6. The pathway analysis revealed that the DEGs were associated with an array of signaling pathways with notable pathways including "Acute Phase Response Signaling", "Complement System", "Atherosclerosis Signaling", "T helper (Th) 1 and Th2 Activation Pathway", "IL-12 Signaling and Production in Macrophages", "Inflammasome Pathway" and "Role of IL-17 in Allergic Inflammatory Airway Disease". Table 4. 7 shows the top 5 signaling pathways from the IPA mapping at each time point. The results of differential gene expression and network/pathway analysis at each time point are provided and discussed in further details in the following sections.

4. 3. 6. Gene expression at 1 week post-infestation

In IPA, the networks represented with week 1 dataset were enriched for genes associated with various diseases and cellular processes including "Antigen Presentation", "Cellular Growth and Proliferation", "Organismal Development", "Cancer" and "Organismal Injury and Abnormalities" (Table 4. 5 and 4. 6).

Table 4. 5. Top gene networks and biological functions from the *IPA mapping associated with gene expression at week 1, 2, 4 and 8 following *S. scabiei* infestation in pigs. Genes entering IPA showed $\geqslant \pm$ 2.0 fold change in expression with FDR corrected p-value of ≤ 0.05 in infested (crusted and ordinary scabies) relative to non-infested (control) pigs. Wpi = weeks post-infestation.

Time	ID	Associated Network Functions	Score
1wpi	1	Antigen Presentation, Cell Death and Survival, Cell-To-Cell Signaling and Interaction	49
	2	Post-Translational Modification, Cell Death and Survival, Cellular Assembly and Organization	42
	3	Cell-To-Cell Signaling and Interaction, Haematological Disease, Metabolic Disease	35
	4	Cellular Function and Maintenance, Molecular Transport, Cell-To-Cell Signaling and Interaction	33
	5	Gene Expression, Protein Synthesis, Cancer	33
2wpi	1	Post-Translational Modification, Carbohydrate Metabolism, Nucleic Acid Metabolism	40
	2	RNA Post-Transcriptional Modification, Cancer, Endocrine System Disorders	40
	3	Cell Cycle, Cellular Assembly and Organization, DNA Replication, Recombination, and Repair	38
	4	Drug Metabolism, Molecular Transport, Small Molecule Biochemistry	38
	5	Cancer, Cell Death and Survival, Organismal Injury and Abnormalities	33
4wpi	1	Cellular Assembly and Organization, Connective Tissue Disorders, Developmental Disorder	54
	2	Lymphoid Tissue Structure and Development, Tissue Morphology, Cell Morphology	34
	3	Lipid Metabolism, Small Molecule Biochemistry, Organismal Injury and Abnormalities	34
	4	Lipid Metabolism, Small Molecule Biochemistry, Cellular Compromise	30
	5	Cellular Assembly and Organization, Cellular Function and Maintenance, Nervous System Development and Function	30
8wpi	1	Cellular Assembly and Organization, Cellular Function and Maintenance, Dermatological Diseases and Conditions	49
	2	Cell Morphology, Embryonic Development, Tissue Development	39
	3	Cancer, Cell Death and Survival, Organismal Injury and Abnormalities	39
	4	Embryonic Development, Organ Development, Organismal Development	37
	5	Organismal Injury and Abnormalities, Renal and Urological Disease, Developmental Disorder	35

*IPA ranks networks according to how relevant they are to the genes in the dataset and assigns a score (numerical value) to each network accordingly. A score of 10 indicates a $p = 10^{-10}$ chance that genes in that network are associated solely by chance. Only gene networks with a score >10 shown.

Table 4. 6. Number of differentially expressed genes (DEGs) involved in diseases and disorders, biological and physiological functions from the IPA mapping associated with gene expression following infestation with *S. scabiei* in pigs at 1, 2, 4 and 8 weeks post-infestation (wpi). The DEGs entering the IPA are those which showed $\geqslant \pm$ 2.0 fold change in expression with FDR corrected p-value of ≤ 0.05 in infested relative to non-infested (control) pigs.

Time	Top Diseases and Bio Functions	#DEGs
1wpi	**Diseases and disorders**	
	Organismal injury and Abnormalities	865
	Cancer	841
	Molecular and Cellular Functions	
	Cellular Growth and Proliferation	388
	Cellular Development	328
	Cellular Function and Maintenance	277
	Physiological Function	
	Organismal Development	338
2wpi	**Diseases and disorders**	
	Organismal injury and Abnormalities	1377
	Cancer	1351
	Dermatological Diseases and Conditions	814
	Molecular and Cellular Functions	
	Cellular Growth and Proliferation	617
	Cell Morphology	416
	Molecular Transport	345
	Physiological Function	
	Tissue Morphology	369
4wpi	**Diseases and disorders**	
	Organismal injury and Abnormalities	443
	Cancer	434
	Metabolic Disease	73
	Molecular and Cellular Functions	
	Molecular Transport	134
	Cell Morphology	112
	Lipid Metabolism	87
	Physiological Function	
	Tissue Morphology	121
8wpi	**Diseases and disorders**	
	Organismal injury and Abnormalities	1039
	Inflammatory Response	358
	Skeletal Muscle Disorders	297
	Inflammatory Diseases	271
	Connective Tissue Disorders	212
	Molecular and Cellular Functions	
	Cellular Growth and Proliferation	513
	Cellular Movement	326
	Cell-To-Cell Signaling and Interaction	245
	Physiological Function	
	Tissue Morphology	313
	Immune Cell Trafficking	221

Table 4. 7. Top 5 (+ 5 immunity related) canonical signaling pathways from the *IPA mapping associated with gene expression at week 1, 2, 4 and 8 following infestation with *S. scabiei.* In 2-way ANNOVA analysis, genes entering the IPA showed $\geqslant \pm$ 2.0 fold change in expression with FDR corrected p-value of ≤ 0.05 in infested relative to non-infested (control) pigs. # = number and Wpi = weeks post infestation.

Time	Canonical Pathway	# Genes in pathway/total # of genes	P-value
1wpi	G-Protein Coupled Receptor Signaling	29/272	5.35E-06
	Protein Kinase A Signaling	35/392	3.30E-05
	IL-12 Signaling and Production in Macrophages	17/146	1.58E-04
	T Cell Receptor Signaling	14/109	2.11E-04
	Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	19/193	5.90E-04
	Immunity related		
	iNOS Signaling	7/44	2.36E-03
	B Cell Receptor Signaling	17/185	2.36E-03
2wpi	Polyamine Regulation in Colon Cancer	9/22	6.53E-06
	Role of JAK2 in Hormone-like Cytokine Signaling	10/34	5.78E-05
	STAT3 Pathway	15/73	9.44E-05
	Complement System	10/37	1.27E-04
	Acute Phase Response Signaling	25/169	1.87E-04
	Immunity related		
	Role Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	36/309	1.06E-03
	NF-κB Signaling	24/180	1.14E-03
	Interferon Signaling	8/36	2.39E-03
4wpi	LXR/RXR Activation	10/121	3.30E-04
	FXR/RXR Activation	10/126	4.19E-04
	Atherosclerosis Signaling	9/127	1.79E-03
	Glucocorticoid Biosynthesis	3/12	1.91E-03
	Fatty Acid -oxidation I	4/32	4.81E-03
	Immunity related		
	Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	10/193	9.53E-03
	IL-12 Signaling and Production in Macrophages	8/146	1.43E-02
	B Cell Receptor Signaling	9/185	1.96E-02
	Oncostatin M Signaling	3/34	3.67E-02
8wpi	Th1 and Th2 Activation Pathway	40/185	4.92E-15
	Th2 Pathway	35/150	2.22E-14
	Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses	33/137	4.63E-14
	Hepatic Fibrosis / Hepatic Stellate Cell Activation	35/183	1.06E-11
	Th1 Pathway	30/135	6.07E-12
	Immunity related		
	Atherosclerosis Signaling	28/127	3.74E-11

* IPA uses a right-tailed Fisher's test to calculate the p-value determining the probability that association between genes in the data set and canonical pathway is due to chance alone.

The top canonical pathways associated were, “Production of Nitric Oxide and Reactive Oxygen Species in Macrophages”, “IL-12 Signaling and Production in Macrophages”, “T Cell Receptor Signaling”, and “iNOS Signaling” (Table 4. 7).

Week 1 showed up-regulation of a number of genes induced during inflammatory response which included intercellular adhesion molecule 3 (ICAM3, 3.11 fold up), nitric oxide synthase 2 (NOS2, 3.71 fold up), C-C motif chemokine receptor (CCR) 3 (2.92 fold up) and oncostatin M (OSM, 3.31 fold up).

Also at 1wpi, adaptive immune response regulator Fc fragment of IgG receptor IIb (FCGR2B) was up-regulated (2.17 fold up). In addition, at week 1, up-regulation of innate immunity genes was observed including complement component (C) 6 (3.27 fold up) and neutrophil cytosolic factor (NCF) 2 (3.19 fold up).

Week 1 also contained a number of transcripts of immunoregulatory mediators being up-regulated including interferon (IFN) -γ (2.26 fold up), forkhead box P3 (FOXP3, 3.87 fold up), and IL (interleukin) -27 (3.02 fold up). On the other hand, week 1 showed down-regulation of a number of factors involved in cell activation and differentiation, antigen processing and presentation, and cell and immunoglobulin mediated immune response including signal transducer and activator of transcription (STAT) 4 (-2.23 fold down), cluster of differentiation (CD) 8α (-3.30 fold down), CD19 (-2.53 fold down), CD70 (-3.49 fold down), CD74 (-2.28 fold down), bone morphogenetic protein receptor type-2 (BMPR2, -2.07 fold down) and CD274 (-3.74 fold down). Therefore, week 1 showed a number of factors up-regulated involved mostly in immune regulation and suppression and also contained several genes down-regulated with roles in innate immune responses and antigen presentation.

4. 3. 7. Gene expression at 2 week post-infestation

Overall week 2 showed a trend of down-regulation with 1212 genes being down-regulated and 293 genes being up-regulated (Table 4. 1). The networks represented here showed enrichment for genes involved in several biological functions and diseases including “Molecular Transport”, “Cellular Growth and Proliferation”, “Cell Morphology, “Cancer”, “Organismal Injury and Abnormalities” and “Dermatological Diseases and Conditions” (Table 4. 5 and 4. 6). In IPA analysis, the top signaling pathways associated with week 2 dataset were, “Role of Janus kinase (JAK) 2 in Hormone-like Cytokine Signaling”, “Acute Phase Response Signaling”, “STAT3 Signaling Pathway” and “Complement System” (Table 4. 7).

At 2wpi, transcription of an array of immune response, cell recruitment and proliferation factors was down-regulated including IL13RA1 (-2.18 fold down), C-X-C motif chemokine ligand (CXCL) 10 (-3.98 fold down) and CXCL13 (-4.22 fold down).

In addition, down-regulation of inflammasome associated molecules NLR family, pyrin domain containing 3 (NLRP3, -2.03 fold down), myeloid differentiation primary response 88 (MYD88, -2.45 fold down), caspase (CASP) 1 (-2.14 fold down), cathepsin (CTS) B (-2.09 fold down) and toll-like receptor (TLR) 4 (-2.12 fold down) was detected.

Transcription of antigen presentation related HLA class II histocompatibility antigen, DM alpha chain (HLA-DMA) was down-regulated (-2.16 fold down) at 2wpi. Week 2 also showed down-regulation of several complement factors including C1R (-2.31 fold down), C1S (-2.49 fold down), C2 (-2.14 fold down) C5 (-2.93 fold down) and CFH (-2.13 fold down) (Figure 4. 2). Conversely C6 was up-regulated at 2wpi.

The other notable immune related transcripts up-regulated at 2wpi included CD81 (2.22 fold up), DNA-binding protein RFX5 (RFX5, 4.21 fold up), CD3g (3.02 fold up) and apolipoprotein (APO) A2 (3.16 fold up).

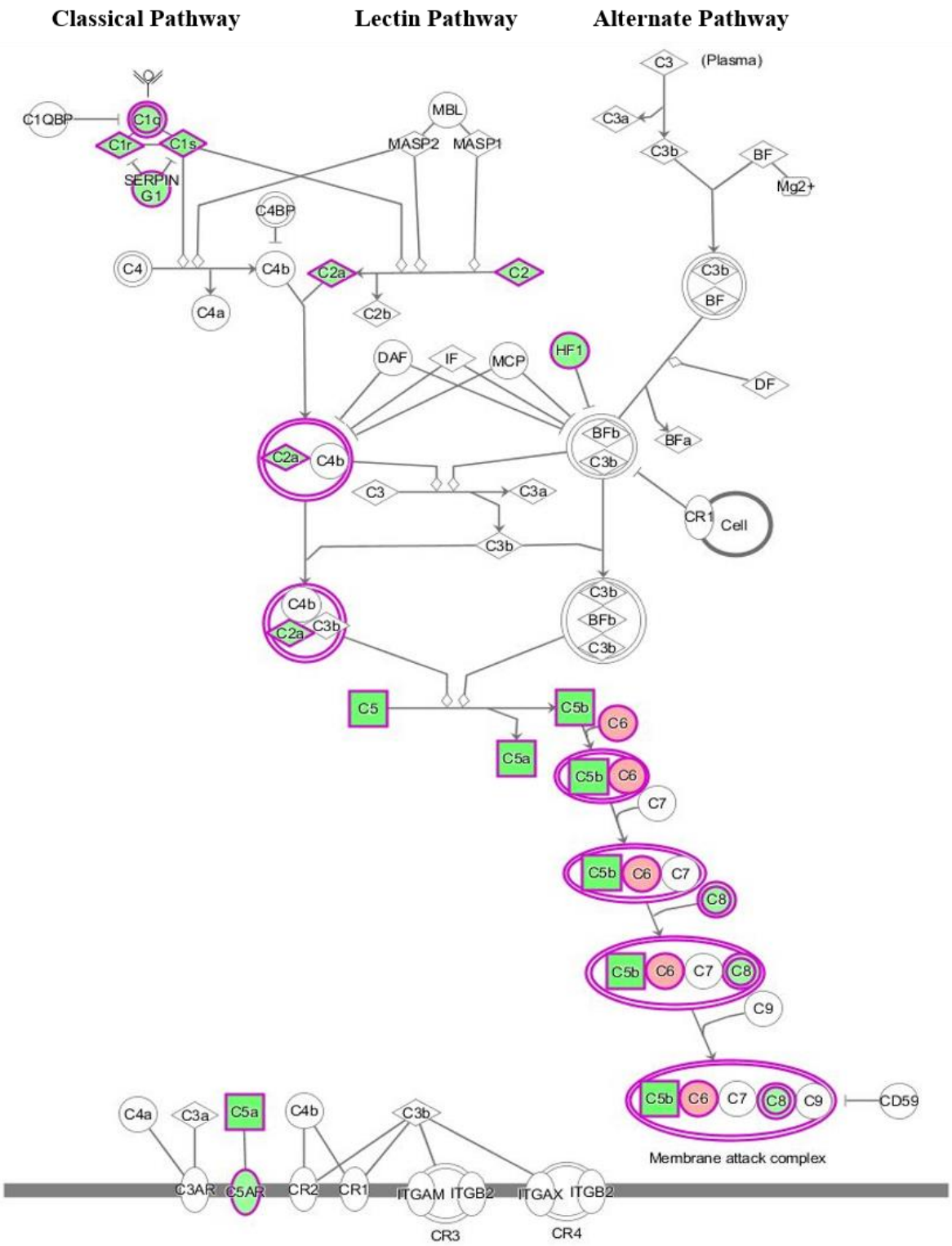

Figure 4. 2. IPA representation of the complement system pathway associated with dataset at week 2 following infestation with *S. scabiei* in pigs. Individual nodes represent protein functions and edges represent relationships. Nodes are coloured by gene expression with green indicating down-regulation, red indicating up-regulation and white indicating gene not differentially expressed but with defined relationship to other genes in the pathway. Arrows indicate directional relationships in the pathway. Genes associated with this pathway had a FDR corrected p-value of $\leqslant 0.05$ and fold change of $\geqslant \pm 2.0$ in infested relative to non-infested (control) pigs.

The networks and pathways represented at week 2 showed enrichment for several molecules (gene expression down-regulated) induced during inflammatory and innate immune responses with roles in the activation and proliferation of leukocytes, cytokine and immunoglobulin production, and complement activation.

4. 3. 8. Gene expression at 4 week post-infestation

Week 4 showed a trend of down-regulation with 286 genes being repressed and 196 genes being up-regulated (Table 4. 1), although fewer genes overall were differentially expressed relative to the other time points. The top diseases associated with the genes expressed at week 4 were, “Cancer” and “Organismal Injury and Abnormalities” (Table 4. 5 and 4. 6). The top signaling pathways were, “LXR/RXR Activation”, “FXR/RXR Activation”, and “Atherosclerosis Signaling” (Table 4. 7). Networks associated with week 4 showed enrichment of genes involved in lipid metabolism, cell morphology, molecular transport and tissue morphology (Table 4. 5 and 4. 6).

The main feature of week 4 was up-regulation of key pro-inflammatory response factors including IL17F (2.74 fold up) and STAT4 (2.14 fold up). Week 4 also showed up-regulation of matrix metalloprotease (MMP) 13 (3.87 fold up) which is involved in the breakdown of extracellular matrix and leukocyte migration (Van Lint and Libert, 2007). At 4wpi, up-regulation of cytotoxic T cell mediated response factor dopachrome tautomerase (DCT) was detected (2.64 fold up). Signaling molecule Mitogen-activated protein kinase kinase 1 (MAP2K1) was also up-regulated (2.74 fold up).

Also at 4wpi, we detected up-regulation (2.48 fold up) of complement C3, but conversely, C6 (-3.07 fold down) was repressed. 6 other notable innate immune and inflammatory response transcripts were down-regulated at 4wpi including CCR3 (-2.13 fold down) and CD36 (-2 fold).

Some of the up-regulated transcripts at week 4 including STAT4, DCT and MAP2K1 were down-regulated early (week 1 and/or 2) in the infestation.

Overall, week 4 dataset contained a number of factors (gene expression up-regulated) mostly involved in the instigation and development of inflammatory response with roles in complement activation and cytokine production, activation and differentiation of circulating leukocytes and triggering their subsequent extravasation into the site of inflammation.

4. 3. 9. Gene expression at 8 week post-infestation

At 8wpi we detected up-regulation of higher number of genes (740) as compared to other time points of the analysis and 405 genes were down-regulated (Table 4. 1). The top biological functions associated with the genes expressed at week 8 included “Organismal Injury and Abnormalities”, “Inflammatory Disease”, and “Connective Tissue Disorders” (Table 4. 5 and 4. 6). In pathway analysis, the top signaling pathways associated with week 8 dataset were, “Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses”, “Th1 and Th2 Activation Pathway”, “Th2 Pathway” and “Th1 Pathway” (Table 4. 7).

The networks represented with week 8 dataset showed enrichment of a large number of genes implicated in inflammatory responses (Figure 4. 3), immune cell trafficking (Figure 4. 4), cell-mediated immune response, cell-to-cell signaling and interaction, cellular development, cellular growth and proliferation (Table 4. 6). In particular, week 8 showed up-regulation of a range of potent proinflammatory cytokines, chemokines, and cell-receptors including IL-1β (5.65 fold up), IL17F (3.34 fold up), IL8 (54.19 fold up), tumour necrosis factor (TNF, 2.49 fold up), IL4 (3.75 fold up), CCL (C-C motif chemokine ligand) 22 (3.11 fold up), CCL27 (2.39 fold up), CCR7 (9.33 fold up), CD40 (2.06 fold up) and CD274 (2.93 fold up).

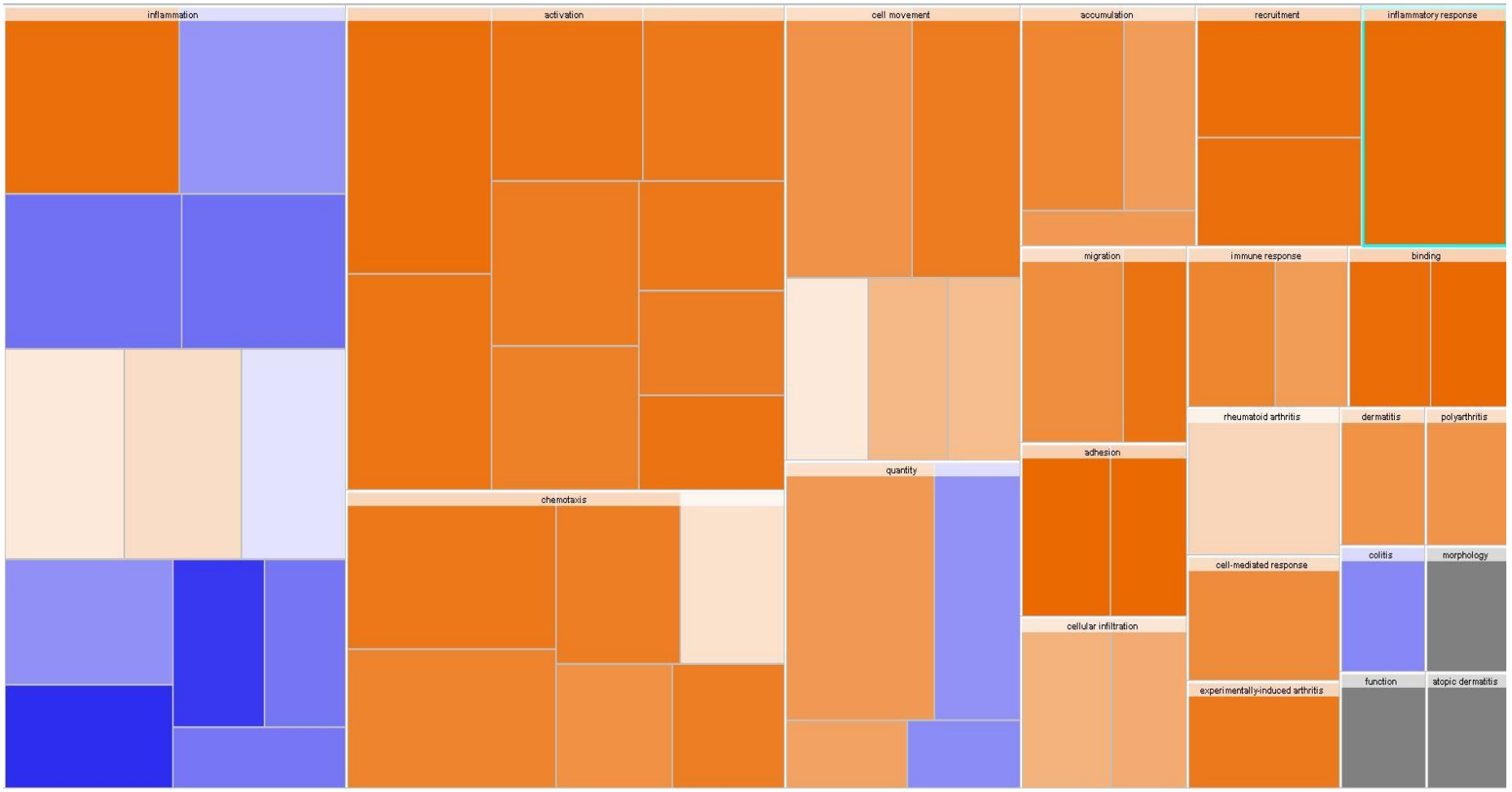

Figure 4. 3. Heat map illustrating inflammatory response related diseases and bio functions associated with gene expression at week 8 following *S. scabiei* infestation in pigs. Each coloured box represents a particular biological function or disease and colour indicates its predicted state: increasing (orange), or decreasing (blue) or could not be predicted (grey). The size of the box represents how many of the genes in the panel are represented in the implicated function or disease. Genes associated with these functions or diseases had a FDR corrected p-value of $\leqslant 0.05$ and fold change of $\geqslant \pm 2.0$ in infested relative to non-infested (control) pigs.

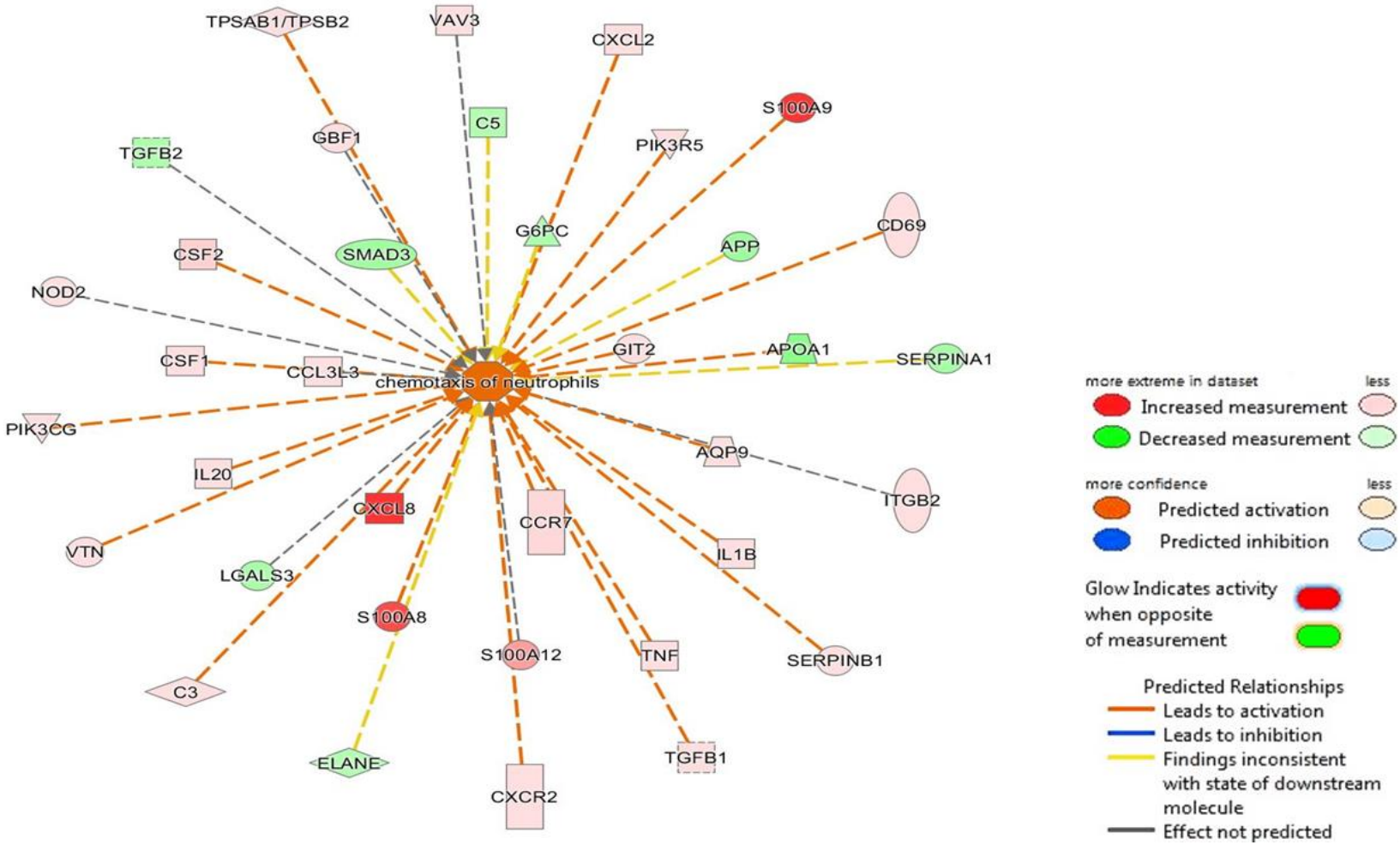

Figure 4. 4. IPA network depicting DEGs associated with the biological function "Immune Cell Trafficking - Neutrophil Chemotaxis" at 8wpi following infestation with *S. scabiei* in pigs. The differentially expressed genes included are those which showed $\geqslant \pm$ 2.0 fold change in expression with FDR corrected p-value of $\leqslant$ 0.05 in infested (crusted and ordinary scabies) relative to non-infested (control) pigs. The notable ones include CSF1, CSF2, S100A8, S100A9, CXCL2, CXCL8/IL-8, CCR7, CXCR2, CSF2, TNF, and IL-1β.

Inflammasomes associated molecules NLRP3 (2.10 fold up), nucleotide-binding oligomerization domain containing (NOD) 2 (3.57 fold up), MYD88 (2.09 fold up), nuclear factor NF-kappa-B p105 subunit (NF-κB1, 2.13 fold up) and CTSB (2.53 fold up) were up-regulated at 8wpi (Figure 4. 5).

Week 8 also showed strong up-regulation of numerous S100 calcium binding proteins including S100A7 (132.92 fold up), S100A8 (44.12 fold up) and S100A9 (50.78 fold up).

This time point also showed enrichment (up-regulation) of inflammation factor granzyme (GZM) B (2.16 fold up). The results also demonstrated up-regulation of metallopeptidases including MMP1 (24.48 fold up), MMP9 (10.22 fold up), MMP12 (23.45 fold up) and MMP13 (2.23 fold up).

In addition, at 8wpi, we detected up-regulation of few immunoregulatory molecules including tumor growth factor (TGF) β1 (2.47 fold up), IL-10 (2.06 fold up) and FOXP3 (2.11 fold up).

At 8wpi the down-regulated genes included complement components C6 (-2.30 fold down) and C5 (-2.09 fold down).

Therefore, week 8 showed enrichment for a number of genes involved in the innate and adaptive immune responses with a focus on up-regulation of a number of key pro-inflammatory factors. In particular, genes involved in instigating, development and further amplification of pro-inflammatory response were enriched at week 8, including factors for T cell activation and differentiation, and leukocyte activation and recruitment to the sites of inflammation.

4. 3. 10. qRT-PCR validation of microarray data

To confirm the validity of the microarray results, nine differentially expressed genes identified in the 2-way ANNOVA with p-value of ≤ 0.05 and FC of > 2.0 were selected randomly and evaluated by qRT-PCR.

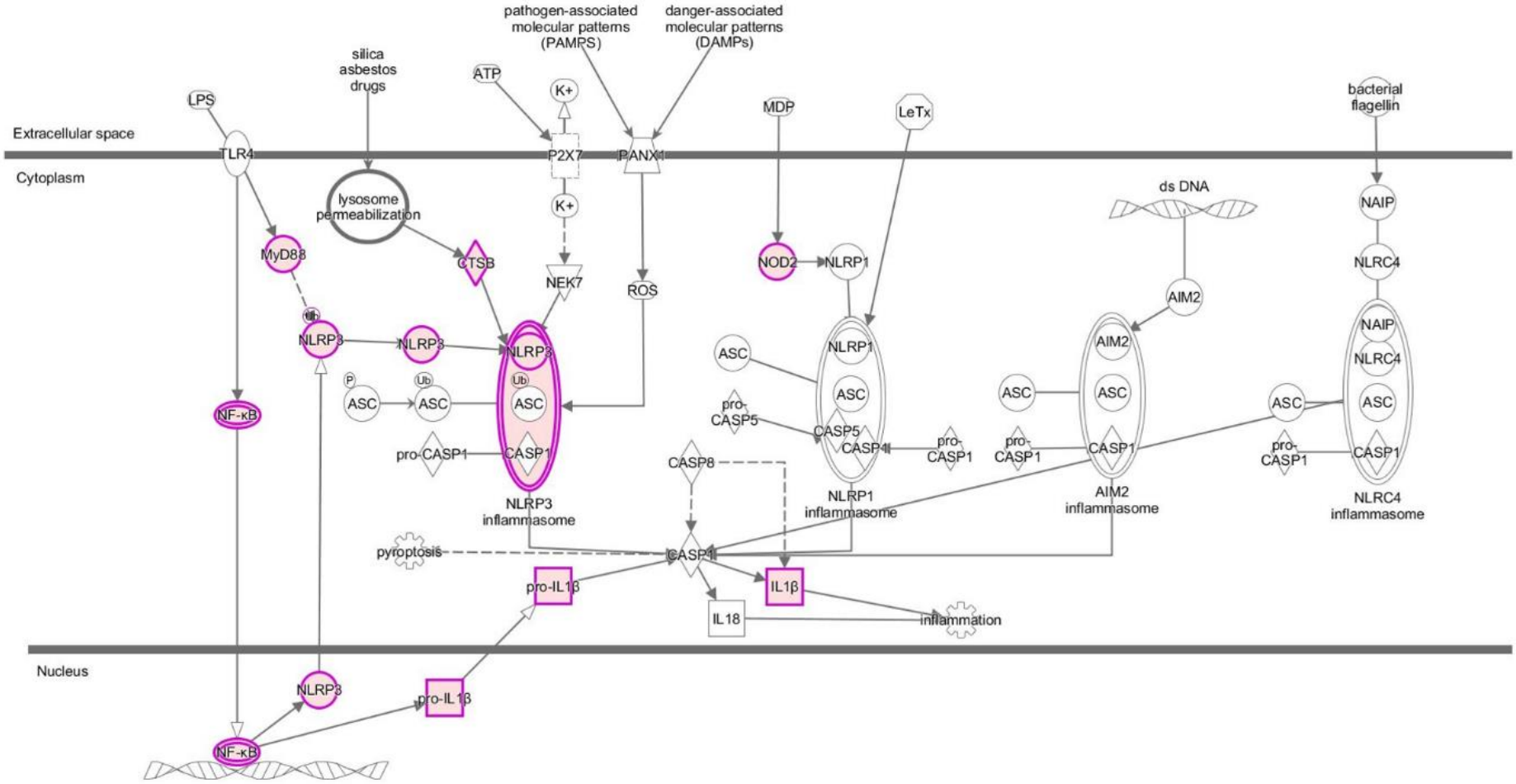

Figure 4. 5. IPA canonical pathway depicting relationships among genes associated with "NLRP3 Inflammasome Pathway" at week 8 following infestation with *S. scabiei* in pigs. The genes included are those which showed $\geq \pm$ 2.0 fold change in expression with FDR corrected p-value of ≤ 0.05 in infested relative to non-infested (control) pigs. Nodes coloured by gene expression. Pink nodes represent up-regulated genes and white indicating not differentially expressed genes. Purple circles and squares highlight roles for IL-1β, NLRP3, MyD88, CTSB, NOD2 and NFκB1.

qPCR assays were performed on the cDNA prepared from the same total RNA samples used for the microarray study. Real-time PCR to validate mRNA expression of IFNγ, IL1β, FOXP3, IL17F, glyoxalase I (GLO1), TGFβ, CD274, NLRP3 and TNF in skin derived from mite infested (OS, n = 4 and CS, n = 4) and non-infested control (C, n = 4) pigs revealed that the expression was confirmed for IFNγ, IL1β, FOXP3, IL17F, GLO1, TGFβ, CD274, NLRP3 and TNF with >3.0 fold higher expression (Table 4. 8 and appendix F, Supplementary Table 3). These selected genes represented a range of genes expressed (up-regulated) at different time points of the study period. qPCR expression of these selected genes over the time course of the infestation was considered to be validated as the fold change measured by both qPCR and microarray were ≥ 2 fold.

Table 4. 8. Confirmation of nine up-regulated genes in microarray data by qPCR. These genes were identified in the 2-way ANNOVA analysis with a p-value of ⩽ 0.05 and FC of > 2.0. Relative quantification of gene expression levels was determined by normalising to the HPRT1 control using the comparative Ct method with the formula $2^{-\Delta\Delta CT}$ and expressed as fold change. Duplicates of each cDNA sample from infested (OS, n = 4; CS, n = 4) and non-infested controls (C, n = 4) were maintained. Wpi = weeks post-infestation.

Gene Symbol	Gene Description	qRT-PCR Validation Fold Change	Microarray Analysis Fold Change	Comparison	Time (Wpi)
IFNγ	Interferon gamma	8.89	2.40	CS vs C	1
IL1β	Interleukin 1 beta	4.50	2.59	OS vs C	1
FOXP3	Forkhead box P3	16.11	3.29	CS vs C	2
IL17F	Interleukin 17F	19.15	2.75	OS vs C	4
GLO1	Glyoxalase 1	5.37	7.93	CS vs OS	4
TGFβ	Transforming growth factor beta	6.29	2.17	CS vs OS	8
CD274	Cluster of differentiation 274	3.40	4.37	OS VS C	8
NLRP3	NOD like receptor protein 3	6.80	2.44	OS vs C	8
TNF	Tumour necrosis factor	9.48	2.45	OS vs C	8

4. 4. Discussion

The aim of the present analysis was to provide a global picture of gene expression profiles and to define and gain insights into the molecular mechanisms and signaling pathways associated with the host inflammatory and immune responses in scabies. The data presented here reveals transcriptional differences in the skin of scabietic compared to control/healthy animals and it was found that infestation with scabies mite influences expression of a large number of genes. In general, these differences are indicative of immunoregulatory and immunosuppressive responses early in the mite infestation. At later time points, the gene expression pattern is indicative of amplified inflammatory and allergic responses.

4. 4. 1. Early immunosuppression in scabies

Consistent with previous studies (Rampton et al., 2013), the clinical manifestations of scabies in this trial became apparent in infested pigs from week 4. Studies suggest that *S. scabiei* may be inhibiting the early immune responses by regulating the production of proinflammatory mediators, immune cells and cytokines (Arlian et al., 2007; Arlian et al., 2003; Arlian et al., 2004b; Arlian et al., 2006; Mika et al., 2012a; Morgan et al., 2013; Swe and Fischer, 2014). In this study it was most evident at week 2, with a substantial number of transcripts down-regulated. Early in infestation, there were also several genes differentially expressed that were associated with immune suppression and / or regulation. For example, IL27 was down-regulated at week 1 which promotes the differentiation of regulatory T cells (Tregs) expressing IFN-γ and IL-10 (Awasthi et al., 2007). Although changes in IL10 transcription at this time point were not seen, it has been demonstrated in PBMCs that scabies mite extract can induce IL-10 expression and by extrapolation, influence Treg activity (Arlian et al., 2006; Walton et al., 2010). Tregs and the anti-inflammatory cytokine IL-10 are efficient regulators of Th1 associated inflammation and contribute substantially to active immune suppression

(Trinchieri, 2007). FOXP3 was up-regulated at week 1 which is an essential transcription factor in the development of Tregs (Miyara and Sakaguchi, 2011; Sakaguchi et al., 2010). Induction of $Foxp3^+$ $CD4^+$ Tregs, and expression of IL-27 and IFN-γ also might promote Treg cell production. This Treg and cytokine induction may be responsible for the suppression of inflammatory and immune responses to the parasite early in the infestation which may explain the delay in symptoms observed in scabies.

Complement system plays an essential role in the innate immunity and targets pathogens through opsonisation, cell lysis and immune cell recruitment (Dunkelberger and Song, 2010). Complement components have also been described as down-regulated in response to *P. ovis* in sheep (Burgess et al., 2010; Burgess et al., 2012), scabies mites in humans (Bergström et al., 2009; Mika et al., 2012a) and house dust mite (HDM) allergens (Maruo et al., 1997). In this analysis, many complement components were repressed early at week 2 following mite infestation and additionally IPA analysis revealed complement system pathways being inhibited at this time point, specifically molecules (6 genes) involved in the classical pathway and membrane attack complex (MAC) (Ricklin et al., 2010).

HDM cysteine protease Der p1 is reported to proteolytically cleave complement component C5, resulting in production of the anaphylatoxin C5a (Maruo et al., 1997). In this analysis, C5 and C5Ra1 were among the many components repressed at 2wpi and it seems a mechanism might also exist in *S. scabiei* to inhibit the complement mediated pathogen killing. C5a and C5aR1 are involved in recruitment and activation of DCs, neutrophils, eosinophils and mast cells, and may contribute further in the pathogenesis of inflammatory and allergic diseases (Maruo et al., 1997). C5a is a constituent of membrane attack complex (MAC) and play a role in MAC-mediated cell lysis and pathogen killing (Parker and Sodetz, 2002).

Down-regulation of complement proteins indicates the potential presence of a transcriptional control mechanism to supress the complement system and the associated inflammation in the

early stages of infestation with *S. scabiei*. This is in agreement with the studies showing *S. scabiei* produces an array of inactivated serine proteases which inhibit complement activation by binding to complement proteins (Bergström et al., 2009; Mika et al., 2012a). These results also suggest that expression of transcription factors or secretion of molecules (proteins) by mites early in the infestation may modulate complement-mediated inflammation or damage at the transcriptional level in the host.

CD5 molecule like (CD5L) is a glycoprotein secreted by tissue macrophages that modulates or enhances inflammatory responses by supporting macrophage survival and phagocytic activity (Sanjurjo et al., 2015). CD5L was repressed early (at week 2) in the infestation indicating that CD5L might be targeted by mite derived factors (proteins) to inhibit innate immune cells and their functions to circumvent host responses and leading ultimately to the disease development.

Signal transducer and transcription activator family members are important in cytokine and growth factor signal transduction and activation of gene transcription mediating cell growth, differentiation, inflammation, and immune response. STATs are primarily activated by Janus family tyrosine kinases (JAK) (O'Shea and Murray, 2008; O'Shea and Plenge, 2012; Schindler et al., 2007). In this analysis, early (at week 2) in the infestation, down-regulation of STAT and JAK genes was detected, which were identified in IPA as members (9 genes) of JAK/STAT signaling pathway. These molecules are also critical for IL-12 and IL-4 signals and, Th1 and Th2 cell differentiation (O'Shea and Murray, 2008; O'Shea and Plenge, 2012). Suppression of STATs and JAKs genes and inhibition of JAK/STAT signaling pathway indicates another mechanism of evading the host defence to facilitate mite growth in the early stages of the disease and to influence the Th cell biased responses. Similarly, Suppressor of cytokine signaling 5 (SOCS5) is involved in the regulation of Th cell differentiation by inhibiting of the IL-4 signaling pathway (Seki et al., 2002). Down-regulation of SOCS5 early

(at week 2) in the infestation by the mites may be another way to manipulate Th1 and Th2 responses and may play role in the disease development.

CD19, CD70 and CD8α gene transcription was down – regulated at weeks 1 and 2 post-infestation indicating an additional pathway/mechanism of inhibiting T cell mediated response, B-cell differentiation and secretion of immunoglobulins which is consistent with the delay in Ig response reported in scabies and lack of detection until disease symptoms appear (Liu et al., 2014b; McCarthy et al., 2004; Rampton et al., 2013). B lymphocyte development, activation, differentiation and function are regulated directly through the B cell receptor (BCR) signaling. CD19, is a cell surface co-receptor and forms a signal transduction complex which modulates B cell receptor (BCR) signaling through the B cell-antigen receptor complex and governs B cell responses and humoral immunity (Tedder et al., 2005). In addition, CD70 is induced upon activation on T and B cells, and is a ligand for the costimulatory molecule CD27. CD27-CD70 interaction contributes to effector, memory T cell formation, B cell responses and is responsible for plasma cell differentiation (Borst et al., 2005; Jacquot, 2000). Cluster of differentiation 8α molecule is a glycoprotein co-receptor found on most cytotoxic $CD8^+$ T lymphocytes and thought to play a role in the process of T-cell mediated killing of an array of pathogens (Harty et al., 2000; Laugel et al., 2007).

Transcription of CD274 was down-regulated at weeks 1, 2 and 4 post-infestation. The CD274 gene encodes an immunoinhibitory ligand (also referred to as Programmed cell death 1 ligand 1 (PD-L1)) expressed by antigen-presenting cells. CD274/PD-L1 acts to dampen immune responses by inhibiting T cell proliferation and cytokine secretion via ligation/interaction with its receptor PD-1. CD274/PD-L1 serves as a negative regulator of immune responses as its loss/absence leads to a breakdown of peripheral tolerance (Freeman et al., 2000; Mazanet and Hughes, 2002). This CD274/PD-L1 suppression may predispose to the development of

disease and enhance T cell proliferation which may contribute to the pathological manifestations in scabies.

4. 4. 2. Acute phase response

The acute phase response is an innate reaction triggered by a local inflammatory response at the site of injury or infection or inflammation. During inflammation neutrophils and macrophages secrete pro-inflammatory cytokines importantly IL-1β, IL-6, IL-8, OSM and TNFα, which stimulate/trigger acute phase protein synthesis (Ebersole and Cappelli, 2000; Eckersall and Bell, 2010; Petersen et al., 2004; Slaats et al., 2016). Increased secretion of these cytokines has been reported in response to *S. scabiei* mites and mite extracts (Morgan and Arlian, 2010; Morgan et al., 2013; Mounsey et al., 2015) indicative of induction of acute phase protein synthesis. Transcription for the gene encoding OSM was up-regulated early in the infestation (at week 1) and accordingly, acute phase response signaling was among the top signaling pathways associated with the genes expressed early in the infestation. This analysis also showed differential gene expression of an array of acute phase proteins including Serum amyloid A (SAA), Transferrin (TF), Albumin, Plasminogen, and Ceruloplasmin. Expression/production of these pro-inflammatory cytokines and acute phase signaling molecules in the skin may stimulate/modulate the production of APPs and may result in systemic acute phase response. Given these results it will be imperative to look into the APP response in these pigs. Acute phase proteins can be used as biomarkers for monitoring inflammatory processes (Almeida Kde et al., 2012; Eckersall and Bell, 2010; Wells et al., 2013), early disease detection and prognosis of scabies in humans and sarcoptic mange in animals.

4. 4. 3. Inflammasomes and IL-1 mediated immune response

The analysis results show enrichment of various genes associated with the inflammasome pathway (Figure 4. 5) (Franchi et al., 2009; Lamkanfi et al., 2007; Latz, 2010). Expression of most of these genes was up-regulated late (week 4 and 8) in the infestation while a few of them were down-regulated earlier (at week 2). Inflammasomes are multiprotein molecular complexes that trigger the maturation and secretion of proinflammatory cytokine IL-1 (Schroder and Tschopp, 2010).

IL-1 induces acute and chronic inflammation through activation of the innate and acquired immune systems by inducing expression of an array of cytokines and chemokines in various cells – keratinocytes, epidermal cells, etc. (Dinarello, 2009) and plays a key role in the pathogenesis of many allergic and inflammatory skin diseases (Nambu and Nakae, 2010). IL1α and IL1β were both expressed at week 8 in this analysis in agreement with previous studies demonstrating increased IL1α and IL1β expression in human skin equivalents stimulated with *S. scabiei* mites (Arlian et al., 1996b; Morgan et al., 2013). IL-1 has also been implicated in the expression of IL-6 and IL-8 in mast cells (Conti et al., 2017; Kim et al., 2010) and they both were up-regulated in this analysis. IL-6 is an important mediator of acute phase response, stimulates production of neutrophils in the bone marrow and plays a key role in differentiation of B cells into Ig- secreting cells. IL-6 is primarily produced at sites of acute and chronic inflammation and stimulates a transcriptional inflammatory response in many diseases such as atherosclerosis, rheumatoid arthritis and cutaneous lupus erythematosus (Nishimoto, 2006; Stannard et al., 2017). As mentioned previously chemokine IL-8 is a potent neutrophil chemoattractant and one of the major mediators of the inflammatory response. IL-6 and IL-8 are also known to activate and stimulate keratinocyte proliferation (Mann et al., 2001). Human skin equivalents (composed of epidermal keratinocytes and dermal fibroblasts) stimulated with *S. scabiei* mites have been shown to

produce IL-6 and IL-8 (Morgan and Arlian, 2010; Morgan et al., 2013). One of the main features of crusted scabies is hyperkeratosis. Up-regulation of IL1α and IL1β, and IL-6 and IL-8 production by fibroblasts and keratinocytes would be consistent with these clinical manifestations and indicates their role in scabies pathology and development of the inflammatory reactions in the vicinity of the scabietic lesions. Also, IL8 was not detected early in the infestation but it was very highly expressed along with IL6 and CSF2 late (week 8) in the infestation which might explain the delay in the appearance of skin lesions and inflammatory responses in scabies. This is in contrast to psoroptic mange, where strong up-regulation of IL8 was seen very early at 3 hours' post-infestation (Burgess et al., 2010).

4. 4. 4. Neutrophil mediated immunity/inflammation

In this analysis, at 8 wpi we saw up-regulated expression of an array of cytokines, chemokines and cell growth factors including CXCL8 (also known as IL8), CXCL2 (also known as IL8R), IL17F, TNF, IL1β and CSF2 which are potent facilitators of neutrophil stimulation and recruitment (Figure 4. 5) to the site of inflammation (Guilloteau et al., 2010; Kolaczkowska and Kubes, 2013). Neutrophils have been detected in skin biopsies of scabies patients (Elwood et al., 2015; Luo et al., 2016) and also in inflammatory infiltrates in the skin of wombats (Skerratt, 2003) and sheep (Dagleish et al., 2007) infested with *S. scabiei*. Neutrophils contribute significantly to local production of inflammatory mediators and in particular they synthesize and secrete IL-8, IL-1, IL-6, TNF-α and OSM, all up-regulated at this time point, which can subsequently activate neutrophils and other immune effector cells (Wright et al., 2010). Furthermore, strong expression of genes encoding calcium-binding proteins S100A8 and S100A9 was detected. These proteins are both expressed at increased levels in psoriatic and atopic skin lesions (Broome et al., 2003; Eckert et al., 2004) and have also been demonstrated (over 50 fold up-regulated) in psoroptic mange (Burgess et al., 2010). S100A8 and S100A9 proteins are highly expressed by circulating neutrophils and are found

at elevated levels in the extracellular milieu during inflammatory conditions. The up-regulation of these cytokines along with S100A8 and S100A9 implicates the neutrophils in an effector function. These findings and those of other groups suggest that neutrophils play a major role in initiating the innate and adaptive responses contributing to scabies immunopathology. These results also help us better understand the role of neutrophils in disease pathogenesis and mechanisms of how local inflammatory responses result in the activation and recruitment of inflammatory cells.

4. 4. 5. Antigen presentation and dendritic cells

Dendritic cells have been detected in the scabietic lesions of dogs (Stemmer et al., 1996), pigs (Mounsey, personal communication) and DCs derived from human PBMCs have been shown to secrete pro-inflammatory cytokines upon stimulation with scabies mite extract (Arlian et al., 2004b). The analysis results showed transcription of pathogen recognition receptor (PRR) molecules expressed on DCs including toll-like receptors and NOD1, NOD2 (Moreira and Zamboni, 2012) up-regulated at 8wpi. In addition, at this time point the analysis revealed expression of several other genes (notable genes include MyD88, NFκB1, CCR7, CD40, CD86 and CSF2) associated with antigen presentation and generation of adaptive responses (Francisco-Cruz et al., 2014; Iwasaki and Medzhitov, 2004; Moreira and Zamboni, 2012; van Vliet et al., 2007). MyD88 and HLA-DMA associated with the process of antigen presentation were supressed early in the infestation. MyD88 is an adaptor molecule, and after TLR binds to its ligand, MyD88 complexes with TLR and triggers a cascade of signaling events (Iwasaki and Medzhitov, 2004). Upon DC activation chemokine CCR7 mediates DC migration from the peripheral tissues to the draining lymph node to present the antigens to naïve T cells (Iwasaki and Medzhitov, 2004). CD40 and CD86 are costimulatory molecules involved in the interaction of the antigen presenting cell to the naïve T lymphocyte (ten

Broeke et al., 2013) leading to activation and differentiation of these T cells. CSF2 stimulates stem cells to produce monocytes which upon migrating to tissues mature into dendritic cells (Conti and Gessani, 2008). In line with this gene expression, IPA revealed several pathways including "TLR Signaling" (6 genes), "CD40 Signaling" (8 genes), "DC Maturation" (21 genes) and "NF-κB Signaling" (20 genes) associated with the dataset at this time point to be activated. These results indicate that DCs are engaged in immune response to *S. scabiei* infestation. The expression of these genes was not seen until later in the infestation, and this may be related to the large amount of antigenic material provided by hyper infestation of mites and inflammation seen/observed at this time point. This is also in concordance with the appearance of clinical signs and inflammation in infested animals which started to appear after week 4. This gene expression further supports why there is a delay in cell and humoral responses in scabies and provides a mechanism of processes/events of/in pathogen recognition, antigen presentation via DCs and adaptive immune and inflammatory response generation via activation of T cells.

4. 4. 6. Th1 and Th2 immune responses

Th1 and Th2 directed immune responses are produced after naïve $CD4^+$ T cells interact with activated antigen presenting cells (dendritic cells and macrophages) laden with antigen-MHC II complex and the cytokine milieu of the microenvironment impacts this differentiation. Th1 cytokine profile has been previously demonstrated in ordinary scabies (Abd El-Aal et al., 2016; Walton et al., 2010) and in this analysis, we observed following *S. scabiei* infestation up-regulation of Th1 cytokines TNFα, LTA and IL2 late in the infestation (at week 8). STAT4 is one of the important transcription factors involved in Th1 cell differentiation and in our analysis, we observed up-regulation of transcription factor STAT4 at week 8 post-infestation. In the pathway analysis, a number of genes up-regulated at week 8 (21 genes)

were related to the Th1 pathway. In contrast, early (at week 1) in the infestation genes CD274, CD8α, and STAT4 associated with Th1 pathway were down-regulated.

Th2 helper cells are main players in immunity against extracellular parasites including helminths (Anthony et al., 2007). IL-4, IL-5 and IL-13 are the main effector cytokines Th2 cells secrete (Luckheeram et al., 2012). These Th2 cells and cytokines induce allergic asthma and, also are involved in driving cell mediated immunity to eliminate Leishmania (Hurdayal and Brombacher, 2014; Seyfizadeh et al., 2015). High levels of IL-4, IL-5 and IL-13 have been detected in crusted scabies patients (Walton et al., 2010) and in animals with sarcoptic and psoroptic mange (Mounsey et al., 2015; Sarre et al., 2015) and consistent with these reports, in this study also we detected expression of IL4 along with interleukin 4 receptor (IL4R) at week-8 post-infestation. IL-2 and IL-6 play an important role in Th 2 lineage differentiation and up-regulation of IL-2 and IL-6 was observed at week 8. Transcription of IL-4, IL-5 and IL-6 was repressed early (at week 2) in the infestation. In further support of Th2 involvement in scabies, we identified a number (28) of genes associated with Th2 canonical pathway being up-regulated at week 8. These results indicate strong Th1 and Th2 responses to the mite infestation. A Th1 profile is often observed in OS (Abd El-Aal et al., 2016; Walton et al., 2010) whilst a Th2 profile is more commonly observed in CS (Walton et al., 2010). In this analysis, CS and OS were not distinguished as we were interested in seeing an overall picture of immune and inflammatory responses in pigs with scabies compared to healthy pigs. The subsequent analysis (Chapter 5) will focus on gene expression profiles in crusted versus ordinary scabies pigs.

Furthermore, at 8wpi IFNγ expression was down-regulated at low levels and we didn't detect IL12 expression but saw up-regulation of IL4 and IL4R which might be one way of directing the Th2 response to scabies mites. DCs when stimulated with Der p 1 also produce less IFN-γ and IL-12, and more IL-4 with Th2 biased response (Ghaemmaghami et al., 2002). These

results indicate that *S. scabiei* infestation induces cytokines, chemokines and transcription factors essential in early events of Th1 and Th2 differentiation. This analysis also reported the mediators of Th1 and Th2 inflammation, and revealed that Th2 lymphocytes and their cytokines contribute to allergic responses similar to what has been seen in other skin inflammatory diseases and allergic disorders mentioned above. This analysis also provides further insights into the mechanisms of these processes and additional mechanisms of inflammation.

4. 4. 7. IL-17 mediated immune response

IL-17 is a highly potent proinflammatory cytokine and plays a critical role in the inflammatory pathology associated with allergic airway inflammation (Figure 4. 6), skin diseases such as psoriasis and atopic dermatitis (Di Cesare et al., 2008; Di Cesare et al., 2009) and parasitic *Leishmania major* and *Schistosoma japonicum* infections (Chen et al., 2013a; Gonzalez-Lombana et al., 2013). Recently our group has shown high IL-17 expression in skin lesions of pigs (Liu et al., 2014b; Mounsey et al., 2015) and increased transcription of IL-17 have also been demonstrated in the skin biopsies of cattle breeds susceptible to psoroptic mange but not in those breeds resistant to Psoroptes infestation (Sarre et al., 2015). In this study, up-regulation of IL17F expression at weeks 4 and 8 post-infestation was detected. Previous studies have also shown increased expression of Th17 associated TGF-β, IL-1β and IL-23 in immune response to scabies mite infestation (Morgan et al., 2013; Mounsey et al., 2015) and these molecules along with IL-6 are key promoters of IL-17 secretion (McGeachy and Cua, 2008). In concordance with these studies, transcription of TGFβ, IL1β and also IL6 was observed suggesting that these molecules might be responsible for IL17 up-regulation here.

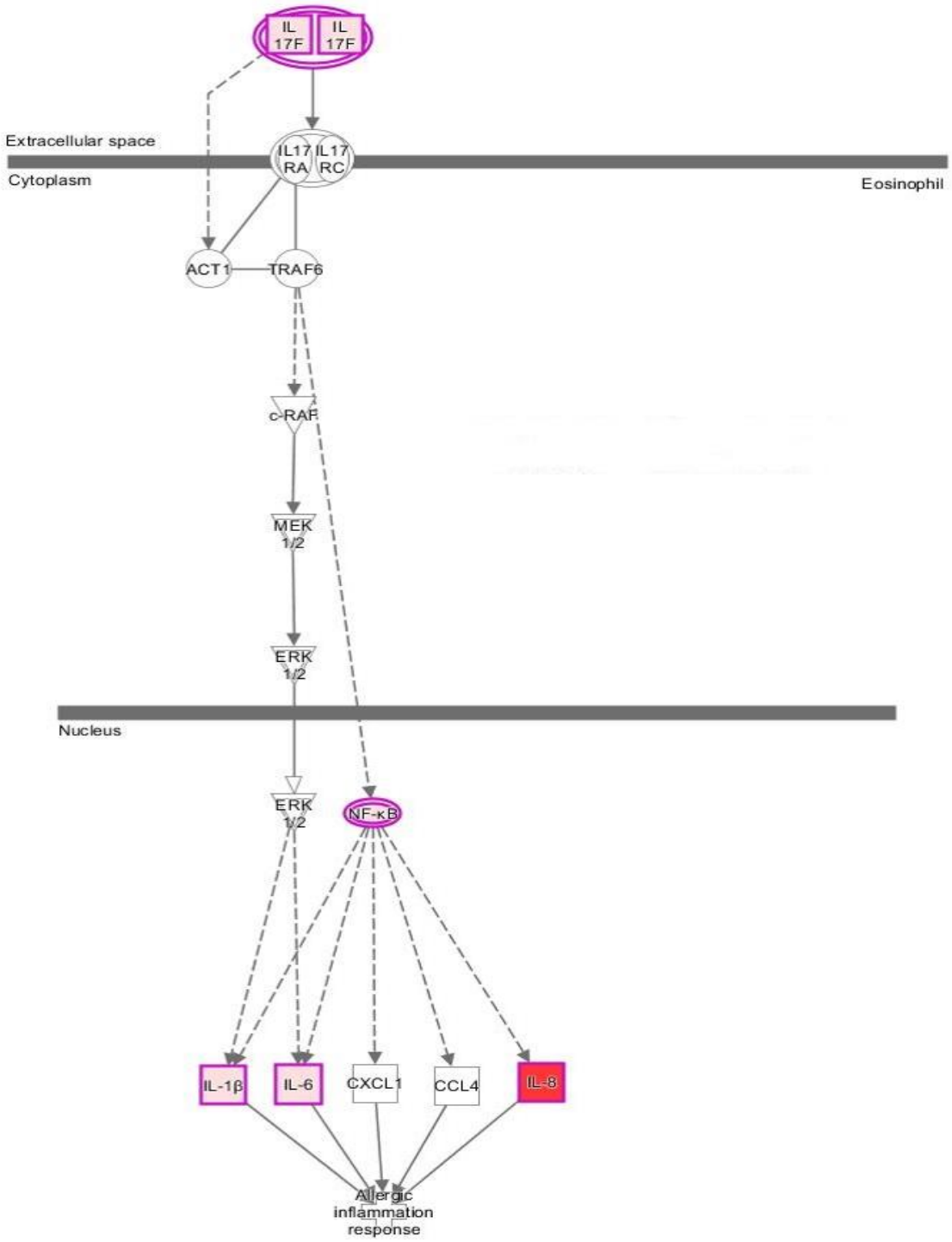

Figure 4. 6. IPA canonical pathway depicting relationships among genes associated with the pathway, "Role of IL-17F in Allergic Inflammation" expressed in pigs infested with *S. scabiei*. The differentially expressed genes included are those which showed ≥ ± 2 fold change in expression with $p \leq 0.05$ in mite infested relative to non-infested pigs. Nodes coloured by gene expression with red (strong) and pink nodes representing up-regulated genes and white indicating genes not differentially expressed but with defined relationship to other genes in the pathway. Pink circles and squares highlight roles for IL-17F, NFκB1, IL-1β, IL-6 and IL-8.

IL-17 is a potent inducer of chemokine ligand CXCL5 (Liu et al., 2011; Ruddy et al., 2004) which upon interacting with its receptor CXCR2, up-regulated here at week 8, stimulates the chemotaxis of neutrophils and recruits them to the site of the inflammation. IL-17 induces another important effector molecule CCL20, up-regulated at 8wpi, involved in the recruitment of the proinflammatory IL-17 producing helper T-cells (Th17) and dendritic cells from blood into inflamed cutaneous tissue and amplifies the cycle of inflammation in psoriasis (Homey et al., 2000; Le Borgne et al., 2006). This up-regulation of potent Th17 cell chemoattractant CCL20, T helper cell marker CD4, Th17 cell differentiation factors TGFβ, IL1β, IL6 and IL17 are indicative of involvement of Th17 cells in scabies. Furthermore, it is suggested (Mudigonda et al., 2012) that IL-17 secretion and further cytokine secretion from the influx of neutrophils, DCs and Th17 cells impact local keratinocytes and leukocytes engaging them in the progression of psoriasis which leads to chronic inflammation. Clinical manifestations in severe form of scabies (CS) such as epidermal hyper proliferation are similar to psoriasis and it seems IL-17 plays a role in the pathogenesis of skin chronic inflammation in scabies.

IL-17 induces expression of granzyme B, which was also up-regulated at week 8. Granzyme B induces and mediates inflammation by stimulating release of pro-inflammatory cytokines IL-1α, IL-1β, IL-18, IL-6 and IL-8 from NK cells (Hiebert and Granville, 2012), all except IL18 are up-regulated at this time point. Granzyme B also possess potent extracellular matrix remodelling activity and can degrade proteins such as fibronectin, laminin in the extracellular matrix resulting in disruption of dermal-epidermal junction and also can degrade decorin resulting in collagen disorganisation (Buzza et al., 2005; Hiebert and Granville, 2012). Granzyme B induced inflammation, lymphocyte migration, extracellular matrix destabilisation and remodelling, and tissue destruction has been linked to allergic diseases such as asthma, contact dermatitis, rheumatoid arthritis and atherosclerosis (Buzza et al.,

2005; Hiebert and Granville, 2012; Tschopp et al., 2006). Up-regulation of granzyme B later in the infestation may contribute to disease pathogenesis in scabies through cytotoxic and extracellular mechanisms.

4. 4. 8. Miscellaneous – other important genes

Other notable differentially expressed genes in this analysis included ARG1, ARG2 AREG, FCER1A and FCGR2B.

We detected strong up-regulation of Arginase 1 (ARG1) and moderate up-regulation of ARG2 late in the infestation. Increased expression of ARG1 has been shown in human asthma patients (North et al., 2009; Salam et al., 2009) and in murine models of ovalbumin-induced airway inflammation (North et al., 2009). Genetic variations in ARG2 have been shown to be associated with childhood allergic asthma risk (Li et al., 2006). Microarray analysis have shown Th2 cytokines IL-4 and IL-13 induce both ARG1 and ARG2 (Zimmermann et al., 2003). The ARG1 and ARG2 expression induced by Th2 cytokines represents another additional pathway which might be related to the inflammatory and allergic response associated pathogenesis in scabies especially in CS cases.

Amphiregulin (AREG) was down-regulated early in the infestation (at week 2). AREG is a member of the epidermal growth factor family, promotes growth of normal epithelial cells and epidermal keratinocytes (Piepkorn et al., 1998). AREG expression is associated with psoriasis-like skin phenotype, neutrophil transmigration (Chung et al., 2005; Cook et al., 1997) and airway remodelling in asthma (Enomoto et al., 2009). It is suggested that AREG possess innate immunogenic or pro-inflammatory capabilities and can stimulate both psoriatic epidermal hyperproliferation and psoriatic inflammation (Chung et al., 2005). In CS cases, disease signs and skin features are similar to as observed in Psoriasis. This AREG down-regulation early in the infestation is another way of explaining why the diseases

manifestation (skin lesions and crusts in CS cases) and skin inflammation are not seen early in the scabies.

Matrix metalloproteinases (MMPs) play roles in the degradation of extracellular matrix and regulatory proteins, immune cell recruitment, and regulate the inflammatory activity of serine proteases, cytokines and chemokines. They are mediators of the pathogenesis of a range of inflammatory and allergic diseases (Kelly and Jarjour, 2003; Le et al., 2007; Van Lint and Libert, 2007). In this analysis, many MMPs were strongly expressed late in the infestation (8wpi) and their expression may influence the progression of various inflammatory processes in scabies.

The results also show up-regulation of high-affinity Fc fragment of IgE receptor Ia (FCER1A, at week 8) which is involved in antibody dependent responses and initiates allergic and inflammatory responses (Sallmann et al., 2011; Semper et al., 2003). It plays key roles in allergic diseases by coupling allergen receptor-bound IgE with mast cells leading to cell activation. This results in the release of inflammatory mediators (histamine) responsible for the manifestations of allergy and secretion of lymphokines, furthering the development of the local inflammatory reaction (Nimmerjahn and Ravetch, 2007). This binding of IgE to FcεRI represents an additional pathway which may trigger the inflammation and be also responsible for the increased IgE responses observed in scabies.

Also, FCGR2B gene expression was detected late in the infestation (week 8). FCGR2B encodes Fc gamma receptor IIb (FcγRIIb), an IgG receptor expressed on various immune cells with an important role in immune regulation. FcγRIIb is involved in the regulation of phagocytosis of immune complexes, cell proliferation, inflammatory cytokine release and, antibody production by B-cells. Polymorphisms/variations in FCGR2B gene are linked with increased susceptibility to skin inflammatory disorder SLE and may contribute to the disease histopathogenesis (Niederer et al., 2010; Tsang et al., 2016; Willcocks et al., 2010). FCGR2B

gene polymorphisms may lead to altered FcγRIIb function resulting in dysregulated FcγR-mediated clearance of immune complexes and control of inflammatory responses which may play a role in the disease development and may be a mechanism in the pathogenesis of scabies.

Skin inflammation and lesions usually appear late in primary mite infestation, as was observed in infested pigs in this study and it is assumed that early in the infestation, the mites may be able to modulate/regulate the immune responses. The results indicate that early in the infestation, the down-regulation may be due to mites secreting factors which modulate gene expression allowing them to establish and grow. Also, this down-regulation of genes by the mites may be the reason that the clinical signs are not seen early in the scabies.
On the other hand, the increased mite numbers at later time points (especially in CS animals) should also be able down-regulate but it seems they are overwhelmed by the amount of response mounted by the host. This might be due to fact that increased number of mites provide larger amount of antigenic material and hence increased immune and inflammatory responses which overcome the inhibitory influence and might be responsible for the severe pathology seen in CS cases.

There were limitations to the design and execution of this study. In this analysis, sample number was small as it is costly to source a large number of piglets and sampling is logistically challenging, despite this our PCA plots demonstrated that samples clustered strongly according to clinical phenotype.

In this current analysis, some of the findings may be conflicting due to the combining of crusted and ordinary scabies samples as we wanted to obtain an overall picture of host responses to mite infestation. For example, expression of various genes including IL4, IL4R, IL6, calcium biding proteins S100s, DCT and chemokines CCL20 and CCL27 was detected which seem to be related to CS as they are expressed and play an important role in the

pathogenesis of inflammatory skin diseases psoriasis and atopic dermatitis, and allergic disorders. These genes are involved in various pathological processes – Th2 immune and allergic response, keratinocyte differentiation, skin depigmentation, cutaneous inflammation, skin crust formation and scaling, and infiltration of immune effector cells - DCs, neutrophils, macrophages and T cells. Psoriasis and atopic dermatitis are clinically more similar to CS and these features have been observed in CS cases. However, the differences in gene expression profiles between crusted and ordinary scabies will be described in the next chapter.

This analysis was carried out on a cohort of pigs with mange (OS and CS) who were not treated with dexamethasone. An advantage of this approach is that, since the objective was to characterize patterns of gene expression associated with immune and inflammatory responses in pigs, the potential confounding effects of dexamethasone on gene expression were minimized.

4. 5. Conclusion

This study is the first comprehensive transcriptomic profiling of host skin responses to infestation with *S. scabiei*. The analysis has demonstrated a large number of genes in skin are differentially expressed in response to burrowing *S. scabiei* mites. The network/pathway analysis has allowed identification of distinct patterns of gene expression in host responses to *S. scabiei* infestation and confirmed and extended also previous findings which accounted for distinct immune and inflammatory events in scabies. This study has provided information of the mechanisms by which mites and mite-derived factors induce/invoke a pro-inflammatory reaction in skin. The initial interactions of the mite antigens with the host skin APCs leads to expression of cytokines, chemokines and adhesion molecules and to activation and trafficking of immune cells – DCs, neutrophils, macrophages, T cells and NK cells to the site of infestation. Our data revealed that the cellular feature of the skin immune and inflammatory

responses in scabies include the infiltration of neutrophils which initiates a mixed Th response as reflected by the up-regulation of various genes associated with these cell type. The findings from this analysis suggest that these cell types play a major role in initiating the innate and adaptive responses contributing to scabies induced skin pathology. Activation of dermal DCs and antigen presentation leads to T cell activation and differentiation and these events drive the immune response towards an allergic Th2 type profile, involving T-cells, B-cells and IgE production with further production of proinflammatory molecules. The subsequent secretion of cytokines and chemokines results in further leukocyte migration and increases vascular permeability which further amplifies the ongoing inflammation, worsening the disease symptoms of inflamed skin and lesions. The study has provided valuable information into the mechanism by which *S. scabiei* supresses the host response earlier in the infection and invokes such a profound inflammatory and allergic responses later in the infestation. It has also highlighted the similarities in host response to a number of similar diseases such as psoriasis and atopic dermatitis and also supported by the histopathological similarities in skin lesions. This identification of distinct patterns of gene expression in scabies will be helpful for the identification of novel methods of disease control and to design specific molecular interventions.

Chapter 5

Gene expression profiling of immune and inflammatory responses in crusted versus ordinary scabies.

5. 1. Introduction

In scabies, a wide range of clinical features are recognised, from the mild type in Ordinary scabies (OS) to rare and severe destructive type in Crusted or Norwegian scabies (CS). In OS, the host skin has a low parasite burden (less than 20 mites). Ordinary scabies cases show an allergic type reaction with skin lesions and intense generalised pruritus (Mellanby, 1944; Wendel and Rompalo, 2002). In contrast, in CS hyper infestation of mites (thousands per gram of skin) is seen and CS patients develop hyperkeratotic thick and scaly skin crusts (Walton et al., 2010). The extremely high mite burden in CS makes it more infectious, and there is a risk of serious secondary bacterial infections. Re-infestations are common in CS cases whereas in OS mite numbers reduce with repeat infestation suggesting development of protective immunity (Hay et al., 2012).

Crusted scabies is caused by the same variant of *Sarcoptes scabiei* mites as those causing OS (Walton et al., 1997) indicating that increased mite virulence does not cause CS. Crusted scabies is relatively rare and it is not yet known what factors lead to the disease development. It is observed that immunosuppression is a predisposing factor associated with CS as it has been demonstrated in those with human immunodeficiency virus (Hulbert and Larsen, 1992), human T-lymphocytic virus 1 infection (Einsiedel et al., 2014; Roberts et al., 2005), in individuals undergoing organ transplantation (Gregorini et al., 2012; Youshock and Glazer, 1981) and also can be induced by corticosteroids (Bilan et al., 2015; Binic et al., 2010;

Marliere et al., 1999). Furthermore, CS has been reported in cases of leprosy (Roberts et al., 2005) and developmental disability, including Down syndrome (Hay et al., 2012). Notably CS is also observed in patients (with 42% of the cases involved in one study) with no identified risk factor or immunodeficiency (Roberts et al., 2005). From these reports, it seems that the susceptibility of this cohort to CS may be due to immune suppression and/or dysregulation, the nature of which is not yet clear.

Different host immune responses have been observed in OS and CS. In OS, the immune response is reported as dominated by a Th1-type cytokine profile – interferon (IFN) γ and tumour necrosis factor (TNF) α, and $CD4^+$ T cells as most prevalent T lymphocytes in the skin (Abd El-Aal et al., 2016; Falk and Eide, 1981; Falk and Matre, 1982; Walton et al., 2010). In contrast, immune response in CS resembles to non-protective T helper (Th) 2 allergic response with elevated Th2 and Th17 cytokine profile interleukin (IL) 4, IL-5, IL-13 and IL-17, immunoglobin (Ig) E levels, and $CD8^+$ T lymphocytes being the predominant effector cells in the skin (Liu et al., 2014b; Mounsey et al., 2015; Walton, 2010; Walton et al., 2008; Walton et al., 2010). The reasons for these distinct host cytokine responses in CS and OS, and the role of $CD4^+$ and $CD8^+$ T cells in the skin are not yet known and the key regulators responsible for such differences in immune responses are yet to be identified.

The observations of host responses in CS so far are collated from limited humoral and cellular studies, with results from human studies limited by the fact that patients often present at an advanced stage of infestation. To gain insights into the immune events leading to this extreme clinical manifestation, analysis of early infestation is required. Patients with crusted scabies also frequently present with co-morbidities which confound the interpretation of study validity. Furthermore, the availability of limited clinical samples and inconsistencies in the assessment of disease severity may further influence the validity of these results (Arlian et al., 1994b; Roberts et al., 2005).

In the first transcriptomic analysis (described in Chapter 4) comparing *S. scabiei* infested with non-infested pigs, we found that early in the infestation, the mites appear to suppress host responses by down-regulating expression of an array of immune and inflammatory genes. In contrast, late in the infestation when the clinical manifestations become evident, the mange positive pigs showed an elevated expression of a large number of inflammatory and allergic factors indicative of a mix of Th1, Th2 and Th17 profiles.

In this analysis, gene expression profiles were compared between pigs with CS and OS, with the hypothesis that these clinical manifestations will be reflected by dramatic differences in gene expression within these hosts. Also the aim was to study gene expression prior to infestation to gain insights into any underlying susceptibility in the absence of infestation and to possibly identify the specific immune factors predisposing the hosts to CS. These early events may provide information about the key regulators in disease progression and development, and these might be used as potential markers for early diagnosis of CS. To better understand the immune, inflammatory and allergic responses we aim to identify further the signaling pathways and networks involved, and the mechanisms behind Th2 allergic and IL-17 responses in CS.

5. 2. Materials and methods

The analysis methods were the same as described in Chapter 3. The detailed description of clinical phenotypes, total RNA extraction and microarray analysis are given in Chapter 3. In this analysis, pig (4 OS and 4 CS) samples were selected randomly from the microarray study.

Within the Partek Genomics Suite, a two-way Analysis of variance (ANOVA) combined with a Fisher's Least Significant Difference (LSD) post-hoc test, was used to compare expression profiles between CS and OS samples at time points zero week pre-infestation, one, two, four

and eight weeks post-infestation (wpi) and lists of significantly differentially expressed genes (DEGs) were generated.

The gene lists were then analysed within the Ingenuity Pathway Analysis (IPA) software to identify the signaling pathways and gene networks associated with the datasets.

Using the Venny (BioinfoGP Service) software a comparative analysis of DEGs of CS vs C and OS vs C comparisons at time points 1 and 8wpi was carried out to elucidate shared and unique genes in CS and OS pigs.

5. 3. Results

Results of total RNA extraction and clinical mange progression are provided in chapter 4. From the experimentally mite infested group total RNA samples from four pigs with CS (lesion score > 4 at more than one time point) and four with OS (lesion score < 4) were used for this analysis.

5. 3. 1. Determination of differentially expressed genes

Here we compared CS with OS samples at time points 0 week, 1, 2, 4 and 8wpi. In Partek, principal component analysis (PCA) showed that CS and OS samples did not group with each other at any time point during the infestation, indicating differences in gene expression between the two groups. Hierarchical clustering of data was in agreement with the PCA showing differential expression of genes in CS compared to OS samples at each time point. Figure 5. 1 shows the scatter plots from PCA while Figure 5. 2 shows the heat maps from hierarchical clustering mapping of CS versus OS samples at 1wpi and 8wpi.

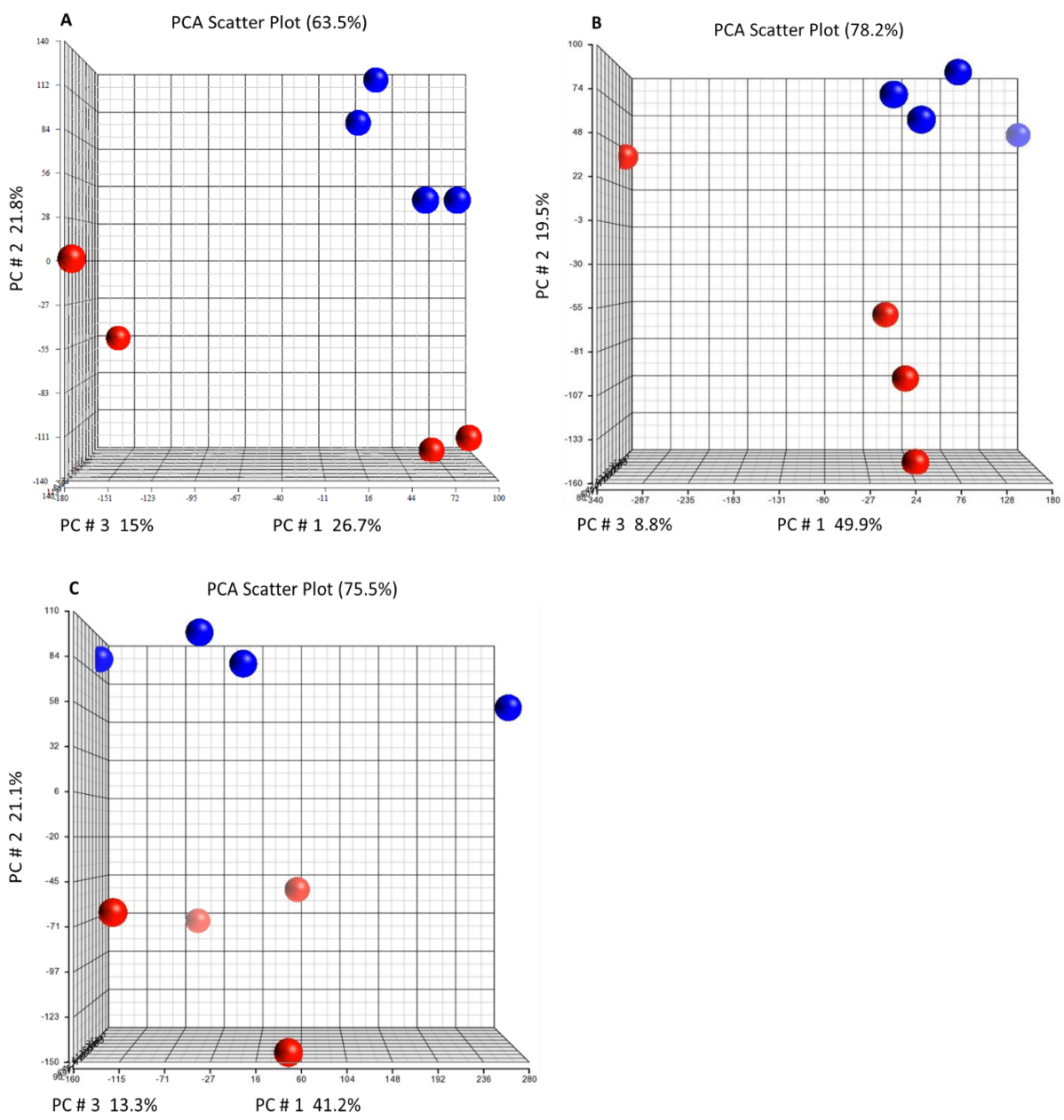

Figure 5. 1. Principal component analysis (PCA) plot comparing gene expression in CS (n=4) with OS (n=4) samples. Samples from week 0 pre-infestation (A), and week 1 (B) and week 8 (C) following *S. scabiei* infestation are plotted. PCA demonstrated that the CS samples grouped separately to OS samples. Each circle represents 1 sample. Red circles indicate CS samples and blue indicate OS samples.

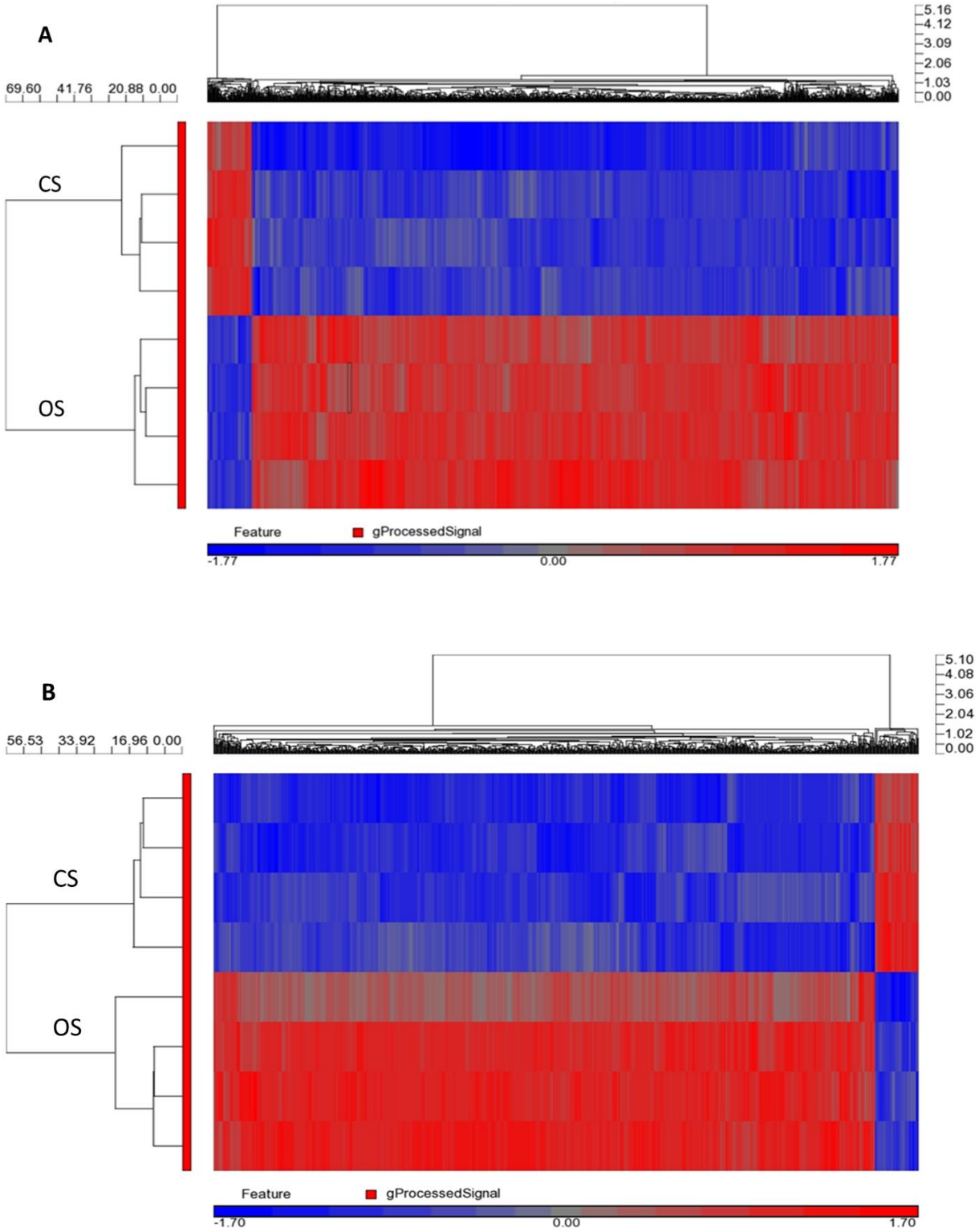

Figure 5. 2. Hierarchical clustering heat map of differentially expressed genes in CS (n=4) relative to OS (n=4) samples. Samples from week 1 (A) and week 8 (B) following *S. scabiei* infestation were mapped. Rows represent samples and column represents a gene/probe. Relative gene expression is colour represented: red indicates higher level expression, blue indicates lower-level expression, and grey indicates no change.

Two-way ANOVA combined with a Fisher's Least Significant Difference (LSD) post-hoc test analysis showed a large number of genes were differentially expressed at each time point of the study except for week 4 (Table 5. 1).

Table 5. 1. Number of differentially expressed genes (DEGs) following infestation with *S. scabiei* in pigs. In Partek, gene expression was carried out with 2-way ANOVA combined with a Fisher's Least Significant Difference (LSD) post-hoc test in CS (n=4) relative to OS (n=4) samples at time points 0 week pre-infestation, 1, 2, 4 and 8 weeks post-infestation (wpi). Number of up (↑) or down (↓) regulated genes with a p-value of ≤ 0.05 and fold change of ≥ ± 2.0 at each time point are indicated.

Time	Partek analysis		
	Number of DEGs	**Up-regulated ↑**	**Down-regulated ↓**
Pre-infestation (week 0)	1319	625	693
1wpi	1327	101	1226
2wpi	1673	199	1474
4wpi	264	262	2
8wpi	1314	263	1051

At week 0, a difference of 68 genes was seen between up-regulated (625) and down-regulated (693) gene probes. Notably, at weeks 1, 2 and 8 a trend of strong down-regulation was observed in CS as up to 80% of all differentially expressed genes (DEGs) were down-regulated at these time points. At 4wpi least number of genes (264) were differentially expressed and a different trend as compared to other time points was observed with almost all (262) genes up-regulated and only two genes down-regulated. Overall, post-mite infestation at each time point except at week 4, a different trend to pre-infestation (at week 0) expression was observed with most of the genes being down-regulated.

5. 3. 2. Venn diagram analysis

A comparative analysis of DEGs of CS vs C and OS vs C comparisons at time points 1 and 8wpi was performed to identify conserved/shared and unique/exclusive transcripts in CS and OS pigs. The corresponding Venn diagrams are shown in Figure 5. 3. The overlapping regions represent the number of conserved/shared DEGs, whereas the non-overlapping regions represent the number of unique/exclusive DEGs.

In this analysis, 946 (230 up and 716 down-regulated) and 688 (505 up and 183 down-regulated) genes exclusively detected in CS compared to OS and C pigs at 1 and 8wpi respectively.

At 1wpi 83% (716) of genes were exclusively found down-regulated in CS compared to OS and C pigs. On the contrary only 22 % (230) genes were exclusively up-regulated in CS at this time point. At 8wpi, a different trend was observed in CS pigs with more number of genes being up-regulated exclusively than at 1wpi.

Comparative analysis results were in line with the Partek results revealing an array of pro-inflammatory mediators – cytokines, chemokines, complement components, cell surface receptors, signaling molecules, growth and transcription factors, etc. exclusively expressed (either up or down regulated) in CS pigs. Some of the notable genes up-regulated at 8wpi included IL17A, IL13, IL19, C-C motif chemokine ligand (CCL) 17, C-X-C motif chemokine ligand (CXCL) 2, CXCL6, toll-like receptor (TLR) 2, and TLR3. CD70, TLR9, CD5L, IL19 and HLA-DOB were the prominent down-regulated genes early (at week 1) in the infestation in CS pigs.

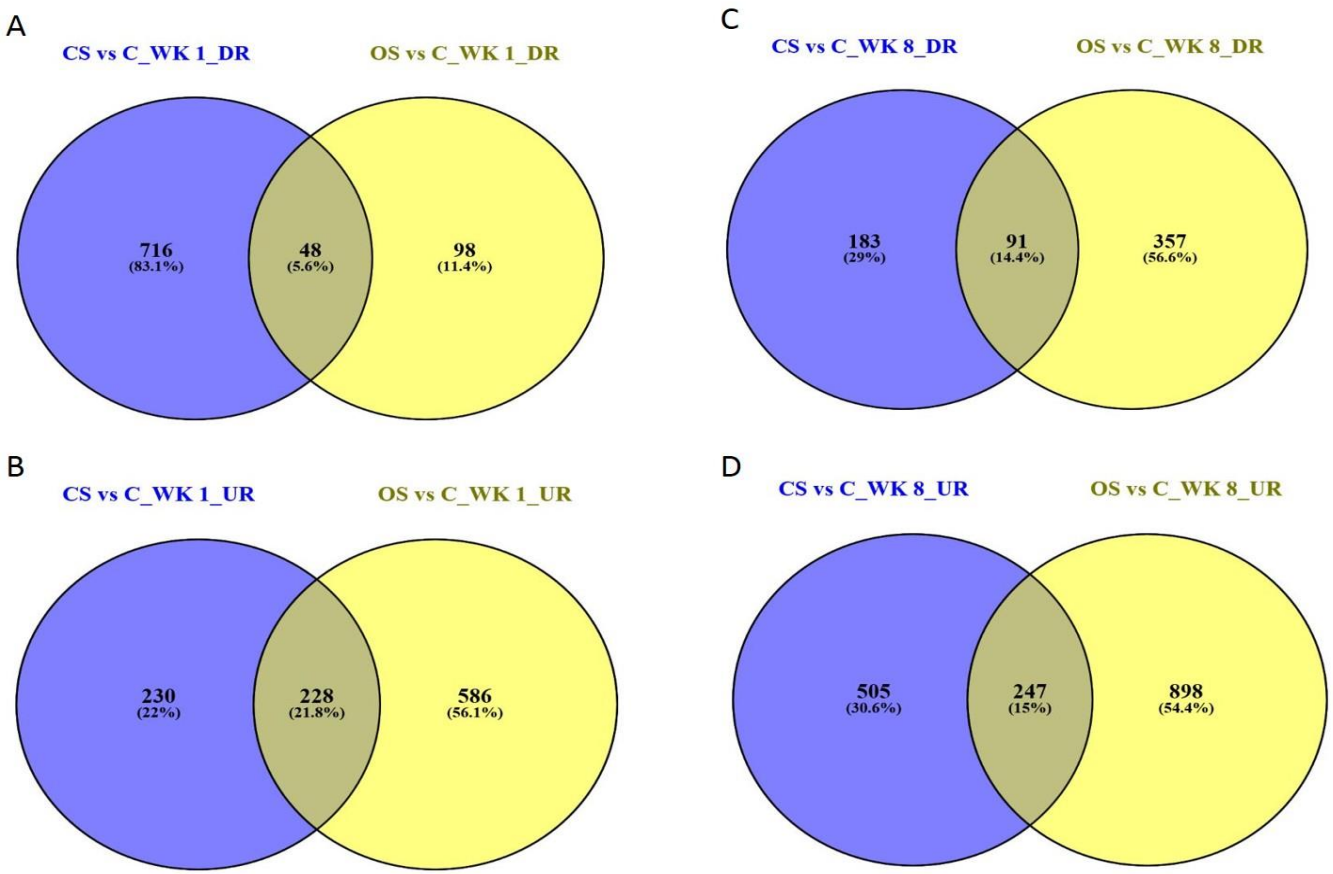

Figure 5. 3. Venn diagrams showing common/overlapping and unique genes between CS vs C and OS vs C comparisons. Up and down regulated genes with a p-value of ≤ 0.05 and fold change of $\geq \pm 2$ were compared at 1 and 8 weeks post-infestation. A: week 1 down regulated genes, B: week 1 up regulated genes; C: week 8 down regulated genes and D: week 8 up regulated genes.

*CS = crusted scabies, OS = ordinary scabies and C = control. 1 = week 1 and 8 = week 8. DR = down-regulated, UR = up-regulated. Blue colour represents CS vs C and yellow colour indicates OS vs C.

5. 3. 3. Gene Network/Pathway analysis of host response to *S. scabiei* infestation in CS versus OS pigs.

Initial analysis of Partek and IPA dataset showed that mite infestation in CS compared to OS animals resulted in the differential expression of an array of immune and proinflammatory mediators – cytokines, chemokines, transcription factors, cell receptors, signaling molecules, enzymes, acute phase and S100A calcium binding proteins over the time course of the infestation (appendix F Supplementary Table 3). The top ten genes with the highest fold change from each of the 5 time points of the analysis are shown in Table 5. 2.

In IPA, pathway analysis showed the DEGs were associated with a number of important signaling pathways over the time course of the infestation. The notable pathways included "Acute Phase Response Signaling", "T Cell Receptor Signaling", "NF-κB Signaling", "IL-12 Signaling and Production in Macrophages", "Th1 and Th2 Activation Pathway", "Role of IL-17A in Psoriasis" and "Atherosclerosis Signaling". The top five canonical signaling pathways associated with each time point are represented in Table 5. 3.

In addition, IPA analysis revealed that the DEGs affected diverse molecular functions and biological processes. The notable ones included "Cellular Growth and Proliferation", "Cellular Movement", "Cell-to-Cell Signaling", "Gene Expression", "Cell-mediated Immune Response", and "Inflammatory and Allergic Response". The DEGs were also associated with various diseases and the prominent ones were "Immunological Disease", "Inflammatory Disease", "Haematological Disease", "Organismal Injury and Abnormalities", and "Connective Tissue Disorders". Further details of gene expression profiles and IPA results are provided in the following sections.

Table 5. 2. Top 10 high fold changing differently expressed genes (DEGs) following infestation with *S. scabiei* in pigs. The DEGs included are those which showed ≥ ± 2 fold change in expression with $p \leq 0.05$ in CS relative to OS pigs at time points week 0 (pre-infestation), 1, 2, 4 and 8 weeks post-infestation (wpi). + is up-regulated and - is down-regulated.

Time	Gene Symbol	Gene Description	Fold Change
Pre-infestation (Week 0)	NAMPT	Nicotinamide phosphoribosyl transferase	50.36
	SEC14L2	SEC14 like lipid binding 2	50.02
	PLA2R1	Phospholipase A2 receptor 1	49.34
	ARPC5L	Actin related protein 2/3 complex subunit 5 like	37.77
	NPFFR1	Neuropeptide FF receptor 1	36.45
	RPS29	Ribosomal protein S29	-23.23
	PDCD7	Programmed cell death 7	-22.9
	ZC3H15	Zinc finger CCCH-type containing 15	-22.38
	TDP1	Tyrosyl-DNA phosphodiesterase 1	-21.4
	PACSIN2	Protein kinase C and casein kinase substrate in neurons 2	-20.97
1wpi	ACTN2	Actinin Alpha 2	164.42
	TLR8	Toll like receptor 8	44.47
	ZKSCAN5	Zinc finger with KRAB and SCAN domains 5	40.9
	CRYGD	Crystalline gamma D	36.38
	KIAA1468	KIAA1468	32.26
	DCT	Dopachrome tautomerase	-33.75
	GTF3C3	General transcription factor IIIC subunit 3	-29.57
	ADIPOQ	Adiponectin, C1Q and collagen domain containing	-24.3
	CTTNBP2NL	CTTNBP2 N-terminal like	-18.06
	RPL8	Ribosomal protein L8	-15.2
2wpi	DCT	Dopachrome tautomerase	18.1
	FAAH	Fatty acid amide hydrolase	15.2
	CYB5B	Cytochrome B5 type B	11.9
	NMUR1	Neuromedin U receptor 1	9.08
	RBM38	RNA binding motif protein 38	8.89
	ANXA2	Annexin A2	-17.41
	APP	Amyloid beta precursor protein	-16.87
	PLA2R1	Phospholipase A2 receptor 1	-16.72
	HOXB13	Homeobox B13	-15.51
	NAMPT	Nicotinamide phosphoribosyl transferase	-15.32
4wpi	SCGB1A1	Secretoglobin family 1A member 1	62.13
	GLRX3	Glutaredoxin 3	21.08
	WDR3	WD repeat domain 3	15.12
	CBX3	Chromobox 3	13.66
	RFX5	Regulatory factor X5	11.7
	PNLIPRP1	Pancreatic lipase related protein 1	-3.73
	FAM63B	Family with sequence similarity 63 member B	-3.01

8wpi	CXCL6	C-X-C motif chemokine ligand 6	64.32
	ARG1	Arginase 1	30.79
	ADGRF1	Adhesion G protein-coupled receptor F1	25.38
	S100A7	S100 calcium binding protein A7	21.52
	S100A9	S100 calcium binding protein A9	21.08
	PACSIN2	Protein kinase C and casein kinase substrate in neurons 2	-12.84
	CARMIL1	Capping protein regulator and myosin 1 linker 1	-12.67
	ADSS	Adenylosuccinate synthase	-10.7
	MFSD6	Major facilitator superfamily domain containing 6	-10.17
	CAPZA3	Capping actin protein of muscle Z-line alpha subunit 3	-9.73

5. 3. 4. Gene expression pre-infestation

A large number of genes were differentially expressed prior to experimental mite challenge at week 0 (Table 5. 1 and appendix F Supplementary Table 3) suggesting inherent differences possibly predisposing pigs to the development of crusted or ordinary scabies.

Genes up-regulated at week 0, in pigs which after mite infection developed CS, included acute phase response proteins, immunomodulatory molecules, chemokines, inflammatory mediators, complement components and innate immunity related genes (appendix F Supplementary Table 3). The prominent genes included haptoglobin (HP, 2.57 fold up), transferrin (TF, 9.67 fold up), forkhead box protein P3 (FOXP3, 3.25 fold up), CCR10 (4.03 fold up), oncostatin M (OSM, 13.78 fold up), nuclear factor kappa B p100 subunit (NFκB2, 2.54 fold up), IFNγ (7.46 fold up), complement component (C) 6 (6.78 fold up), signal transducer and activator of transcription (STAT) 3 (4.58 fold up) and interleukin-1 receptor-associated kinase 1 (IRAK1, 3.04 fold up). Week 0 also showed down-regulation of various key pro-inflammatory response related molecules including IL17F (-4.26 fold down), TNF (-3.36 fold down), STAT4 (-4.1 fold down), T-cell surface glycoprotein CD8 alpha chain (CD8α, -3.41 fold down), myeloid differentiation primary response protein (MYD88, -6.65 fold down) and transcription factor p65 (RELA, -2.36 fold down).

Table 5. 3. Top 5 (+5 immunity related) canonical signaling pathways from the IPA mapping at each time point following infestation with *S. scabiei* in CS vs OS pigs. # = number and Wpi = weeks post infestation.

Time	Canonical Pathways	# Genes in pathway /total # of genes	p-value
Pre-infestation (Week 0)	Acute Phase Response Signaling	28/169	8.26E-07
	FXR/RXR Activation	23/126	1.40E-06
	LXR/RXR Activation	21/121	9.04E-06
	AMPK Signaling	28/189	7.79E-06
	IL-12 Signaling and Production in Macrophages	24/146	5.66E-06
	Immunity related		
	MIF Regulation of Innate Immunity	10/41	1.13E-04
	iNOS Signaling	10/44	2.12E-04
	Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	24/193	5.23E-04
	Atherosclerosis Signaling	18/127	5.40E-04
1wpi	Axonal Guidance Signaling	54/450	1.13E-06
	Adipogenesis pathway	23/134	5.08E-06
	Thyroid Cancer Signaling	11/40	1.69E-05
	PPAR/RXR Activation	25/178	7.02E-05
	Human Embryonic Stem Cell Pluripotency	21/143	1.37E-04
	Immunity related		
	Role of Osteoblasts, Osteoclasts and Chondrocytes in Rheumatoid Arthritis	28/232	3.74E-04
	T Cell Receptor Signaling	16/109	8.28E-04
	STAT3 Pathway	12/73	1.33E-03
	PKCθ Signaling in T Lymphocytes	17/132	2.50E-03
	NF-κB Signaling	21/180	2.89E-03
2wpi	Acute Phase Response Signaling	38/169	9.49E-10
	G-Protein Coupled Receptor Signaling	43/272	3.42E-06
	cAMP-mediated Signaling	36/223	1.30E-05
	FXR/RXR Activation	23/126	6.80E-05
	TR/RXR Activation	19/98	1.24E-04
	Immunity related		
	IL-12 Signaling and Production in Macrophages	24/146	2.58E-04
	Role of MAPK Signaling in the Pathogenesis of Influenza	15/72	2.76E-04
	STAT3 Pathway	15/73	3.23E-04
	iNOS Signaling	10/44	1.40E-03
	Role of JAK family kinases in IL-6-type Cytokine Signaling	7/25	2.03E-03
4wpi	Nur77 Signaling in T Lymphocytes	6/59	8.03E-05
	Cytotoxic T Lymphocyte-mediated Apoptosis of Target Cells	4/32	5.88E-04
	Granzyme B Signaling	3/16	8.86E-04

	Mitochondrial Dysfunction	8/171	1.20E-03
	cAMP-mediated Signaling	9/223	1.70E-03
	Immunity related		
	iNOS Signaling	4/44	1.98E-03
	CTLA4 Signaling in Cytotoxic T Lymphocytes	5/99	7.25E-03
8wpi	LXR/RXR Activation	25/121	4.08E-08
	Aryl Hydrocarbon Receptor Signaling	25/140	7.71E-07
	Role of IL-17A in Psoriasis	7/13	3.31E-06
	T Cell Receptor Signaling	20/109	6.25E-06
	Glucocorticoid Receptor Signaling	37/287	8.55E-06
	Immunity related		
	Regulation of IL-2 Expression in Activated and Anergic T Lymphocytes	16/79	1.43E-05
	CD28 Signaling in T Helper Cells	20/131	9.83E-05
	IL-8 Signaling	26/197	1.21E-04

The differentially expressed genes in pigs at week 0 that went on to develop crusted scabies were associated with various biological functions and the notable ones include “Cell-To-Cell Signaling and Interaction”, “Organismal Injury and Abnormalities”, and “Cell-mediated Immune Response”.

In pathway analysis, the top canonical pathways associated with the dataset were, “Acute Phase Response Signaling”, “FXR/RXR Activation” and “AMPK Signaling”. Some of the genes expressed at week 0 were then differentially expressed over the infested period including HP, TF, IRAK1, CCR10, STAT3 and STAT4 (at 2wpi), RELA (at 8wpi), FOXP3 and MYD88 (at 1 and 2wpi), OSM and IFNγ (at 2 and 8wpi), NFκB2 (at 1, 2 and 8wpi).

5. 3. 5. Gene expression post mite infestation

In this analysis, exclusive expression of a number of genes (Table 5. 4) compared to the first analysis (scabies versus control animals, described in Chapter 4) was detected.

Table 5. 4. Differentially expressed genes in Crusted vs Ordinary Scabies pigs upon infestation with *Sarcoptes scabiei*. Genes that differed significantly in expression (either up or down regulated) levels upon mite infestation over the time course of first 8 weeks. These genes had a FDR corrected p-value of ≤ 0.05 and fold change of ≥ ± 2.0. Genes detected overlapping and non-overlapping in CS vs OS compared to CS and OS vs C analysis are indicated. FC = Fold change.

Gene	Gene Description	Infested vs Non-infested				Crusted vs Ordinary Scabies (CS vs OS)			
		Early gene expression		Late gene expression		Early gene expression		Late gene expression	
		Week 1 FC	Week 2 FC	Week 4 FC	Week 8 FC	Week 1 FC	Week 2 FC	Week 4 FC	Week 8 FC
	Pro-inflammatory molecules								
IFNα4	Interferon alpha 4						-2.98		
IFNγ	Interferon gamma	2.26					-4.86		2.19
IL1RL1	Interleukin 1 receptor like 1				2.14				
IL1α	Interleukin 1 alpha				2.61				
IL1β	Interleukin 1 beta				5.65	-6.01			
IL12β	Interleukin 12 subunit beta						-2.43		
IL12Rβ1	Interleukin 12 receptor subunit beta 1								2.3
IL12Rβ2	Interleukin 12 receptor subunit beta 2						-2.75		
IL13	Interleukin 13								2.35
IL17A	Interleukin 17A								4.67
IL17F	Interleukin 17F			2.74	3.34				
IL17RB	Interleukin 17 receptor B		-2.7						4.89
IL19	Interleukin 19					-7.51			7.89
IL20	Interleukin 20				2.82				9.05
OSM	Oncostatin M	3.31					-6.67		3.42
	Immunoregulatory molecules								
FOXP3	Forkhead Box P3	3.87			2.11	3.11	-3.85		
IL27	Interleukin 27	3.02					-5.91		
TGFβ1	Transforming growth factor beta 1				2.47	-4.31			2.17
	Complement components								
C3	Complement C3			2.48	6.45	-2.08	-2.36		-2.63
C4A	Complement component 4A						-6.47		2.38
CFD	Complement factor D						-2.43		
C6	Complement C6	3.27	2.68	-3.07	-2.3	5.01	-9.12		
	Cell surface receptors and ligands								
CD3ε	CD3 molecule, epsilon				2.23				3.43
CD3γ	CD3 gamma molecule		3.02		3.42	20.56		8.86	3.57

CD5L	CD5 antigen-like		-2.44			-6.92	-4.97		
CD40L	CD40 ligand								3.58
CD274	CD274 molecule	-3.74	-2.59	-3.01	2.93	-5.02	4.08		
CD82	CD82 molecule					-3.1			
CD70	CD70 molecule	-3.49	-2.78			-8.21	3.24		
CD8α	CD8 alpha molecule	-3.3	-2.07			-3.04	2.93		
	Chemokines- receptors and ligands								
CCL3L1	C-C motif chemokine ligand 3 like 1								2.14
CCL4	C-C motif chemokine ligand 4								2.36
CCL5	C-C motif chemokine ligand 5						-2.73		
CCL17	C-C motif chemokine ligand 17								2.38
CCL20	Chemokine (C-C motif) ligand 20				6.16				9.05
CCL27	Chemokine (C-C motif) ligand 27				2.39				3.24
CCR10	C-C motif chemokine receptor 10						-2.26		
CXCL2	C-X-C motif chemokine ligand 2								6.14
CXCL6	C-X-C motif chemokine ligand 6								64.32
ICAM3	Intercellular adhesion molecule 3	3.11			2.71		-6.19		3.31
SELPLG	Selectin P ligand						-4.08		3.9
	Pathogen recognition, cell signaling and transcription – receptors and factors								
TLR2	Toll-like receptor 2						-4.17		
TLR3	Toll-like receptor 3					-2.09			
TLR8	Toll-like receptor 8					44.47			2.84
STAT1	Signal transducer and activator of transcription 1		-2.36					3.77	
STAT3	Signal transducer and activator of transcription 3						-3.55		
MYD88	Myeloid differentiation primary response 88		-2.45		2.09	-9.71	3.92		
	Miscellaneous – growth factors, enzymes, signaling molecules, etc.								
ARG1	Arginase 1				52.45				30.79
ARG2	Arginase 2				2.22	-2.39			4.03
GZMB	Granzyme B				2.16				3.52
RFX5	Regulatory factor X5		4.21				-3.52	11.70	
CSF3	Colony stimulating factor 3								-2.8
CSF3R	Colony stimulating factor 3 receptor								-2.73
IL15	Interleukin 15					-2.73			

IL33	Interleukin 33						-3.05		
CLDN6	Claudin 6								-2.47
CLDN11	Claudin 11								-2.86
CDH1	Cadherin 1						-2.44		-2.18
PIK3R5	Phosphoinositide-3-kinase regulatory subunit 5							2.88	3.49
DCT	Dopachrome tautomerase	-11.84	-4.51	2.64		-33.75	18.1		-6.40
HLA-3	MHC class I antigen/ SLA-3 histocompatibility antigen, class I								2.6
HLA-5	MHC class I antigen/ SLA-5 histocompatibility antigen, class I					-6.1			
HLA-1	MHC class I antigen/ SLA-1 histocompatibility antigen, class I		2.85						
HLA-DOB	SLA class II histocompatibility antigen, DO beta chain					-2.81			

In addition, genes (both up and down-regulated) showing a different expression trend was seen with mostly strong down-regulation early and up-regulation late in the infestation compared than in the first analysis. In this chapter/analysis, the main focus was on these genes.

5. 3. 5. 1. Early in the infestation

In this analysis, early (at week 1 and 2) in the infestation an array of genes which are generally produced during inflammation were down-regulated in CS. Complement component C3 was down-regulated in CS at weeks 1 and 2 (-2.08 and -2.36 fold down respectively), and C4A (-6.47 fold down), complement factor D (CFD, -2. 43) and mannose binding lectin 2 (MBL2, -3 fold down) were also down-regulated at week 2. Conversely C6 was up-regulated in CS (5.01 fold up) at week 1 but down-regulated at week 2 (-9.12 fold down).

Partek and comparative analyses revealed pro-inflammatory cytokines IL-19 and oncostatin M (OSM) and IL-19 being down-regulated (-7.51 and -6.67 fold down respectively) in CS pigs in pre-clinical infestation.

While some toll like receptors (TLRs) were down-regulated at week 1 (TLR3, -2.09 and TLR9 -3.18 fold down, only in CS), TLR8 was up-regulated by 44.47 fold. At week 2, TLR2 and TLR5 were also down-regulated in CS (-4.17 and -3.11 fold down respectively).

Antigen presentation related genes were down-regulated in CS including HLA-5 (MHC class I antigen 5, -6.1 fold down at 1wpi), CD82 (-3.1 fold down at 1wpi), HLA-DOB (Major histocompatibility complex, class II, DO beta, -2.81 fold down at 1wpi) and DNA-binding protein RFX5 (RFX5, -3.52 fold down at 2wpi).

Expression of molecules associated with leukocyte migration down-regulated at 1wpi in CS included IL15 (-2.73 fold down), IL19 (-7.51 fold down, only in CS down) and IL33 (-3.05 fold down). At 2 wpi, transcription of molecules associated with immune cell recruitment and

trafficking was down-regulated including CCL (C-C motif chemokine ligand) 5 (-2.73 fold down), CCR (C-C motif chemokine receptor) 10 (-2.26 fold down), selectin P ligand (SELPLG, -4.08 fold down) and intercellular adhesion molecule 3 (ICAM3, -6.19 fold down).

Th cell activation and differentiation associated molecules were down-regulated in CS pigs at 1wpi including CD70 (-8.21 fold down, exclusively in CS), MAP2K1 (-9.5 fold down) and NFKB2 (-2.6 fold down). While CD3G was strongly up-regulated in CS (20.56 fold up) and down-regulated in OS (-6 fold down) at week 1, other T cell markers were down-regulated in CS, including CD8A (-3.04 fold down, only in CS), CD247 (-3.22 fold down) and CD4 (-2.11 fold down). Lymphocyte antigen 9 (LY9), an immunomodulatory receptor promoting Th17 differentiation was down-regulated only in ordinary scabies (-3 fold). Th1 pathway was predicted to be inhibited in CS skin at week 2, with its related genes including IFNγ (-4.86 fold down), IL12B (-2.43 fold down) and IL27 (-5.91 fold down) being down-regulated. Transcription of certain immunoregulatory mediators was down-regulated in CS skin including IL27 (-5.91 fold down at 2wpi) and transforming growth factor (TGF) β1 (-4.31 fold down at 1wpi). FOXP3 was initially up-regulated at week 1 (3.11 fold up) but then down-regulated (-3.85 fold down) at 2wpi. The immunoinhibitory CD274 (PDL1) was decreased (-5.02 fold down) only in CS at 1wpi, but increased (4.08 fold up) at 2wpi.

In IPA, consistent with the type of gene expression detected, the biological processes and diseases associated were, “Cell-To-Cell Signaling”, “Immune Cell Trafficking”, “Lipid Metabolism” “Cellular Growth and Proliferation”, “Inflammatory Response” and “Immunological Disease”. In pathway analysis, the notable immune related canonical pathways predicted to be inhibited in CS pigs early (at weeks 1 and 2) in the infestation included, “STAT3 Pathway”, “Th1 Pathway” and “T Cell Receptor Signaling”.

In comparative analysis, using Venn diagrams, comparing CS vs controls, and OS vs controls at 1wpi revealed that Dopachrome Tautomerase (DCT, -33.75 fold down) and Adiponectin (ADIPOQ, -24.3 fold down) were specifically down-regulated in CS. Melanocyte pigmentation pathway was inhibited, although curiously DCT was up-regulated (18.1 fold up) in CS at 2pwi.

In summary, in pre-clinical CS infestation down-regulation of Th1, innate immune and inflammatory response, and antigen presentation related genes was detected indicating the presence of transcriptional control mechanisms to repress Th1 biased and innate immune responses, and delay antigen presentation early in the infestation in pigs with CS. There was early evidence to suggest promotion of Th2 and Th17 responses in CS.

5. 3. 5. 2. Late in the infestation

At week 4 post-infestation, clinical signs of scabies first became evident (Figure 4. 1, Chapter 4). The trend of gene expression was different to other weeks, with a much lower proportion of differentially expressed genes and few logical networks or pathways. Significant canonical pathways were related to apoptosis (Nur77 and Granzyme B signaling) (Table 5. 3). Of the genes differentially expressed in CS at week 4, ones of possible relevance included CD3G (8.86 fold up), STAT1 (3.77 fold up), and the MHC II transcriptional activator Regulatory Factor X5 (RFX5, 11.70 fold up). Fc fragment of IgG IIb (FCFR2B) was increased (2.4 fold up), along with LY96 (4 fold up, also increased in CS pre-infestation). Antigen presentation related major histocompatibility complex class I antigen (HLA-1) was also up-regulated at week 4 (5.13 fold up).

At week 8, clinical phenotypes of CS or OS were readily discernible (Figure 4. 1, Chapter 4). In contrast to the gene profiles observed in weeks 1 and 2, later crusted scabies infestation was associated with up-regulation of genes associated with inflammatory responses, antigen

presentation and cell trafficking. Pathway analysis revealed a large number of signaling pathways associated with this gene set and the notable ones include, “Role of IL-17A in Psoriasis” (Figure 5. 4), “T Cell Receptor Signaling” and “Th2 Pathway” (Table 5. 3). IPA revealed an array of genes at this time were associated with processes – “Immune Cell Trafficking, “Cellular Growth and Proliferation”, “Inflammatory Response”, “Organismal Injury and Abnormalities”, “Inflammatory Diseases”.

A number of canonical pathways were related to IL-17A mediated inflammatory processes. In particular, psoriasis associated pathway genes were strongly represented in CS (Figure 5.4), with high transcription of IL17A (4.67 fold up), S100 calcium binding proteins S100A7 (psoriasin 1, 21.52 fold up), S100A8 (calgranulin A, 19.85 fold up) and S100A9 (calgranulin B, 21. 07 fold up); CCL20 (9. 05 fold up), CXCL6 (64 fold up) and IL8 (13. 89 fold up). Venn analysis comparing CS vs control and OS vs control samples indicated that several of these, and other IL-17A associated genes were exclusively up-regulated in CS, including IL8, CXCL6, CCL20, IL17RB, CCL27, OSM, IL19 and IL-20. Conversely, at 8wpi IL17F was only up-regulated in OS, and OSM was down-regulated in OS pigs. Moreover, strong up-regulation of Arginase 1 (30.79 fold up) and Arginase 2 (4.30 fold up) was observed, which have been found to be associated with high IL-17A expression.

Genes associated with antigen presentation in crusted scabies were evident at week 8, with HLA-3 up-regulated (2.6 fold up), whereas this gene was exclusively up-regulated at week 1 in ordinary scabies.

Transcription of an array of chemokines with roles in immune cell activation and trafficking was up-regulated at 8wpi in CS including CCL3L1 (2.14 fold up), CCL4 (2.36 fold up), CCL17 (2.38 fold up), CXCL2 (6.14 fold up), selectin SELPLG (3.9 fold up) and ICAM3 (3.31 fold up).

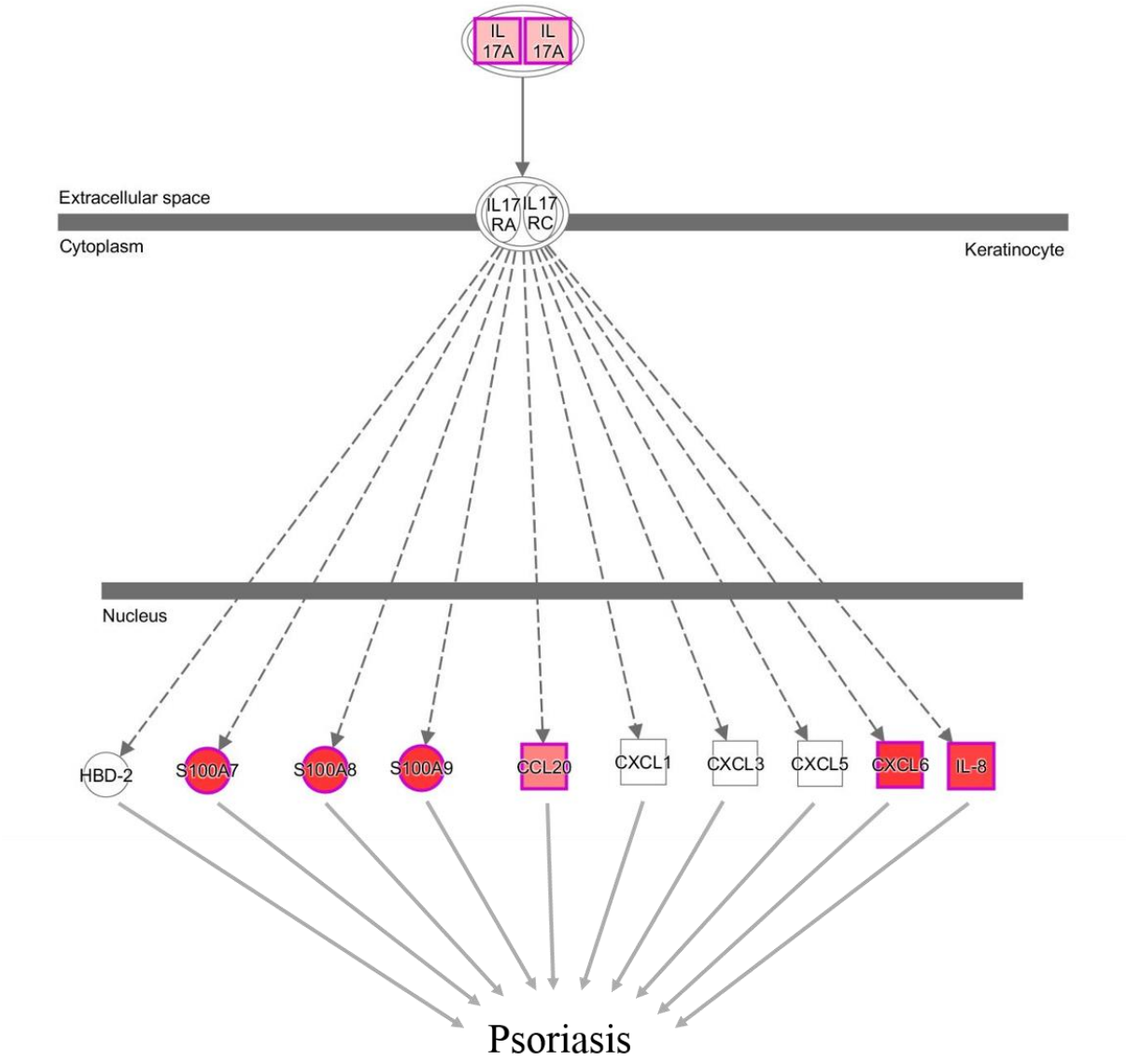

Figure 5. 4. IPA canonical pathway depicting relationships among genes associated with Role of IL-17A and S100s – calcium binding proteins in Psoriasis. The differentially expressed genes included are those which showed >2-fold change in expression with $P < 0.05$. Nodes coloured by gene expression with red (strong expression) and pink nodes representing up regulated genes and white indicating not differentially expressed genes. Pink squares and circles highlight the roles for IL-17A, S100A7, S100A8, S100A9, CCL20, CXCL6 and IL-8.

As observed in previous weeks, CD3 markers were up-regulated (CD3E, 3.43 fold up, CD3G, 3.57 fold up). Other differentially expressed immune cell surface markers at this time point included CD4 (2 fold up) and CD40L (3.58 fold up). Conversely, the antigen presenting cell associated marker CD86 was down-regulated in CS (-3 fold down), and exclusively up-regulated in OS vs control at week 8. As well as the strong IL-17A response mentioned previously, an association of Th2 type cytokines in week 8 of CS infestation was evident, including IL-13 and (2. 35 fold up), IL-5 (exclusively in CS vs control, 3 fold up).

The other genes showing down-regulation late in the infestation at week 8 included C3 (-2.63 fold down), granulocyte colony-stimulating factor (CSF3, -2.8 fold down) and CSF3R (-2.73 fold down).

5. 4. Discussion

This is the first comprehensive analysis of gene expression profiles comparing crusted to ordinary scabies in a porcine model of scabies. The aim of this analysis was to examine the gene expression profiles in immune and inflammatory responses in CS compared to OS. Another aim was to investigate the predisposing factors for CS and key regulators in disease development and progression. Microarray analysis was carried out to identify genes expressed in the lesional skin and to gain insight into the expressional repertoire in the host responses to scabies mite infestation in CS.

The DNA-microarray gene expression analysis clearly differentiated CS from OS pigs, and demonstrated significant differences in gene expression between the two groups/phenotypes. In general, gene expression post-mite infestation in CS pigs is associated with an array of biological pathways including chemokine signaling, immune cell trafficking, T cell differentiation and proliferation, organ inflammation, calcium activation and inflammatory response with a focus on up-regulation of a number of pro-inflammatory and allergic

mediators. Repression of immune mediators associated with Th1 responses and down-regulation of Th2 and Th17 response inhibitors was also observed. In addition, early in the infestation our results show repression of factors which included those involved in certain aspects of the inflammatory response.

5. 4. 1. Gene expression pre-infestation

One of the aims of this analysis was to investigate the factors involved in possible underlying susceptibility to CS and as such, gene expression profiles were compared in pigs prior to infestation. Principal component analysis demonstrated that CS pigs grouped separately from the OS pigs indicating differences in gene expression. In gene expression analysis, at week 0 prior to mite infestation, we detected differential expression of genes which play roles in immune and inflammatory responses and acute phase reaction. Expression of acute phase reaction related molecules such as OSM were up-regulated in CS pigs, as were other inflammatory molecules including chemokines CCL25, CCR3 and CCR10, adhesion ICAM3 and selectin SELPLG, signaling molecules NFκB2 and STAT3, and complement components C4A and C6. When interpreting these results, it is important to acknowledge both the young age (3 weeks old) of the pigs and the events surrounding this time point. Firstly, it is acknowledged that 3-4 weeks represents a “critical window” in immune development in piglets, and high variation in immune parameters is expected at this time (Butler et al., 2006; Nguyen et al., 2016). Indeed, early studies during the establishment of this porcine model for scabies showed that early age was necessary to facilitate successful mite infestation (Mounsey et al., 2010). Additionally, all piglets at this time point were subjected to several stressors including separation from the mother, early weaning, and movement to experimental facility, handling, and adjusting to a different physical environment (Campbell et al., 2013; Wang et al., 2008). Enhanced pro-inflammatory response and acute phase

responses at this time point may be indicative of a heightened stress response associated in individual pigs, subsequently predisposing pigs less equipped to deal with such stressors to more severe infestation upon mite challenge.

Pigs that went on to develop CS had transcriptional profiles suggestive of potential dysregulation in macrophage associated pathways. Observations of no or very few macrophages have been noted previously in biopsies collected from CS patients (Falk and Eide, 1981; Walton et al., 2010). The down-regulation of MYD88, a key regulator of innate and adaptive immunity, is notable, as is suppression of CD27/70 signaling, suggesting possible impaired T-cell proliferation and survival in CS pigs. Conversely, transcription of immunoregulatory FOXP3 was up-regulated in pigs which later developed CS, which may be why mites can more successfully establish in these pigs. It has been previously demonstrated that *S. scabiei* extracts stimulate T regulatory (Treg) cells to produce immunosuppressive cytokines in PBMCS, further attesting to this (Arlian et al., 2006). IL-17F was down-regulated in pigs that later developed CS, an observation that was maintained throughout, suggesting baseline differences in Th17 differentiation pathways between clinical phenotypes.

5. 4. 2. Early gene expression

Expression of particular genes early in the infestation might be a way of modulating the specific aspects of immune and inflammatory responses and might play a role in disease susceptibility and development.

Toll-like receptors (TLRs) play a fundamental role in pathogen recognition and via TLR signaling activate innate immune responses, and mediate the production of cytokines necessary for the downstream activation of effective cell-mediated immunity (Nilsson et al., 2012; Pasare and Medzhitov, 2004). In this analysis, TLR2 and TLR3 were found down-

regulated early in the infestation in CS compared to OS pigs. Also, comparative analysis revealed TLR9 being exclusively down-regulated at week 1 post-infestation in CS compared to OS and C skin. In line with this, pathway analysis showed 7 down-regulated genes in our dataset involved in TLR signaling pathway. TLR2 mediates the innate immune response to bacterial pathogens and regulates also adaptive immunity by inducing Th1 cytokine secretion (Lancioni et al., 2011). The house-dust-mite allergen, Der p 2, promotes airway hypersensitivity reactions in diseases such as asthma. Der p 2 shares structural similarities with MD-2 (Derewenda et al., 2002; Ohto et al., 2007) also known as Lymphocyte antigen 96 (LY96), which was exclusively up-regulated in OS at week 1 and 2 post-infestation. LY96 enables TLR2 to respond to bacteria and their components, and enhances the responses of TLR2 which in turn enhances LY96 expression, and activates the NF-κB signal transduction pathway that induces transcription of chemokine genes (Dziarski and Gupta, 2000; Dziarski et al., 2001). TLR3 is expressed on epidermal keratinocytes and signals primarily through NF-κB leading to IFN production in innate immune response (Dai et al., 2006). TLR9 is a nucleotide-sensing TLR and mediates cellular response to unmethylated cytidine-phosphate-guanosine (CpG) dinucleotides and like TLR3 activates NFκB to mount an innate immune response (Takeshita et al., 2004). The absence of TLR2, TLR3, TLR9 and LY96 in early CS may thus indicate impaired innate immune signaling, favouring mite proliferation. Also, LY96 gene expression in OS pigs indicates a differential mechanism of pathogenesis between OS and CS pigs.

TLR8 was strongly up-regulated at 1wpi and is an important component of innate and adaptive immunity. Tregs express a relatively high level of TLR8 and TLR8 signaling has been shown to play a role in reversing the suppressive function of Tregs (Peng et al., 2005). TLR8 up-regulation may be a mechanism for controlling Treg cell function resulting in the shift of functional balance between Treg and effector T cell through TLR8 signaling pathway

and may be playing a role in CS disease pathogenesis. In addition, TLR8 gene polymorphisms have been implicated in the susceptibility to allergic disorders such as asthma and allergic rhinitis, and associated with increased IgE response (Moller-Larsen et al., 2008; Nilsson et al., 2012). TLR8 gene associated mutations may be responsible for dysregulated host response predisposing individuals to CS and may be also responsible for increased IgE production in CS. Gene polymorphism have not yet been studied in scabies but it might be of future interest to investigate the genetic variation associated with CS in Toll-like receptor genes.

In our analysis, we detected complement components C3, C4A, C6 and CFD being down-regulated early (at week 2) and C6 was also up-regulated initially at week 1 in the infestation. Previously, in patients with CS low levels of C3 and C4 have been reported (Roberts et al., 2005). Pathway analysis demonstrated that 7 genes, down-regulated in our dataset, were associated with "Complement System" pathway. C3 activates the complement system – both classical and alternative pathways and is a mediator of inflammatory responses (Gullstrand et al., 2009). The C4A gene encodes complement C4 and is essential for the propagation of the classical complement pathway and is a mediator of local inflammatory process (Gullstrand et al., 2009). Low expression or the absence of the C4 protein coincided with disease activities of systemic lupus erythematosus (SLE) and is the strongest genetic risk factors for SLE or lupus-like disease (Yang et al., 2004). C6 is one among the five constituents (complement components five to nine) of the membrane attack complex (MAC) that plays a key role in the innate immune response. Absence of any one component leads to failure to form the MAC and lack of complement pathway function could make the host more susceptible to infections (Sarma and Ward, 2011; Varela and Tomlinson, 2015). C6 gene defects and mutations have also been found to be an important factor associated with susceptibility to various bacterial infections (Orren et al., 2012). CFD is involved in the complement alternate pathway

activation and deficiency of CFD protein is associated with an immunologic disorder characterized by increased susceptibility to bacterial infections (Sprong et al., 2006). C4A deficiency might be a disease susceptibility factor in scabies and its strong repression early in the infestation might play a role in CS development. C3, C6 and CFD repression early in the infestation in CS pigs might abolish alternative and classical pathway dependent complement activation by mites allowing them to establish and multiply in large numbers in CS cases. Also, C6 up-regulation at 1wpi indicates that there is some sort of host complement response to the mites but it seems the mites are able to overcome this response by suppressing C6 and other complement gene expression at week 2 post-infestation. The JAK/STAT pathway is an important signaling event controlling processes like cell differentiation and secretion of cytokines in both adaptive and innate immune cells in inflammation (Cai et al., 2015; Coskun et al., 2013; Migita et al., 2013). Various cellular players and cytokines contribute to JAK/STAT signaling (Jostins et al., 2012). IL12B positively regulates T cell proliferation, and Th1 and Th17 type immune responses. IL12 receptor β2 subunit (IL12Rβ2) receptor for interleukin-12 is expressed on Th1 but not on Th2 clones (Rogge et al., 1997). With STAT4 being the major effector molecule, IL12Rβ2 via JAK2 promotes differentiation of T cells towards Th1 phenotype by strongly inducing IFNγ (Amsen et al., 2009). Selective expression of the IL12Rβ2 might be responsible for Th1/Th2 differentiation and the down-regulation of IL12B, IL12Rβ2, IFNγ, STAT4 (exclusively in CS) and JAK2 early in the infestation may be one of the reasons why we see a limited Th1 type immune response in CS compared to OS. In addition, pathway analysis in IPA revealed 9 genes associated with JAK/STAT signaling pathway (Figure 5. 5) being down-regulated which are expected to be up-regulated in its activation state. This gene down-regulation might result in the inhibition of the JAK/STAT signaling pathway and the inflammatory response and might be one way of driving pathogenesis in CS.

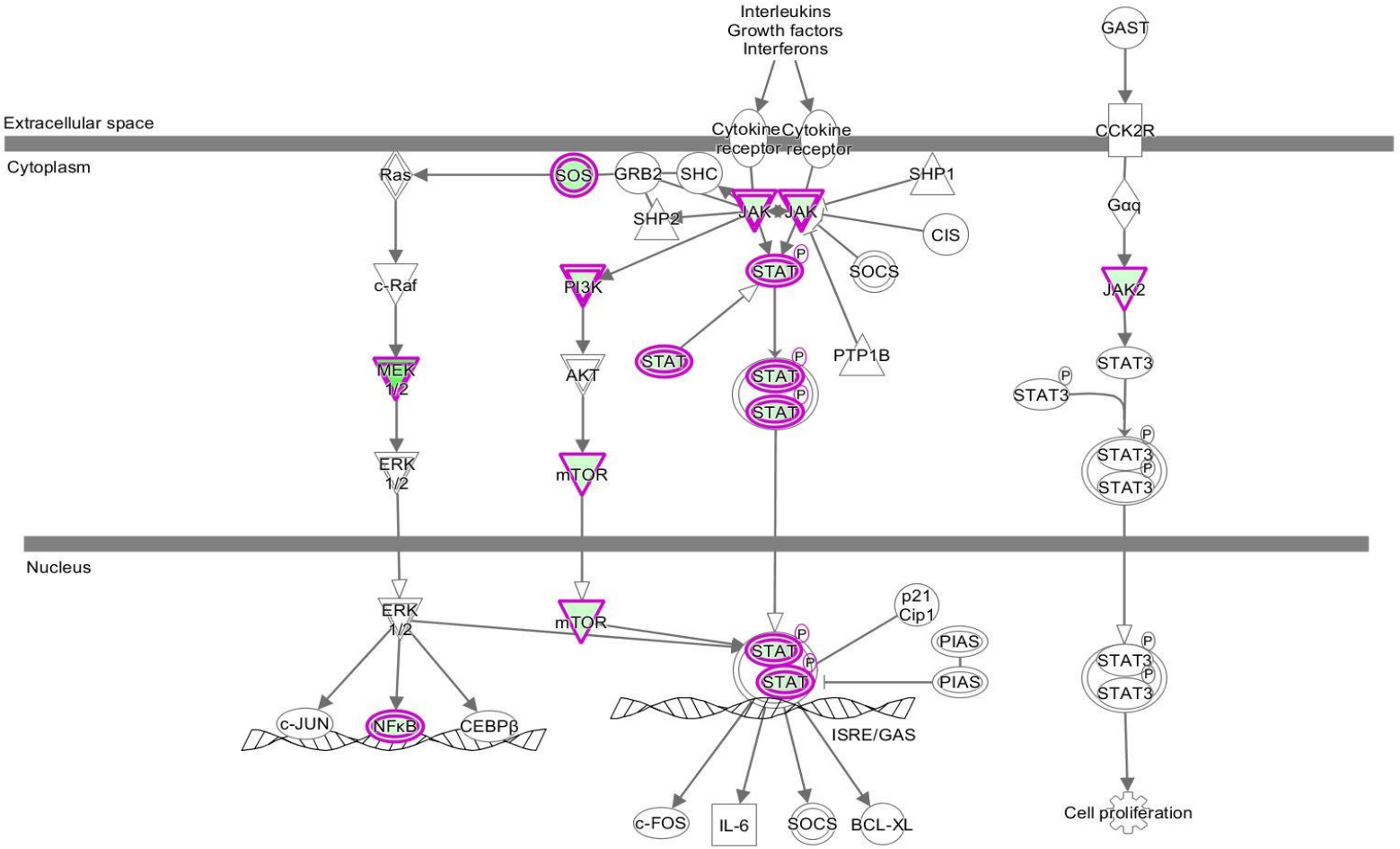

Figure 5. 5. IPA canonical pathway depicting relationships among genes associated with "JAK-STAT Signaling pathway" at week 1 post-infestation in Crusted versus Ordinary scabies pigs. The differentially expressed genes included are those which showed ≥ ± 2 fold change in expression with $P \leq 0.05$. Individual nodes represent protein functions with relationships represented by edges. Nodes coloured by gene expression with green down-regulated genes and white indicating not differentially expressed genes. Pink triangles and circles highlight the roles for JAK2, SOS2, MTOR, STAT2, STAT6, STAT5A, TLR9, NFκB2 and MAP2K1.

In this analysis, STAT3 gene was down-regulated at 2wpi and in line with this in IPA, 12 genes associated with STAT3 signaling pathway were down-regulated. STAT3 pathway is involved in cell proliferation and differentiation, and important in modulating both innate and adaptive responses (Harris et al., 2007; Hillmer et al., 2016). STAT3 is an essential factor for the production of Th17 cells which are critical for host defense to bacterial and fungal pathogens (Hirahara et al., 2010). STAT3 inhibits production and function of DCs which have important phagocytic and antigen presenting activities (Hillmer et al., 2016). In addition, STAT3 regulates proliferation and migration of neutrophils upon pathogen (bacterial or fungal) invasion during host innate response to increase the number of neutrophils to help contain infection (Hillmer et al., 2016). STAT3 down-regulation early in the infestation in CS pigs may result in low number of granulocytes – neutrophils and DCs leading to reduced antigen presentation which might result in diminished effector T cell production and adaptive immune responses. This may also explain absence of/ limited defence/resistance to the mites in CS cases which may result in the host's inability to clear the mites allowing them to grow in number. Furthermore, STAT3 gene mutations have been associated with immunodeficiency, autoimmunity, recurring bacterial infections of skin, increased circulating immunoglobulin E (IgE) and severe eczematoid rash (Hillmer et al., 2016; Holland et al., 2007). STAT3 gene variations are also associated with increased predisposition to psoriasis and multiple sclerosis (Hillmer et al., 2016). Possibly STAT3 gene mutations or repression may be responsible for increased IgE response seen in CS and may play a role in disease predisposition.

Although a few STAT molecules were down-regulated early, up-regulation of STAT1 was detected late in the infestation. STAT1 is a key molecule in IFNγ mediated signaling events and STAT1 gene mutations have been associated with impaired IFNγ mediated immunity and inflammation, and susceptibility to severe mycobacterial (Bustamante et al., 2014) and yeast

(Sampaio et al., 2013) infections. STAT1 gene mutations might be responsible for impaired host immune responses and associated with CS diseases susceptibility.

The transcription factor NF-kappa-B (NF-κB) signal transduction pathway is well known for its role in many biological processes such as inflammation, immunity, cell differentiation and apoptosis (Chan et al., 2006; Hayden and Ghosh, 2011; Hayden and Ghosh, 2012; Lawrence, 2009). NF-κB regulated genes have been shown to greatly influence the development of a cutaneous inflammatory response to mite *Psoroptes ovis* in sheep (Burgess et al., 2011). In this analysis, we saw down-regulation of innate immune gene NFκB2 in CS pigs both early and late in the infestation. NF-κB2 is an essential subunit of the transcription factor complex NF-κB and associated with the NF-κB pathways and functions in peripheral lymphoid organ development, B cell development, antibody and cytokine production, defects in T and B cells and impaired NK-cell cytotoxic activity. Also, defects in NFκB2 gene have been associated with primary immunodeficiency and autoimmunity (Chen et al., 2013b; Lougaris et al., 2015; Shi et al., 2016). In IPA, early in the infestation (at week 1 and 2) a number (up to 19) of genes associated with NF-κB signaling pathway (Figure 5. 6) were down regulated. This down-regulation of innate immune genes might cause disruption of NF-κB pathways, in this manner might affect T cell and NK-cell activity, and impair innate and adaptive immune responses. Also, these results suggest a role of NF-κB in disease pathogenesis and NFκB2 early gene suppression might affect susceptibility to CS disease.

Down-regulation of RFX5, tetraspanin CD82, HLA-5 and HLA-DOB was detected at week 2 post-infestation in CS pigs. In addition, in comparative analysis, HLA-DOB was uniquely down-regulated early in the infestation in CS. RFX5 is a transcription regulator which plays a pivotal role in the transcriptional activation of MHC-II genes (Hanna and Etzioni 2014).

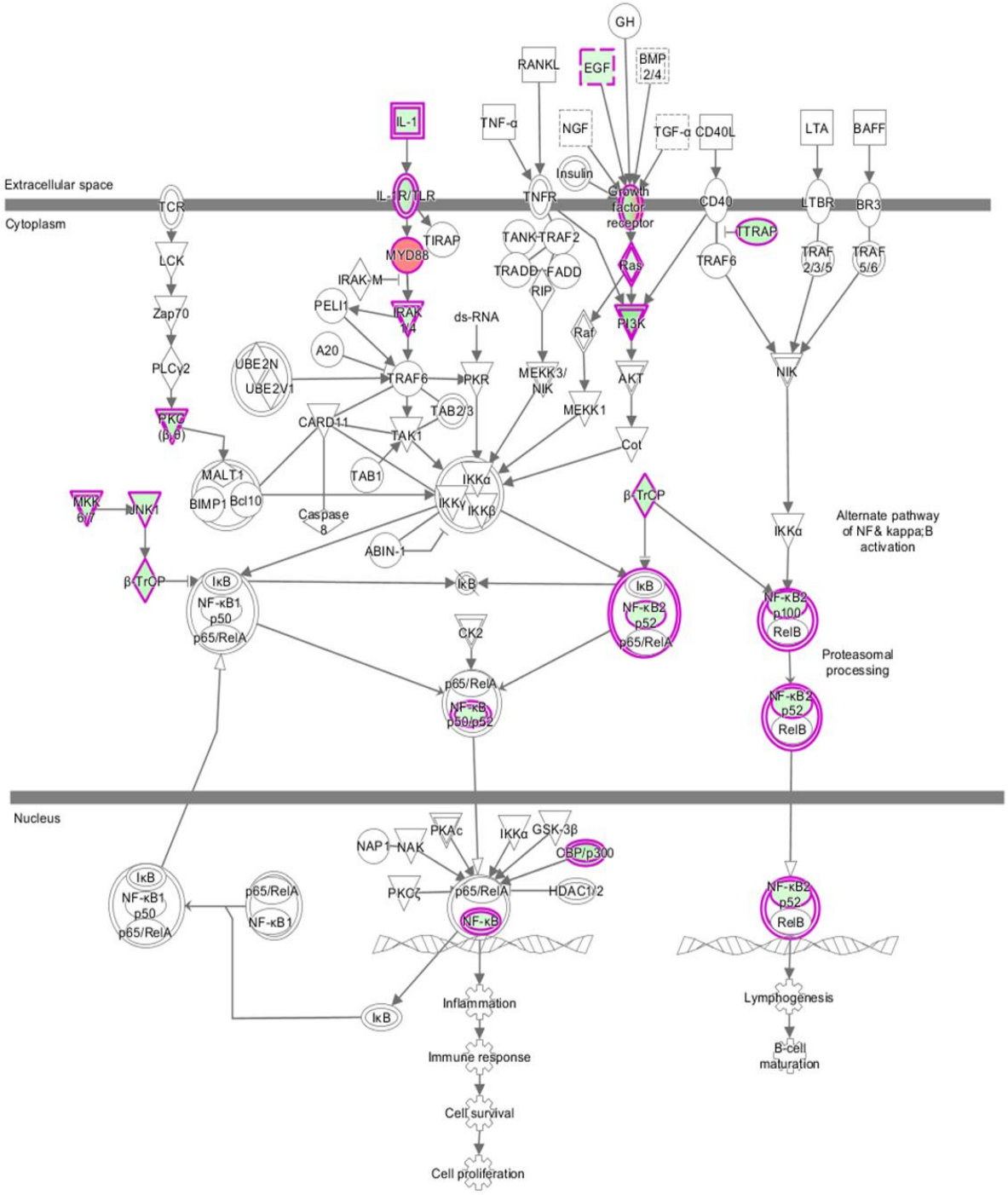

Figure 5. 6. IPA canonical pathway depicting relationships among genes associated with "NF-κB Signaling pathway" at week 2 post-infestation in Crusted versus Ordinary scabies pigs. The differentially expressed genes included are those which showed ≥ ± 2-fold change in expression with $P \leq 0.05$. Individual nodes represent protein functions with relationships represented by edges. Nodes coloured by gene expression with green down-regulated genes, orange/red up-regulated and white indicating not differentially expressed genes. Pink triangles, squares and circles highlight the roles for EGF, MYD88, TLR2, TKR5, IRS1, IRAK1, PIK3C2B, PRKCB, EP300, NFκB2, MAP2K6 and MAPK8.

CD82 molecularly associates with MHC II, the peptide-editing/loading mediators' HLA-DM and HLA-DO and regulates antigen - processing and presentation machinery in DCs response (Hammond et al., 1998; Kropshofer et al., 2002). HLA-DOB is involved in antigen processing and presentation via MHC class II and HLA-5 is part of MHC class I protein complex. MHC molecules are essential in the adaptive immune system and regulation of their gene transcription is critical for efficient activation and development of T cells, antibody production and maintenance of immune tolerance (Janeway et al., 2001c; Neefjes et al., 2011). Early suppression of RFX5, CD82, HLA-5 and HLA-DOB might be responsible for low antigen presentation and hence affecting adaptive responses. In support of this, pathway analysis revealed 16 genes, down-regulated in our dataset, involved in DC maturation pathway. DCs are critical antigen presenting cells and their maturation is essential for efficient antigen processing and presenting and thereby regulating T-cell responses (Adams et al., 2005). Down-regulation of genes involved in DC maturation may impact the antigen presentation leading to impaired or dysregulated immune responses early in the infestation which might play a role in disease susceptibility. This early suppression of genes in CS may also be a mechanism to control adaptive immune responses orchestrated by DCs. Also, we observed up-regulation of some of these mentioned earlier and few other genes related to antigen presentation late in the infestation which are discussed later in this chapter.

Immune regulation is important to limit inflammatory responses, pathology and maintain immune homeostasis during infection and inflammation. In our analysis, we found transcription of TGFβ1 (exclusively) down-regulated at week 1, and IL27 at week 2 following mite infestation in CS compared to OS pigs. Also, FOXP3 was initially up-regulated at week 1 and then strongly repressed at week 2 in CS pigs. TGF-β1 is an important growth factor in Treg development and it also regulates gene expression of various essential cell growth factors including IFN-γ and TNF-α which play key roles in immune and

inflammatory responses (Hadaschik and Enk, 2015; Sakaguchi et al., 2010; Sakaguchi et al., 2008). IL-27 is a potent inhibitor of Th2 and Th17 cell development and of IL-4, IL-5 and IL-17 production (Stumhofer and Hunter, 2008). FOXP3 is a transcriptional regulator and an essential molecule for the production and immunosuppressive function of regulatory T-cells (Sakaguchi et al., 2010; Sakaguchi et al., 2008) and *P. ovis* mite infestation in sheep has been shown to result in the infiltration of $Foxp3^{(+)}$ T cells into the skin (McNeilly et al., 2010). Our results suggest that the suppression of these immunoregulatory genes in the skin may result in the immune and inflammatory responses characteristic of CS. In addition, IL27, FOXP3 and TGFβ1 down-regulation may be some of the mechanisms to enhance and mediate the local T cell responses and inflammation, and hence may play a role in disease pathogenesis in CS. This gene expression might also explain the increased Th2 and Th17 responses observed in CS.

In this analysis, CD70 was strongly down-regulated at week 1 compared to the first analysis (described in chapter 4), and 7 genes, associated with the "CD27 Signaling in Lymphocytes" (Figure 5. 7) were also down-regulated. In addition, comparative analysis using venn diagrams also demonstrated CD70 being uniquely down-regulated at week 1 post infestation in CS compared to OS and C pigs. CD70, a ligand for CD27 is a co-stimulatory glycoprotein expressed by activated DCs, T-Cells and B-Cells. The contribution of CD27 to the immune response is dependent upon CD70 expression. CD70 signals into DCs upon engagement with CD27 on antigen activated T cells and condition the DCs to promote T cell proliferation (Borst et al., 2005). CD27-CD70 interactions provide key contributions in promoting effector and memory $CD8^+$ T cell formation and survival hence enhance $CD8^+$ T responses (Keller et al., 2008). Also, this CD27-CD70 interaction augments the activity of $CD4^+$ T cells (Hashimoto-Okada et al., 2009). By controlling CD70 gene expression, scabies mite antigens may control CD27 function and hence affect primary T cell responses.

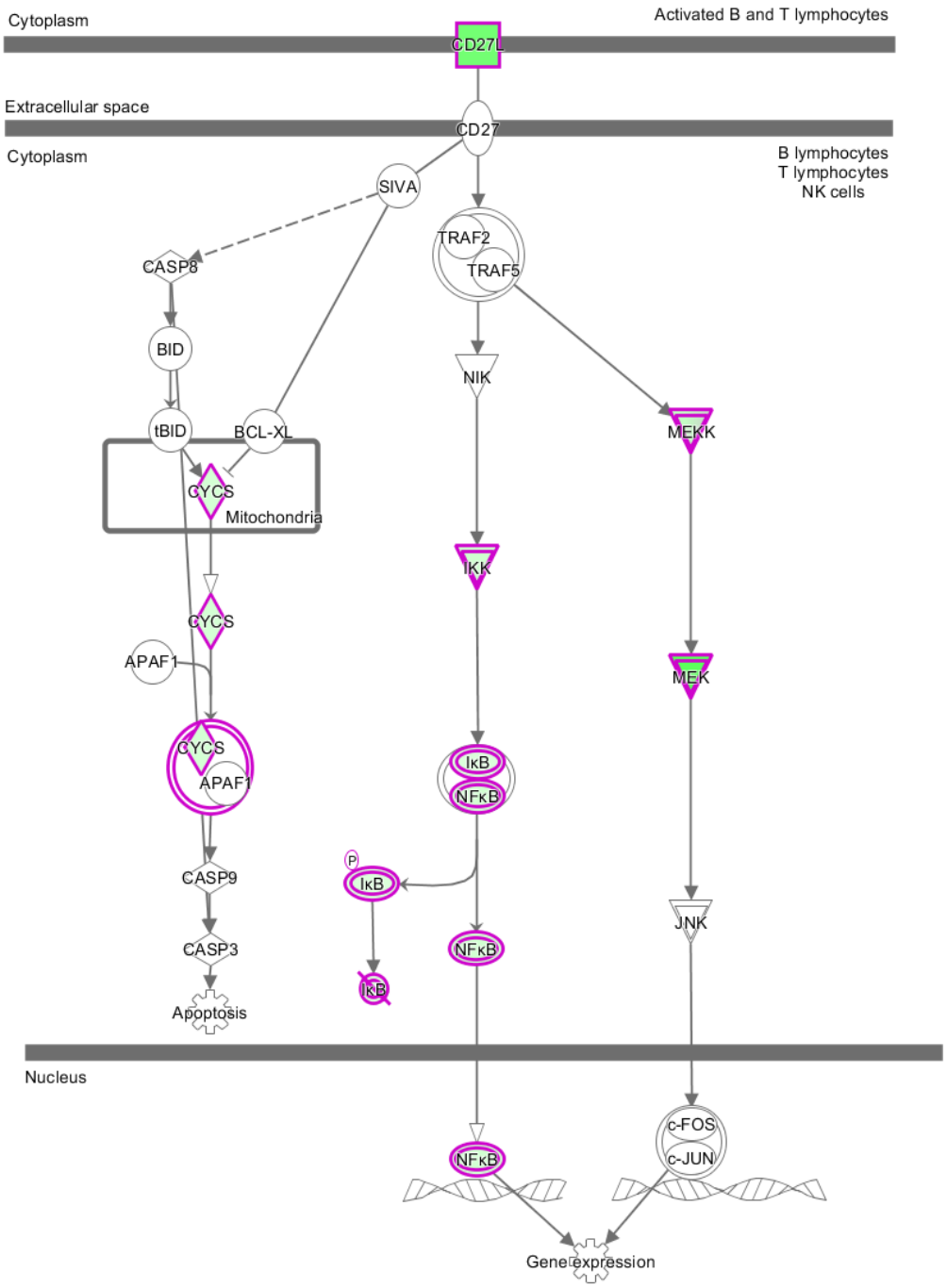

Figure 5. 7. IPA canonical pathway depicting relationships among genes associated with "CD27 Signaling in Lymphocytes pathway" at week 1 post-infestation in Crusted versus Ordinary scabies pigs. The differentially expressed genes included are those which showed ≥ ± 2 fold change in expression with P ≤ 0.05. Individual nodes represent protein functions with relationships represented by edges. Nodes coloured by gene expression with green down-regulated genes and white indicating not differentially expressed genes. Pink triangles, squares and circles highlight the roles for CD4, CD27L/CD70, CD247, CD3G, IKBK, HLA-DOB, TLR9, NFκB2, MAP2K1.

Possibly this transcriptional down-regulation of CD70 at this time (week 1) in infestation may be due to fact that not enough antigenic material and activated T cells expressing CD27 are available to induce its expression. Also, CD70 gene and CD27 signaling pathway associated gene down-regulation early in the infestation may provide a mechanism via CD27-CD70 interaction and CD27 signaling in lymphocytes pathway of inhibiting T cell mediated immunity against the mites.

Selectins and chemokines (ligands and receptors) play an important role in immune and inflammatory responses, immunomodulation and have been implicated in various inflammatory skin diseases (Nedoszytko et al., 2014). We detected down-regulation of selectin SELPLG, chemokines CCL5 and CCR10 compared to the first analysis. CCR10 expressing circulatory T cells are attracted/recruited by cutaneous T cell-attracting chemokine (CTACK/CCL27) predominantly expressed in the skin by keratinocytes during the local immune response (Xiong et al., 2012). CCL5 (also referred as RANTES) is responsible for the recruitment of leukocytes and T-lymphocytes during inflammation and immune response (Nedoszytko et al., 2014). Selectin P ligand (SELPLG) gene encodes a glycoprotein which mediates leukocyte trafficking during the initial steps in inflammation. Aberrant SELPLG expression results in defective innate and adaptive immune responses (Somers et al., 2000). This down-regulation of chemokines and selectins early in CS might inhibit trafficking of immune effector cells in the skin, development of essential T cells and inflammatory responses. This early suppression may also impair the local tissue immunity allowing the mites to grow in number, promote the progress of the disease and be responsible for the clinical outcomes seen at later stages of the disease.

In our study, we found CD5L gene expression was down-regulated early in the infestation (at weeks 1 and 2). Also, in Venn diagram analysis, we detected CD5L exclusively down-regulated at week 1 post-infestation in CS skin. CD5L affects the expression of pro-

inflammatory genes and its up-regulation inhibits the pathogenic function of Th17 cells (Gaublomme et al., 2015; Wang et al., 2015). Our results suggest that CD5L down-regulation early in the infestation in CS might be another mechanism of promoting the activities of IL17 producing cells in CS.

Dopachrome tautomerase (DCT) was strongly down-regulated at week 1 (exclusively) and up-regulated at week 2, and then down-regulated again moderately at week 8 post-infestation. As described in chapter 4 (infested versus non-infested), DCT is an antigen recognized by $CD8^+$ T cells (O et al., 2003; Palermo et al., 2001) and its strong down-regulation might be a way to avoid cytotoxic T cell mediated responses. Vitiligo is an inflammatory disorder characterised by depigmentation of skin and decreased DCT expression has been detected in skin lesions in patients with Vitiligo (Gottschalk and Kidson, 2007). Dysregulated DCT expression may be associated with disrupted melanin synthesis in CS resulting in destruction of melanocytes and depigmentation of skin often observed in CS.

In Venn diagram comparative analysis, a greater number (over 300%) of genes were exclusively down-regulated than up-regulated counterparts early (at week 1) in the infestation in CS as compared to OS and C pigs (Figure 5. 3). In addition to the earlier mentioned genes down-regulated early in the infestation in CS compared to OS and C skin, a few more important genes involved in various aspects of immune and inflammatory responses were detected. Transcripts of CD274/PDL1, TAGAP and CD8α were among the unique strongly down-regulated which are associated with T cell activation, proliferation, immunoregulatory cytokine production and play a role in the process of T-cell mediated killing. In addition, down-regulation of inflammatory mediators NLRP3, IL5 and IL8 was exclusively observed in CS pigs early in the infestation. Given that the same number of mites were used for infestation in all experimental animals, this increased gene down-regulation early in infestation in CS animals may likely reflect the early age related genetic mechanisms/changes

existing in these pigs. This might explain the inability to mount an effective response by these animals to the mites allowing them to grow unchecked and increase in number manifold resulting in hyper infestation as is observed in CS. Also, this early gene down-regulation may impair/dysregulate the immune and inflammatory responses which might be responsible for disease development and progression in CS.

On the contrary, in OS skin, early (at week 1) in the infestation a large number of genes were exclusively up-regulated. The prominent genes included cell receptor CD180, MHC molecules HLA-DQA1 and HLA-DOB, signaling molecules LY96, ICOS, FADD and MAL, immunoregulatory factors STAT5A/B and TGFβ1, cytokine IL1β, chemokine CCL27 and selectin SELP.

CD180 and LY96 mediate and initiate innate immune signaling and responses, involved in B cell proliferation and antibody production hence defense against mite infestation (Akashi-Takamura and Miyake, 2008; Diacovich and Gorvel, 2010; Dziarski and Gupta, 2000; Dziarski et al., 2001). HLA-DQA1 and HLA-DOB are involved in and mediate the presentation of antigens during infections to T cells influencing the generation of adaptive immune responses (Janeway et al., 2001c; Neefjes et al., 2011). Molecules ICOS, FADD and MAL are important in and enhance T-cell signal transduction, early T cell activation and proliferation, hence play a role in T cell responses to foreign antigens. ICOS also provides effective help for antibody secretion by B-cells (Simpson et al., 2010; Tourneur and Chiocchia, 2010). IL-1β is a potent proinflammatory cytokine induces T and B cell activation and cytokine and antibody production (Dinarello, 2009). STAT5A/B and TGF-β1 are critical for the differentiation and inhibitory function of Treg cells (Hadaschik and Enk, 2015; O'Shea and Plenge, 2012). Tregs modulate the expansion and function of effector T-cells, and may be controlling excessive T cell-mediated inflammation in OS cases by producing TGF-β1. CCL27 and SELP mediate leukocyte and lymphocyte recruitment to the sites of infection

during inflammation and immune responses (Xiong et al., 2012; Zarbock et al., 2009) which may intensify the killing of invading mites and stop their spread.

Ordinary scabies pigs seem to mount an early response against the mites as is indicated by up-regulation of a greater number of genes as compared to CS and C pigs. This gene expression may be controlling/limiting the mite spread/growth early in the infestation and be the reason for low mite burden observed in OS cases. In addition, up-regulation of immunoregulatory molecules early in the infestation may supress the pathogenic inflammatory T cells which may limit the damage/injury to the host.

5. 4. 3. Late gene expression

In our study, the infested animals started to show clinical signs and inflammation after week 4 from the initial contact with mites, and at this time we also detected expression of an array of genes with majority of them involved in inflammatory and pro-allergic Th2-type responses more specific to CS.

5. 4. 3. 1. Antigen presentation associated gene expression

In this analysis, we detected early in the infestation down-regulation of few genes as described earlier which might affect antigen presentation and adaptive responses. Antigen processing and presentation is an essential process in order to engage the key elements of adaptive immunity. MHC class I and II molecules found on the surface of antigen-presenting cells (APCs) facilitate antigen presentation and present peptides at the APC surface to $CD8^+$ and $CD4^+$ T cells, respectively (Janeway et al., 2001c; Neefjes et al., 2011). This early gene down-regulation may be because the mites low in number at that time might not provide enough antigenic stimulus or it might be that in CS pigs genetic changes occurring early might be responsible for this antigen presentation related gene suppression. On the other hand, late in the infestation RFX5 (strongly) and HLA-3 gene transcription was up-regulated

in CS compared to OS. Also, in Venn diagram analysis, we found HLA-DRA, HLA-DMB, HLA-DQA1 and HLA-DQB1 genes uniquely up-regulated at this time in CS compared to OS and C pigs suggesting delayed and/but increased antigen presentation in CS. Late in the infestation the higher mite numbers provide enough antigenic stimuli which might be triggering their expression leading to increased MHC activation and host humoral and cell mediated responses as are seen at this time in infestation in CS. In addition, as mentioned earlier RFX5 mediates transcriptional activation of MHC class II genes and mutations in RFX5 gene also affect the expression of MHC-II at the transcriptional level (Hanna and Etzioni, 2014). These RFX5 mutations are associated with severe immunodeficiency syndrome characterised by impaired MHC class II transcription and immune responses and result in significant susceptibility to severe infections (Hanna and Etzioni, 2014). Impaired/dysregulated MHC class II transcription via RFX5 gene expression might be related to increased susceptibility to CS and might also be responsible for secondary bacterial skin infections seen in CS. In addition, MHC II molecules are involved in $CD4^+$ T cell differentiation (Luckheeram et al., 2012; Neefjes et al., 2011) and this might also be a reason for reduced $CD4^+$ T cell count in CS. HLA-3 gene encodes MHC class I molecules and they present peptides at the cell surface to $CD8^+$ T cells (Neefjes et al., 2011). HLA-3 expression may be related to the increased $CD8^+$ T cell numbers observed in CS (Walton et al., 2008; Walton et al., 2010).

5. 4. 3. 2. Chemokines and immune cell trafficking in CS

Chemokines – ligands and receptors are highly important molecules in modulating immune and inflammatory responses. An array of chemokines has been implicated in the pathogenesis of various skin inflammatory conditions. Chemokine ligand CCL27 (also referred to as cutaneous T cell-attracting chemokine, CTACK), was up-regulated here late in the infestation (at week 8) in CS pigs and previously scabies mite extract has been show to induce CCL27 in

human cell lines (Morgan and Arlian, 2010). CCL27 is predominantly expressed in the skin by keratinocytes and upon interacting with its specific receptor CCR10 mediates the recruitment of skin-associated memory T-lymphocytes. CCL27 also plays a role in the homing of T-lymphocytes to cutaneous tissue and the CCL27-CCR10 interaction is thought to play a key role in T cell-mediated skin inflammation (Homey et al., 2002; Xiong et al., 2012). Notably, specific responses to common skin-associated allergens such as house dust mites are restricted to such T cells (Santamaria et al., 1995).

Up-regulation of another chemokine ligand CCL17 (also referred to as thymus and activation regulated chemokine, TARC) was also detected at week 8. CCL17 is produced by dendritic cells (DCs), endothelial cells, keratinocytes and fibroblasts at the site of inflammation. CCL17 displays chemotactic activity for T lymphocytes and plays also a role in activation of mature T cells (Yoshie and Matsushima, 2015). In addition, CCR4, a specific receptor for CCL17, is predominantly expressed by skin-homing T cells and Th2 cells (Yoshie and Matsushima, 2015). These $CCR4^{+}$ T cells play a pathogenic role and have been demonstrated in increased numbers in various inflammatory and allergic disorders (Pilette et al., 2004; Vijayanand et al., 2010; Yang et al., 2004; Yoshie and Matsushima, 2015).

In alignment with this, the comparative (Venn diagram) analysis revealed CCL17, CCL22 and CCL27 exclusively up-regulated at week 8 post-infestation in CS compared to OS and C animals. High numbers of T cells have been demonstrated in CS skin (Liu et al., 2014b; Walton et al., 2010), CCL17, CCL22 and CCL27 might be the factors mediating the thymocyte extravasation into the skin and contributing to the local inflammation and tissue damage. Upon reaching the inflamed skin, T lymphocytes may encounter their mite specific antigen and secrete immune effector mediators inducing more and different chemokines and cytokines to maintain a state of inflammation that further enhances the development of skin lesions and finally leads to disease phenotype. Although we didn't detect receptors CCR4 and

CCR10 expression in our analysis, the increased expression of CCL27 and CCL17 may play a role in the pathogenesis of CS.

TARC/CCL17 levels are highly elevated in the blood of patients with allergic diseases such as AD, asthma and allergic rhinitis (Horikawa et al., 2002; Yoshie and Matsushima, 2015). Furthermore, due to increased CCL17 serum levels in AD patients it is now regarded as a biomarker for the disease and a serum TARC/CCL17 ELISA kit has been approved for AD diagnosis (Kataoka, 2014) and it would useful in future to explore serum levels of CCL17 in scabies.

In this analysis, chemokines CXCL2, CXCL6 and CCL4 were also exclusively up-regulated in CS pigs. CXCL2 is expressed at sites of inflammation and contributes to neutrophil activation, and is suggested to play a role in the pathogenesis of airway remodelling in asthma (Al-Alwan et al., 2013; Rouault et al., 2013).

CXCL6 (also known as granulocyte chemotactic protein 2, GCP-2) complements the activity of IL8 (exclusively up-regulated at week 8) as a neutrophil chemoattractant and activator, and contributes to the inflammatory response (Jovic et al., 2016). This strong up-regulation (over 64 fold up) of CXCL6 in CS may be due to increased inflammation at this time in infestation. This CXCL6 expression might further amplify trafficking of immune effector cells in the skin and development of essential T cells promoting the progress of the disease and exacerbated inflammation seen at this time in the infestation and is logical considering the up-regulation of IL8.

Chemokine CCL4 is a suppressive and chemoattractive factor and its expression on activated B cells and APCs recruits Tregs to initiate immune responses (Bystry et al., 2001). TGFβ1 marker for Tregs was also up-regulated here at this time (8wpi) point. CCL4 and TGFβ1 up-regulation is indicative of involvement of Tregs in CS which might be a way of regulating T cell and humoral responses to maintain immune system homeostasis. CCL4 expression also

provides a mechanism of how Tregs might be recruited during an immune response in CS. Also, even though Tregs may be present and function normally, effector T cells ($CD8^+$ T and Th17 cells) may resist/overcome the inhibitory effect of Tregs as has been seen in other inflammatory skin diseases (Tao et al., 2017). Increased $CD8^+$ T cells have been reported in CS cases (Walton et al., 2008; Walton et al., 2010) and in this analysis, we detected strong expression of a number of genes (prominent ones include IL17A and ARG1) indicative of involvement of Th17 cells.

Strong up-regulation of CCR7 was detected in this and earlier (scabies versus control animals, described in chapter 4) analysis. CCR7 chemokine receptor is involved in the trafficking of neutrophils and dendritic cells (DCs) from the periphery to the lymph nodes and may participate in the processes of antigen presentation and T cell proliferation and in this way, regulate adaptive immune responses (Beauvillain et al., 2011; Moschovakis and Forster, 2012; Riol-Blanco et al., 2005). Expression of CCR7 may facilitate neutrophil and DC migration to lymph nodes where they present antigens in an MHC-II dependent manner to naïve T cells and stimulate the proliferation of antigen-specific T cells (Beauvillain et al., 2011; Moschovakis and Forster, 2012; Riol-Blanco et al., 2005). Neutrophils are reported to cross-prime naive $CD8^+$ T lymphocytes and increased numbers of $CD8^+$ T cells have been reported in CS (Walton et al., 2008; Walton et al., 2010). This might be another pathway of immune cell trafficking and CCR7 signaling-driven mechanism mediating T-cell induced inflammation and may play a role in CS pathogenesis.

In line with this inflammatory chemokines gene expression, IPA analysis revealed various networks associated with late gene expression involved in inflammatory response generation and immune cell trafficking. This increase in many of the inflammatory factors indicates the strong influence of these activities late in the infestation. It is possible that expression of these

genes enhances the extent of inflammation and tissue damage that occurs upon infestation in CS.

5. 4. 3. 3. Pro-inflammatory response gene profile

Oncostatin M is a highly potent inflammatory cytokine and OSM gene expression was down-regulated early and then up-regulated late in the infestation in CS compared to OS pigs. It seems late in the infestation in CS, given the huge parasite load host cells are presented with strong antigenic stimuli and that might be triggering the OSM expression. OSM modulates the production of potent proinflammatory molecules and has been implicated in the pathogenesis of various cutaneous inflammatory and allergic diseases such as rheumatoid arthritis, asthma, allergic rhinitis, psoriasis and atopic dermatitis (Boniface et al., 2007; Cross et al., 2004; Kang et al., 2005; Richards, 2013). In addition, OSM is an important cytokine involved in keratinocyte activation and proliferation (Boniface et al., 2007; Rabeony et al., 2014). Psoriasis and atopic dermatitis are skin disorders associated with altered keratinocyte function. OSM ligand for oncostatin-M receptor (OSMR) is expressed on epidermal keratinocytes and the expression of their transcripts is enhanced in skin lesions from both psoriatic patients and those with atopic dermatitis. OSM also induces IL-4, a Th2 cytokine and supresses the Th1 response (Gazel et al., 2006) and IL-4 is produced in CS (Walton et al., 2010). Inducing OSM might be one way of facilitating/directing Th2 responses in CS and may explain why Th1 responses are not reported in CS. These results also suggest that OSM expression might play an important role in CS pathogenesis by effecting keratinocytes and might be involved in leukocyte trafficking to the inflammatory skin lesions, thereby contributing to the local cutaneous inflammation and tissue destruction in CS especially late in the infestation.

In Partek analysis, at 8wpi, expression of powerful inflammatory mediators including IL17A, CXCL6 and CCL20 were exclusively up-regulated in CS compared to OS skin samples. IL-17A is more proinflammatory compared to IL-17F which was detected in our first analysis (scabies versus control animals, described in chapter 4) and in OS skin. Also, in this and the first analysis, strong up-regulation of IL8 and its receptor IL8R was detected. In line with this, Venn diagram analysis revealed IL8, CXCL6, and CCL20 exclusively strongly up-regulated in CS compared to OS and C pigs at week 8 post-infestation. These molecules have been implicated in pathogenesis of psoriasis (Figure 5. 4) and contribute to the psoriatic condition by increasing the migration of effector immune cells to the sites of inflammation (Chiricozzi, 2014; Duan et al., 2001; Girolomoni et al., 2012; Lynde et al., 2014; Schutyser et al., 2003; Sticherling et al., 1999). The defining features of psoriatic skin are scaly raised plaques, cutaneous inflammation and infiltration of various immune effector cells - DCs, neutrophils, macrophages and T cells. Similarly, in CS, patients develop thick scaly crusts of skin and this inflammatory cell infiltrate has been demonstrated in CS skin. These results are indicative of involvement of these inflammatory molecules in disease pathogenesis and similar immune responses in CS as in Psoriasis. In addition, CCL20 exhibits antimicrobial activity (Yang et al., 2003), since secondary bacterial infections have been seen associated with CS, production of CCL20 may be important for host defense against bacterial pathogens.

5. 4. 3. 4. Th2 and allergic response gene profile

Increased production of the Th2 cytokines, IL-4 and IL-13, IgE and eosinophilia have been reported in CS (Mounsey et al., 2015; Roberts et al., 2005; Walton et al., 2010). Th2 biased and allergic responses have been implicated in the pathogenesis of various inflammatory skin disease such as Psoriasis, systemic lupus erythematosus (SLE), Atopic dermatitis (AD) and Rheumatoid arthritis (RA).

CD40 and CD40L exclusive up-regulation at week 8 in the skin of CS compared to OS and C animals was observed. CD40L expressed on the surface of T cells is a ligand for costimulatory protein CD40 which is expressed on APCs and primarily on B cells. CD40-CD40L interactions are essential for development of CD4 T-cell-dependent effector functions (Elgueta et al., 2009). CD40L upon cross-linking with CD40 generates a costimulatory signal which results in T and B cell proliferation, antibody isotype switching in B cells and enhances production of IL-4 (gene expression up-regulated here) and IgE (Elgueta et al., 2009). The Partek and Venn diagram comparative analysis revealed expression of IL13 only in CS at 8wpi which wasn't detected in the first analysis (chapter 4, scabies versus control samples). In addition, exclusive up-regulation of IL5 late in the infestation in CS compared to OS and C skin was detected. These type 2 cytokines cause eosinophil and B-cell maturation, differentiation and recruitment, and promote IgE isotype switching in B cells (Ngoc et al., 2005). IL-13 and IL-5 play an essential role in the pathogenesis of asthma and allergy (Ngoc et al., 2005). This exclusive IL13 and IL5 up-regulation observed here in CS skin is in line with increased Th2 activity, eosinophil and IgE and IgG responses seen in CS previously. Also, polymorphisms in IL13 gene are associated with increased IgE levels, asthma and AD (Granada et al., 2012; Paternoster et al., 2011; Wu et al., 2010), and IL13 might be the susceptibility locus for the dysregulation of IgE and allergy in CS.

In comparative analysis (using Venn diagrams), exclusive up-regulation of complement C3 at week 8 post-infestation in CS skin was observed. C3a, derived from degradation of C3, is a mediator of local inflammation, and activates and recruits' neutrophils, eosinophils, and mast cells in chronic inflammation and contributes to pathological processes in inflammatory and immunological diseases (Khan et al., 2015; Zhang and Kohl, 2010). Recently, C3a was shown to mediate the production of IL-17A by Th17 cells and control the disease severity in allergic asthma (Lajoie et al., 2010). Previously increased eosinophils have been detected in

CS cases and in this analysis, we detected IL17A expression in CS skin. This C3 up-regulation may be associated with complement-mediated inflammation via recruitment of eosinophils and neutrophils and through this mechanism may trigger the skin tissue injury, allergic immune responses and contribute to pathogenesis in CS. In addition, complement-mediated regulation of IL-17A may play a role in diseases severity in CS.

Furthermore, Partek and Venn diagram comparative analyses demonstrated CD40L along with CD3γ (T-cell receptor T3 gamma chain), CD3ε (T-cell receptor T3 epsilon chain) and iCOS, associated with iCOS-iCOSL signaling pathway, exclusively up-regulated at this time in infestation in CS pigs. iCOS-iCOSL signaling pathway is critical for adaptive immunity, allergy and in infectious diseases and stimulates T-cell activation, differentiation, effector responses, and T cell–dependent B-cell responses. It is also essential in CD40-CD40L mediated class switching of Ig isotypes and IL-4 production (Elgueta et al., 2009; Simpson et al., 2010).

Reported for the first time, IL19 gene was exclusively down-regulated early and then up-regulated late in infestation. IL-19 is a newly discovered cytokine produced under inflammatory conditions which induces Th2 cytokines on activated T cells and alters the balance of Th1/Th2 cells in favour of Th2. IL-19 has been reported in the serum of patients with asthma and might play an important role in the pathogenesis of asthma (Gallagher et al., 2004; Liao et al., 2004). Increased IL19 expression has also been reported in skin lesions of psoriatic patients and IL19 overexpression was reflected by elevated IL-19 serum levels that correlated with psoriasis severity. IL-19 is induced in keratinocytes by IL-17A (gene expression up-regulated here as well) and keratinocytes were found to be important targets of IL19. IL-19 has been shown to amplify the production of S100A7/8/9 and also pro-inflammatory cytokines IL-1β and IL-20 (gene expression strongly up-regulated here as well) (Witte et al., 2014). IL19 expression might contribute to the chronicity of the pathologic

interactions between immune and tissue cells. By inducing S100s and pro-inflammatory molecules it may support the infiltration of immune cells into the skin and may amplify the local inflammation, contributing to the CS epidermal alterations.

In addition, in Partek and Venn diagram comparative analyses, IL17RB was up-regulated exclusively and it is reported for the first time in CS. IL-17RB is the specific receptor for the proinflammatory cytokines IL-17B and IL-17E. IL-17B stimulates the release of TNF-α and IL-1β (Iwakura et al., 2011). IL-17E amplifies Th2 immune responses by inducing Th2 cytokine secretion and is also able to induce IgE production and eosinophilia (Iwakura et al., 2011). Expression of IL17RB may be a way of enhancing Th2 immune responses and might explain the increased IgE production and eosinophilia seen in CS, contributing to the disease pathophysiology.

These results provide insights about the mechanisms of Th2 and allergic responses and might explain the increased Th2 and antibody response in CS. This also highlights the bias towards a Th2 based immune response to infestation with *S. scabiei* in CS. In addition, Th2 responses with increased production of IL-4 and IL-13, can damage skin barrier integrity by supressing expression of genes which regulate skin barrier function (Howell et al., 2009; Kim et al., 2008). In addition, it has been demonstrated that mite *P. ovis* infestation in sheep results in the disruption of the epidermal barrier causing significant alterations in the expression of critical barrier components and epidermal pathology (Stoeckli et al., 2013). This analysis results and those observed by others in CS (Walton et al., 2010) indicate that the Th2 response might play a role in epidermal barrier disruption leading to increased pathogen invasion and allergen sensitization, and amplifying the inflammatory response thereby further augmenting the disease state. Also, this barrier disruption might to lead to secondary bacterial skin infections which have been seen associated with CS.

5. 4. 3. 5. Hyperkeratosis/skin pathology associated gene expression in CS

During the later stages of infestation in CS, hosts show thickened skin and scaly crusts and it is suggested that keratinocytes along with other inflammatory mediators - cytokines, immune cells and chemokines play a role in skin inflammation, lesion and crust development (Mounsey et al., 2013; Walton, 2010). In Partek and comparative analyses (using Venn diagrams), IL-20, a proinflammatory cytokine produced by keratinocytes, monocytes and endothelial cells, was found uniquely up-regulated at week 8 post infestation in CS skin. Scabies mites have been previously demonstrated to induce IL-20 in human skin equivalents (Morgan et al., 2013). IL20 mRNA expression in psoriatic skin lesions is strongly up-regulated and IL-20-treated keratinocytes have demonstrated up-regulation of disease-related and IFN-γ-induced genes (Otkjaer et al., 2005; Wang et al., 2006) and play a key role in the pathogenesis of psoriasis. During inflammation IL-20 mediates the keratinocyte proliferation and IL-20 is implicated in epidermal hyperplasia (Sabat et al., 2007). Furthermore, skin of transgenic mice overexpressing IL20 displays histological changes of thickened epidermis, hyperkeratosis, compact stratum corneum and elevated markers of hyperproliferation (Blumberg et al., 2001). This up-regulation of IL20 in CS might play a role in keratinocyte proliferation and development of thickened epidermis, scales and crusts seen in CS.

The S100 factors S1000A7, S100A8 and S1000A9 are calcium - activated signaling proteins. These proteins are important molecular mediators of acute and chronic inflammatory diseases such as psoriasis, atopic dermatitis, arthritis, atherosclerosis and microbial infections (Broome et al., 2003; Eckert et al., 2004; Goyette and Geczy, 2011; Oesterle and Bowman, 2015). Similar to other transcriptional studies (Burgess et al., 2010), in this analysis strong enrichment (up-regulation) of transcripts for S1000A7 (Psoriasin), S100A8 (calgranulin A) and S100A9 (calgranulin B) was observed in CS suggesting a role for these proteins in disease pathogenesis. These molecules were also seen up-regulated in our first analysis

(chapter 4). Psoriasin is an important effector molecule of the cutaneous barrier and is abundantly expressed in psoriatic and atopic dermatitis keratinocytes and it was found that epidermal barrier disruption significantly enhanced psoriasin expression (Broome et al., 2003; Glaser et al., 2009; Martinsson et al., 2005). Calgranulins S100A8 and S100A9 are markers of keratinocyte activation and have been demonstrated to play roles in hyper-proliferation and abnormal differentiation of keratinocytes in psoriasis (Benoit et al., 2006; Martinsson et al., 2005) and also result in the development of severe crusted lesions in sheep scab (Burgess et al., 2010; Burgess et al., 2012). S100A8 and S100A9 are proposed to act as damage-associated molecular patterns engaged in innate immunity and initiate pro-inflammatory responses. They regulate processes leading to leukocyte adhesion and transmigration, which further exacerbates the inflammatory responses (Goyette and Geczy, 2011; Oesterle and Bowman, 2015; Ryckman et al., 2003). IL-17A (gene expression up-regulated here as well) promotes S1000A7, S100A8 and S1000A9 expression (Glaser et al., 2009; Kido et al., 2005). This increased expression of S100 genes in mite infested skin could further amplify an already prolific inflammatory response and these results also indicate their role in the development of skin lesions and crusts observed in CS cases.

In Venn diagram comparative analysis, late in the infestation the unique up-regulated genes were more in number than down-regulated genes in CS pigs. Also, late in the infestation (at week 8) the unique up-regulated genes detected were more in number compared to week 1. This gene expression likely reflects the host response to the mites and may be associated with clinical disease signs and inflammation observed at this time in the infestation.

The number of genes down-regulated at week 8 was lesser (down by 6 times) compared to week 1. This gene down-regulation seems to be age related as the pigs are more mature at this time point. Down-regulation also seems to play a role in mite growth and in CS disease susceptibility as early in the infestation more genes were down-regulated in CS pigs. Possibly

as the clinical disease and inflammation have appeared at this time point in the infestation so down-regulation is less or not any more required.

In contrast, in comparative analysis, in OS compared to CS and C pigs, a greater number of genes (898) were up-regulated at week 8 post-infestation (Figure 5. 3) which may reflect protective response observed in OS at this time point onwards. Up-regulation of few inflammatory molecules including IL6, IL17F and IL15, and down-regulation of STAT3 and AGP here may be related to the inflammation observed late in the infestation in OS cases (Cheung et al., 2008; Hochepied et al., 2003; O'Shea and Plenge, 2012; Read et al., 2016). IFNγ and C6 down-regulation may be responsible for mite infestation and clinical signs in OS (Schoenborn and Wilson, 2007; Varela and Tomlinson, 2015). Venn diagram comparative analysis also revealed CD70 and CD207 exclusively up-regulated in OS skin, which play roles in immune response generation via antigen processing and presentation, B-cell activation, cytotoxic function of natural killer cells and antibody production (Borst et al., 2005; Hunger et al., 2004). In addition, up-regulation of SAA1 at this time in infestation may down-regulate the inflammatory process by modulating neutrophil function (Gatt et al., 1998). Furthermore, down-regulation of potent proinflammatory molecule OSM (Boniface et al., 2007; Richards, 2013) late in the infestation (week 8) indicates another defence mechanism of controlling the inflammatory responses and hence the disease in OS.

5. 4. 3. 6. Miscellaneous – other important genes

Intercellular adhesion molecule 3 is important in primary immune response and plays a significant role in clearance of apoptotic immune cells during phagocytosis (Kristof et al., 2013). The analysis results show ICAM3 related to apoptotic cell clearance is strongly repressed early in the infestation. Lack of ICAM3 would result in decreased clearance of apoptotic cells which can result in the release of the intracellular content of apoptotic cells

and might contribute to the pathophysiology of recurring inflammations and disease pathogenesis (Nagata et al., 2010; Nathan and Ding, 2010). Partek and Venn diagram comparative analysis then revealed ICAM3 exclusively up-regulated at week 8 post-infestation in CS skin. ICAM3 up-regulation late in the infestation indicates the immune response related to phagocytosis and clearance of the apoptotic cells, and might be a way to maintain tissue homeostasis.

Increased up-regulation of ARG 1 and moderate ARG2 was detected in this and earlier (scabies versus control animals, chapter 4) analysis. Arginase is involved in the pathogenesis of schistosomiasis as enhanced peripheral blood mononuclear cell ARG1 expression and increased arginase activity in peripheral blood has been demonstrated during murine and human *Schistosoma mansoni* infection (Barron and Wynn, 2011; Getaneh et al., 2015; Grzych et al., 1991; Rotondo et al., 2011). Furthermore, ARG1 has been shown in peripheral blood of patients with active autoimmune disease systemic lupus erythematosus (SLE). Myeloid-derived suppressor cells (MDSCs) are thought to contribute to SLE pathogenesis via arginase 1 as increased numbers of these cells were detected in patients with SLE, and MDSCs produced high levels of arginase 1 correlating positively with the disease severity (Wu et al., 2016). Th17 cells producing IL-17 are effector immune cells in various inflammatory diseases and MDSCs are highly efficient in stimulating Th17 cell differentiation and promote tissue inflammation and pathogenesis (Guo et al., 2016; Wu et al., 2016). Furthermore, MDSCs from SLE patients displayed increased capacity to drive Th17 cell differentiation in an Arginase 1-dependent manner (Wu et al., 2016). Previously, arginase 1 was modestly induced by scabies mites in human skin equivalents (Morgan et al., 2013). In this analysis, along with strong up-regulation of ARG1, IL17A expression was also up-regulated at this time (8wpi). These results indicate that ARG1 production may further exacerbate inflammation by promoting Th17 cell differentiation and IL-17 production in CS,

and also provide a mechanism of disease pathogenesis in CS. Our results further implicate that Th2 cytokine regulated arginase as a possible inflammatory pathway with significant ramifications on the manifestations of the disease as Th2 specific cytokines IL-4, IL-5 and IL-13 (gene expression up-regulated here as well) induce ARG1 and ARG2 gene transcripts, associated with pathogenesis of allergic asthma (Li et al., 2006; Zimmermann et al., 2003).

IL-10 is a potent immunosuppressive cytokine and helps to diminish the collateral damage caused by exaggerated inflammation. IL-10 down modulates inflammation and T cell responses during antigen-specific immune responses by preventing APCs from producing pro-inflammatory cytokines, and up-regulating molecules involved in antigen presentation and lymphocyte activation (Iyer and Cheng, 2012; Moore et al., 2001). Given that Tregs and Th2 cells are a source of IL-10 and up-regulation of CCL4 and TGFβ1, IL19, IL17RB, CD40L, CD3γ, CD3ε and iCOS genes at 8wpi is indicative of involvement of Tregs and Th2 here in CS pigs. Interestingly in this analysis, IL10 gene expression was not detected and also reduced IL-10 secretion in both PBMCs and lesional skin of CS cases has been reported (Walton et al., 2008; Walton et al., 2010). Deficiency of IL-10 leads to inflammation due to excessive activation of TLR signaling pathways, dendritic cells (DCs) and also increase IL-17-secreting T cells resulting in a Treg/Th17 dysfunctional immune response and severe pathology (Gonzalez-Lombana et al., 2013). It seems mites suppress the IL10 gene expression especially late in the infestation which might exaggerate inflammation and also result in exacerbated pathology and tissue damage via IL-17 production as IL17A gene expression was detected at week 8. Also, this lack/absence of IL-10 may be an essential contributing factor in the inflammatory response and immunopathology observed late in the infestation particularly in CS.

5. 5. Limitations

In this analysis, at all-time points of the study over 1000 genes were differentially expressed except at week 4 (264 genes). The quality (RIN values) and quantity of week 4 total RNA and cRNA samples were congruent with the recommendations and were in similar range as of other time point samples. The protocols during the experimentation were also followed strictly as recommended. Furthermore, during the differential gene expression and statistical analysis same parameters were applied for each comparison and time point data analysis.

For differential gene expression, FC cut-off of ≥ 2 to eliminate false positives was applied and also with the assumption that larger FC cut-offs will preferentially detect/select the genes with the most biological significance, and that are actually influencing the disease. Genes expressed with minimal/low FC (e.g., IL22 and IL23) in various inflammatory skin diseases (Coda et al., 2012; Suarez-Farinas et al., 2010) which might have not passed the FC cut-off might prevent the identification of significant associations with the host responses and disease related pathways.

In this analysis, gene expression results were collated from combining the samples (n=4) and variation in the fold changes for particular genes on the individual animal level may exist. Also, it is acknowledged that the lack of replicates is a source of variation in transcriptomics performed as only 1 skin sample per animal each time was examined. The numbers of biopsies collected from the pig ears also has ethical considerations being mindful that additional biopsies were collected for cellular immunology and histology studies in the overall trial related to this work.

5. 6. Conclusion and future directions

In the first analysis, comparing scabies vs control animals (described in Chapter 4), early (at week 2 and 4) down-regulation and then at week 8 increased gene up-regulation was observed which is in line with previous findings in primary infestations.

In this analysis, comparing CS to OS pigs, post mite infestation mostly a trend of gene down-regulation was observed. Given the less parasite burden early in the infestation, gene down-regulation was still evident, and disease manifestations and inflammation did not show till week 4. It seems in CS cases, early in the infestation the host seems to be more susceptible and is in part responsible for this gene down-regulation which might be playing a role in disease pathogenesis and development. Late in the infestation (week 8), it seems the mites which are grown in number are also down-regulating/supressing the gene expression. In addition, the differences in gene expression prior to infestation suggests that genetic susceptibility might be associated with the molecular and pathologic diversity seen in CS. Interestingly, in this analysis, at week 8 post-infestation, among the genes expressed, a large number of the up-regulated genes were involved in pathogenic inflammatory and allergic responses in CS. On contrary/the other hand, in OS cases, week 8 onwards it has been seen that host protective responses start to develop (Walton, 2010). This up-regulation of inflammatory genes in CS may be in response to the large antigenic material provided by the mites grown in number at this time in infestation, and responsible for the disease phenotype (hardened skin and crusting).

In summary, this transcriptomic analysis of the lesional skin in CS relative to OS has revealed enrichment of many genes possibly associated with the pathophysiologic pathways implicated in psoriasis, rheumatoid arthritis, AD, asthma, SLE, Th2 and Th17 pronounced responses. The analysis has provided further insights in the events which drive the host

response towards an allergic Th2 type profile, involving T-cells, B-cells and IgE production and mechanisms by which mites induce increased antibody production in CS. It has provided key insights into the broader proinflammatory and allergic responses in CS and role of various immune effectors cells, molecules and associated pathways in the development of the crusted pathology.

Early in the infestation in CS pigs, *S. scabiei* mites and host seem to influence the process of antigen presentation by transcriptional control of MHC class II molecules via down-regulating expression of RFX5 and by inhibition of expression of several genes involved in the process of DC maturation. This inhibition of MHC class II antigen and DC mediated presentation via transcriptional regulation of MHC and DC function associated molecules presents an additional mechanism to control or subvert host immune responses in CS.

CD70 is acquired upon DC maturation during infection or inflammation and via CD27-CD70 interactions regulate the generation of adequate T cell responses (Borst et al., 2005; Hashimoto-Okada et al., 2009; Keller et al., 2008). The analysis results indicate that the CD70 gene and CD27 signaling associated gene down-regulation early in the infestation may impact/influence the formation of $CD4^+$ and $CD8^+$ effector T cell pools via CD27-CD70 interaction at local tissue sites in primary responses in CS cases. The results present an opportunity to look into CD70 molecule as it may be a good target for immunotherapy and further clarifications of the mechanism of CD27-CD70 are warranted in CS. Apart from using anti-CD70 therapy in stimulating the anti-scabietic immune response, other opportunities to exploit CD27 signaling and CD27-CD70 interactions in CS disease treatment present themselves.

Expression of skin associated inflammatory chemokines CCL27 and CCL17, late in the infestation when the disease signs and inflammation had occurred, may play a role in both skin homeostasis and the initiation and enhancement of skin inflammation by mediating T

cell recruitment to the skin lesions as seen in inflammatory skin diseases (Homey et al., 2002; Xiong et al., 2012; Yoshie and Matsushima, 2015). CCL27 and CCL17 chemokines mediate lymphocyte recruitment upon interaction with their receptors – CCR10 and CCR4. The expression patterns and the molecular mechanisms underlying these receptors are unclear in CS and in future CCL27 - CCR10 and CCL17 – CCR4 interactions may be targets for the development of novel and selective therapies for CS.

IL-17A, hallmark cytokine of Th17 cells, is a highly potent inflammatory mediator which profoundly participates in the pathogenesis of multiple immune mediated diseases (Fouser et al., 2008; Jin and Dong, 2013). Cytokine IL-23 is one among the various factors essential/important in Th17 cell differentiation and IL-17A production (Fouser et al., 2008; McGeachy and Cua, 2008). Chronic/increased Th17 cells and expression of IL-17A can be deleterious in the context of skin inflammation. Studies by our group have demonstrated IL-17 and IL-23 secretion in the skin of pigs with CS (Liu et al., 2014b; Mounsey et al., 2015) and in this analysis up-regulation of IL17A supports the involvement of Th17 cells and IL-17A in CS cases. IL-23 and IL-17 are promising targets and their antagonism might be a promising therapy for the treatment of CS. Biologic immune based therapies are currently in clinical trials for various inflammatory diseases and efficacy has been seen in clinical trials in psoriasis. Antibodies that block IL-23 have been demonstrated to prevent the infiltration and expansion of Th17 cells in the skin. IL-12/IL-23p40 neutralizing antibodies have significantly reduced IL-17A, IL-17F, and IL-22 transcripts in the skin of animals (Beroukhim et al., 2015; Campa et al., 2016).

Arginase 1 and 2, induced in myeloid cells, myeloid-derived suppressor cells (MDSCs), macrophages and neutrophils, by pro-inflammatory cytokines, are among the main arginine-catabolizing enzymes involved in inflammatory immune responses during pathological conditions through mechanisms that use mediators of this unique metabolic pathway

(Munder, 2009; Rodriguez et al., 2017). Elevated ARG1 expression in SLE patients facilitate Th17 cell differentiation from naïve $CD4^+$ T and augmenting inflammatory Th17 response and SLE disease activity (Wu et al., 2016). These results indicate that the up-regulation of ARG1 and ARG2 may promote immune dysfunction via depriving T cells of essential metabolites such as arginine. The inflammation observed in CS pigs late in the infestation may cause expansion of MDSCs which may result in expression of ARG1 promoting Th17 cell proliferation and thereby enhance the inflammation and disease activity in CS. A better understanding of arginine metabolic pathways within the inflammatory environments in CS cases will facilitate the development of novel therapeutic interventions in CS. Also, ARG1 expression in Th17 proliferation instructed by arginine metabolism can be targeted for the therapeutic approaches.

This study has refined our understanding of CS pathogenesis and provided information about various pro-inflammatory mediators and molecules in pathways which could be used as markers for disease severity and therapeutically targeted for patients with CS.

CCL17 could be used as biomarker in the diagnosis of CS or in tracking its progress. Increased expression of molecules such as S1000A7 (Psoriasin), OSM, IL17A, IL20, IL19, CXCL6, CCL20, CD70, CCL27, ARG1 in this analysis and increased expression also of these mediators in human and animal inflamed tissues support the assumption that they play an essential role in the immunopathogenesis of CS. For this reason, it is postulated that these mediators can be used as promising therapy targets of an inflammatory cascade in CS.

CCR7, CD27-CD70, CCL27-CCR10 and CCL17-CCR4, CD40-CD40L, iCOS-iCOSL, JAK-STAT signaling pathways can also be targeted to prevent immunopathology.

Th17 and $CD8^+$ T cells are a primary cellular source of proinflammatory cytokines and mediate immune cell activation and tissue inflammation in a range of inflammatory and immune-mediated diseases. The increased expression of genes associated with Th17 and

$CD8^+$ T cell proliferation and recruitment to inflamed tissue indicates their role in the pathogenesis of CS. The infiltration of Th17 and $CD8^+$ T cells in the skin and their proliferation can be prevented by using antibodies against molecules such as CCL17, CCL27 and IL23 to alleviate disease activity in CS.

STAT1, TLR8, IL13, RFX5 gene mutations or polymorphisms have been demonstrated as susceptibility loci for the immune dysregulation in various inflammatory and allergic diseases. In future, these loci can be an area of investigation to ascertain the association of these genetic risk factors in immune dysregulation and CS pathogenesis. In addition to this, genetic variants in STAT6, high affinity receptor for IgE (FCER1A) and HLA-DRB1 genes have been found to influence susceptibility to various infectious and immune-mediated diseases (Backes et al., 2013; Okada et al., 2014; Qian et al., 2014), could be potential determinants of impaired immune response and IgE concentration in CS.

Also at the same time further investigations through genome-wide replicative studies and the studies of additional genes and pathways are required. IL-22, IL-6 and CCR4 are expressed by inflammatory cells infiltrating the skin and play a crucial role in the pathogenesis of cutaneous inflammation and tissue damage in various inflammatory diseases such as psoriasis, SLE and AD. Although their expression wasn't detected in this analysis, given their importance and association with inflammatory responses and immune mediated diseases, these molecules warrant an investigation in CS.

Transcriptional profiling at a tissue level or measuring cell-specific transcriptome allows analysis of genome wide targets and assessment of the impact of the various signaling pathways and molecules. This can also be helpful in the development of tools to elucidate the molecular mechanisms/signatures of CS disease with diagnostic and possibly prognostic values. Furthermore, the cohort of crusted skin associated DEGs should provide information to focus future candidate gene sorting for the precise localization of disease susceptibility,

pathogenesis or progression loci. In future genetic analyses the gene candidates search can be narrowed by focussing on transcriptionally dysregulated genes particularly within the reported susceptibility loci like RFX5, TLR8, IL13, etc. as mentioned earlier.

Gene expression has been shown to be dramatically influenced by DNA chromatin organization and epigenetic modifications, and studies in this regard could reveal specific genes regulated by such mechanisms and modifications in the context of immune and inflammatory responses underlying diseases susceptibility and pathogenesis.

Chapter 6

Acute phase protein response in pigs with sarcoptic mange.

6. 1. Introduction

The acute phase protein response is an innate reaction rapidly triggered by external or internal challenges such as infection, tissue injury or stress (Gabay and Kushner, 1999; Moshage, 1997). The acute phase protein response consists of substantial alterations to the serum concentrations of acute phase proteins (APPs), a diverse group of highly conserved proteins (Eckersall and Bell, 2010; Petersen et al., 2004). Generally, APPs are present at very low levels in healthy animals and any variation is suggestive of underlying infection, injury or inflammation (Cray et al., 2009). APPs are mainly secreted by hepatic cells upon stimulation from pro-inflammatory cytokines such as transforming growth factor (TGF) β, tumor necrosis factor (TNF) α, interferon (IFN) γ, interleukin (IL) 1, oncostatin M (OSM), IL-8, IL-10, IL-6 and IL-6 type cytokines (Ebersole and Cappelli, 2000; Eckersall and Bell, 2010; Petersen et al., 2004; Slaats et al., 2016). APPs are involved in a range of supportive immune functions including chemotaxis, coagulation, opsonization, immunomodulation, iron regulation, wound healing and angiogenesis. These proteins are also involved in anti-inflammatory, antioxidant and protease inhibitory activities (Ceciliani et al., 2002).

The production of APPs can either increase (positive APPs) or decrease (negative APPs) in response to inflammatory stimuli (Eckersall and Bell, 2010; Petersen et al., 2004). APPs are classified on the basis of magnitude of their increase. Major APPs are rapidly produced upon stimulation and show very high increases (100 – 1000-fold), peak at 24–72 hours and then decrease rapidly during the recovery phase. Moderate APPs rise to 5 – 10-fold on stimulation,

peak after 3-5 days and then decline slowly. While minor APPs rise gradually between 1–2-fold and remain elevated for weeks (Cray et al., 2009; Petersen et al., 2004).

In pigs, some of the main APPs include haptoglobin (HP), transferrin (TF), serum amyloid A (SAA), major acute phase protein (MAP), C-reactive protein (CRP), and alpha-1-acid glycoprotein (AGP) (as reviewed in Eckersall and Bell, 2010; Petersen et al., 2004).

Transferrin is an iron-binding protein and likely restricts availability of ferric ions to bacteria and parasites which may inhibit their growth providing protection against infection (Ceciliani et al., 2012; Law, 2002; Yang et al., 2003). It must be noted that TF is a negative APP and usually decreases in inflammation. The levels of host transferrin in scabies infestation in pigs or humans have not yet been determined.

Haptoglobin binds hemoglobin and is the principal scavenger of free hemoglobin in blood. HP is usually anti-inflammatory dampening the inflammatory responses by inhibiting neutrophil activity and suppressing also T cell differentiation through the inhibition of T helper (h) 2 cytokine secretion (Arredouani et al., 2005; Ceciliani et al., 2012; Oh et al., 1990).

Autoantibodies to TF and HP have been demonstrated in mange positive pigs, which is suggested to occur as a mechanism of sequestering transferrin in transferrin-antibody complexes and thereby further limiting iron to the mites (Toet et al., 2014; Zalunardo et al., 2006). Alternatively, TF autoantibody stimulation could be due to antigenic cross-reactivity as similar antigenic determinants (epitopes) between *Sarcoptes scabiei* TF molecules and pig TF might exist. This might result in stronger immune response and may complicate further inflammatory conditions (Kurosky et al., 1980; Zalunardo et al., 2006).

Serum amyloid A is involved in chemotactic recruitment of leukocytes to the site of infection and implicated in the pathogenesis of inflammation (Badolato et al., 1994; Xu et al., 1995).

SAA has been demonstrated as a more sensitive indicator of acute than of chronic inflammation (Alsemgeest et al., 1994; Horadagoda et al., 1999). Further activity of SAA includes a role in opsonization and acts as a pattern recognition protein increasing macrophages and neutrophil phagocytosis of bacteria (Ceciliani et al., 2012).

Alpha-1-acid glycoprotein (also known as orosomucoid or ORM) has anti-inflammatory properties, dampens neutrophil activation (Fournier et al., 2000) and can potentially inhibit neutrophil migration to the site of infection (Barroso-Sousa et al., 2013; Mestriner et al., 2007). AGP also inhibits lymphocyte proliferation (Itoh, 1989), natural killer cell activity (Okumura et al., 1985) and increases the secretion of IL-1 receptor antagonist by macrophages (Fournier et al., 2000). AGP was generally described as an archetypical positive acute phase protein as increased AGP production was demonstrated during experimental infection with bacteria and virus in pigs (Greiner et al., 2001; Son et al., 1996; Tecles et al., 2007) but others have shown no change in its concentration during inflammation (Asai et al., 1999; Eckersall et al., 1996; Lampreave et al., 1994). However, recent studies have shown AGP behaving as a negative acute phase protein during a range of experimental infections and aseptic inflammation in pigs (Heegaard et al., 2013; Kahlisch et al., 2009; Skovgaard et al., 2010; Yang et al., 2012).

There are no reports regarding APP response to *S. scabiei* infestation in pigs or humans. There are a few reports of APPs in sarcoptic mange in Ibexes (Raez-Bravo et al., 2015; Rahman et al., 2010), in pigs with *Streptococcus suis* infection (Sorensen et al., 2006) and in sheep with mite *Psoroptes ovis* infestation (Wells et al., 2013). SAA, AGP and HP were all significantly increased in the serum of sarcoptic mange positive ibexes (Raez-Bravo et al., 2015; Rahman et al., 2010). SAA and AGP were classified as major APPs, and HP as a minor APP (Rahman et al., 2010) while others considering them all as moderate APPs (Raez-Bravo et al., 2015). Also, these studies reported higher APP response (AGP and SAA) in severely

mange affected compared to less affected ibexes indicating a relation between disease severity and APP response (Raez-Bravo et al., 2015; Rahman et al., 2010).

In experimental infestation of sheep with *P. ovis*, significant levels of HP (10-fold increase) and SAA (1000-fold increase) were detected at 4 weeks post-infestation (Wells et al., 2013). The study authors suggested HP and SAA can be useful as early disease biomarkers, potentially in combination with antibody testing, which would be a highly specific test for detecting pathogen specific infestation and current disease status (Wells et al., 2013).

From the above reports, it is highly likely that acute phase protein responses in pigs occurs after the mite infestation, but it is not clear yet at what time point in infestation it starts to occur. Even though increased APP production is correlated with clinical signs and pathological lesions (Raez-Bravo et al., 2015; Rahman et al., 2010), APP gene expression (detected in our microarray analysis) occurred prior to onset of clinical disease reflecting some degree of difference in the response. Overall APPs have not been sufficiently studied in scabies and the variability of the results makes it difficult to draw any conclusions and therefore, the dynamics of acute phase response in scabies is not well understood.

In the gene expression analysis (described in Chapter 4 and 5), significantly differentially expressed genes (DEGs) were found to be associated with acute phase signaling pathways at several time points (week 0, 1, 2 and 4) pre-and post-infestation in mite infested vs non-infested (control) pigs. In addition, microarray analysis revealed up-regulation of transcripts of OSM and interferon (IFN) γ at week 0 and 1wpi, and as previously mentioned these cytokines are potent stimulators of the APP production.

Diagnosis of *S. scabiei* infestation is currently based on the observation of clinical signs and skin lesions, confirmed by the isolation and identification of the mites. Accurate diagnosis of scabies can be challenging as affected humans and animals are asymptomatic during early

infestation, and clinical signs appear late (after 4-6 weeks post mite infestation) in first exposure to mites and can be easily missed. Asymptomatic affected humans and animals represent a source of infection to healthy counterparts and play a significant role in disease transmission. The disease burden and difficulties in disease diagnosis emphasize the need for a sensitive and specific test which would allow early detection of infestation and determine current disease status.

This study investigates quantitative serum levels of AGP, SAA, TF and HP over time in the same pigs used for microarray analysis (described in Chapter 4 and 5) during experimental infestations with *S. scabiei,* to further characterize acute phase protein response patterns and elucidate differences in response patterns. These proteins were selected because they are among the strongly reacting APPs in most species (Eckersall and Bell, 2010; Petersen et al., 2004) as have been demonstrated following experimentally induced inflammation or infection, including with *S. scabiei* (Gonzalez et al., 2008; Raez-Bravo et al., 2015; Rahman et al., 2010). Importantly initial microarray analysis revealed differential gene expression of some of these proteins including HP, TF and SAA (Table 6. 1) which was prompting and called forth for further investigation in the APP response in these pigs. It was decided to analyze the APPs in early time point samples to attempt to identify disease markers early in the infestation which could be used to diagnose the condition early and facilitate effective management and treatment of scabies prior to specific disease outcomes. The findings of this study will add to our knowledge base regarding the regulation of APPs, response dynamics and may be used to guide in the potential identification of appropriate APPs for use as a diagnostic and prognostic tool in scabies.

Table 6. 1. Gene expression of acute phase response proteins. In Partek, gene expression was carried out with 2-way ANOVA combined with a Fisher's Least Significant Difference (LSD) post-hoc test across infested CS (n=4), OS (n=4) and non-infested C (n= 4) samples at time points 0 week pre-infestation, 1, 2, and 4 weeks post-infestation. Genes with a p-value of ≤ 0.05 and fold change of ≥ ± 2.0 at each time point are indicated. CS = Crusted scabies, OS = Ordinary scabies, C = Control, FC = Fold change.

Comparison	Gene	Gene Description	Week 0 (FC)	Week 1 (FC)	Week 2 (FC)	Week 4 (FC)
CS vs OS	HP	Haptoglobin	3.89		-3.12	
	SAA	Serum amyloid A	-5.43	-3.02	2.66	2.23
	TF	Transferrin	9.67		-3.12	
CS vs C	HP	Haptoglobin	2.08		-2.2	
	SAA	Serum amyloid A	-5.48	-4.30	2.27	
	TF	Transferrin	5.6	2.70		
OS vs C	SAA	Serum amyloid A			-3.54	
	TF	Transferrin			2.02	-2.44

6. 2. Materials and methods

6. 2. 1. Sample collection

The samples used in this analysis were from the same 12 pigs (4 CS, 4 OS and 4 controls) described/used in the microarray study (chapter 4 and 5). One control sample at week 2 was substituted with another control sample of the same time point because of the non-availability of plasma from this animal. Blood was collected from each animal at several time points during the trial. For the acute phase protein analysis, we analyzed three time points: baseline (week 0 pre-infestation), weeks 2 and 4 post-infestation. Approximately 10mL of blood was collected from the cephalic vein or anterior vena cava into Lithium Heparin CPT Tubes (BD Biosciences, Australia) or Serum Tubes (ThermoScientifc, Australia). Plasma was prepared by centrifuging whole blood at 1,000 g for 15 min at room temperature. Plasma samples were then aliquoted and stored at −80 °C until use.

6. 2. 2. ELISA analysis

The concentration of HP, SAA, TF and AGP was measured by using commercially-available ELISA kits (pig HP #AB205091 and AGP #AB205068 from Abcam, Australia; multispecies SAA #KAA0021 from Life Technologies Pty Ltd, Australia and TF #CSB-E13764P from Cusabio, China) in accordance with the manufacturer's instructions. Prior to the assay optimal dilutions were determined using pooled plasma from three pigs with CS at week 8 post-infestation. For the final assay, all plasma samples were diluted in the provided kit buffer as per manufacturer's instructions (1: 100 for TF; 1: 500 for SAA; 1: 10,000 for HP and 1: 20,000 for AGP). The TF assay was a competitive inhibition enzyme immunoassay technique in which the microtiter plate was pre-coated with TF instead of/ with anti-transferrin antibodies. In the end of this assay the measured intensity of colour is inversely proportional to the amount of TF in the samples. Samples were tested in duplicate alongside standards and the absorbance was read at 450 nm using 630nm as a reference. The blank standard absorbance value was subtracted from all other sample and standard absorbance values. The absorbance values of replicates of all samples and standards were averaged. Standard curves (Figure 6. 1) were generated from the average absorbance values of standards using CurveExpert Basic (Version 1.4, www.curveexpert.net) and a best fit curve through the points on the graph was drawn. CurveExpert computes correlation coefficient (r) using Pearson Correlation to infer linear relationship between the variables. The closer r is to 1 the better the approximation and usually an $r > 0.97$ is recommended for a good linear approximation. To determine the concentration of APPs in the plasma samples, the absorbance values of the samples were interpolated against the standard curve, again using the CurveExpert software. The concentration was then multiplied by the sample dilution factor for each protein accordingly.

The acute phase protein concentrations were compared and tested for statistical significance in Graph Pad Prism (Version 7.0, Graph Pad Software Inc.) using repeated measures two-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test. Differences with $p < 0.05$ were considered as significant.

6. 3. Results

6. 3. 1. Quantitative analysis of acute phase proteins

The standard curves generated for HP, SAA, TF and AGP standards showed r values of > 0.99 (Figure 6. 1) indicating a good association/relationship between the variables. In the 2-Way ANOVA analysis, the source of variation in the means was attributed to several factors including interaction, time, phenotype (group) and animals (matching).

TF and AGP showed increases in all groups at week 2 and 4 compared to pre- week 0 levels (Figure 6. 2B and 6. 2C). However, no significant differences were observed in SAA and TF concentration in plasma when comparing CS with OS, CS with C and OS with C pigs at any time point of the study period. AGP and HP showed significant difference ($p = 0.0187$ and $p = 0.0311$) upon comparing CS with C and OS with C samples respectively at week 4 (Figure 6. 2C and 6. 2D).

TF levels detected were the highest among the APPs analyzed. The TF levels in plasma of CS pigs were 8.66 mg/mL at week 0 which increased to 21.83 mg/mL at week 2 and 18.97 mg/mL at week 4. TF levels in OS and C animals showed a trend similar to CS animals during the experimental period (Table 6. 2).

HP levels in plasma of CS pigs showed a mean of 0.38 mg/mL at week 0 which decreased to 0.22 mg/mL at week 2 post-infestation and returned to around pre-infestation levels at week 4. On the other hand, in OS samples HP levels at baseline were 0.56 mg/mL, which declined

sharply to 0.06 mg/mL at week 2 and then a slight increase was seen (0.11 mg/mL) at week 4. Control samples showed HP levels of 0.10 mg/mL, 0.19 mg/mL and 1.11 mg/mL at week 0, 2 and 4 respectively (Table 6. 2). In the control group one of the sample showed a higher mean (3.11mg/mL) than the other three which might have contributed to the difference observed between OS versus C at week 4.

AGP increased from a week 0 concentration of 0.42 mg/mL to 1.84 mg/mL at week 2 and elevated further to 2.08 mg/mL at week 4 in CS pigs. In AGP, time (p 0.0001) and clinical phenotype were both significant contributing factors for the mean variation. In OS animals, a trend similar to CS animals in AGP response was observed at baseline and week 2 but the levels were a little lesser at week 4 compared to CS counterparts. Healthy (C) animals showed levels similar to OS pigs at all-time points of the study (Table 6. 2). A significant difference in mean values of AGP (p .0187) was detected at week 4 in CS compared to C pigs.

The SAA concentration in plasma of pigs with CS post-infestation at week 2 (9.51 μg/mL) dropped 5-fold as compared to pre-infestation levels (49.30 μg/mL). Between weeks 2 and 4 post-infestation SAA levels increased around 2.5-fold (from 9.51 μg/mL to 24.09 μg/mL). In contrast, OS pigs showed around 2-fold increase in SAA levels (21.17 μg/mL) at week 2 post-infestation from pre-infestation levels (11.66 μg/mL). These values returned to around pre-infestation levels (12.92 μg/mL) at week 4. Control pigs showed a trend similar to CS animals (Table 6. 2).

In this analysis, large errors in the mean in SAA in CS (SEM ± 37.5) and C (SEM ± 25.27) samples at week 0 was observed and this was often caused by an outlier pig in the groups (Figure 6. 2A).

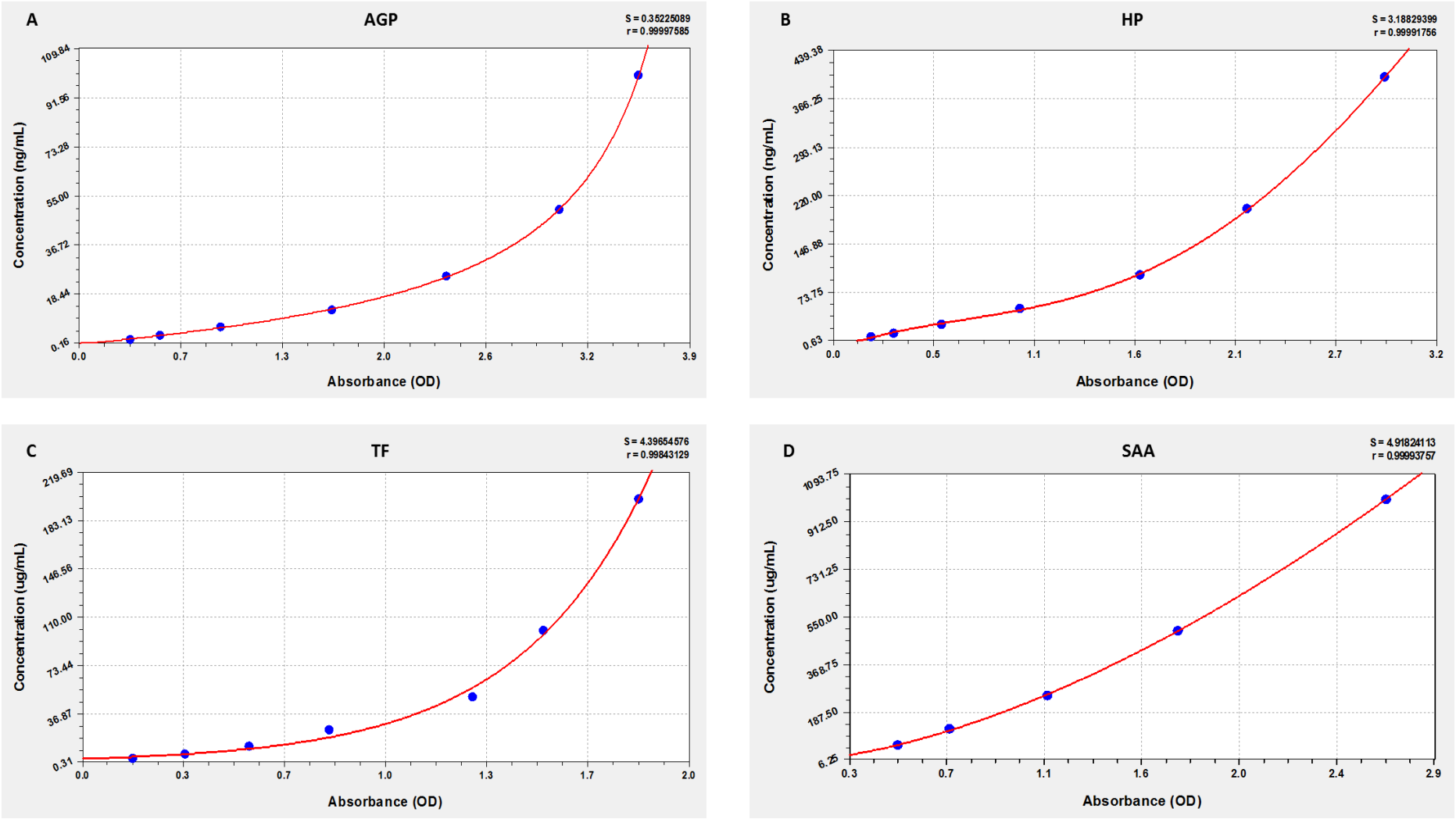

Figure 6. 1. Standard curves of pig SAA, HP, TF and AGP standards. Standards were tested in duplicates and the absorbance was read at 450 nm using 630nm as a reference. Absorbance values were averaged and using CurveExpert Basic (Version 1.4, CurveExpert Software) software standard curves were generated. A = AGP, B = HP, C = TF and D = SAA standard curve. r = 0.99.

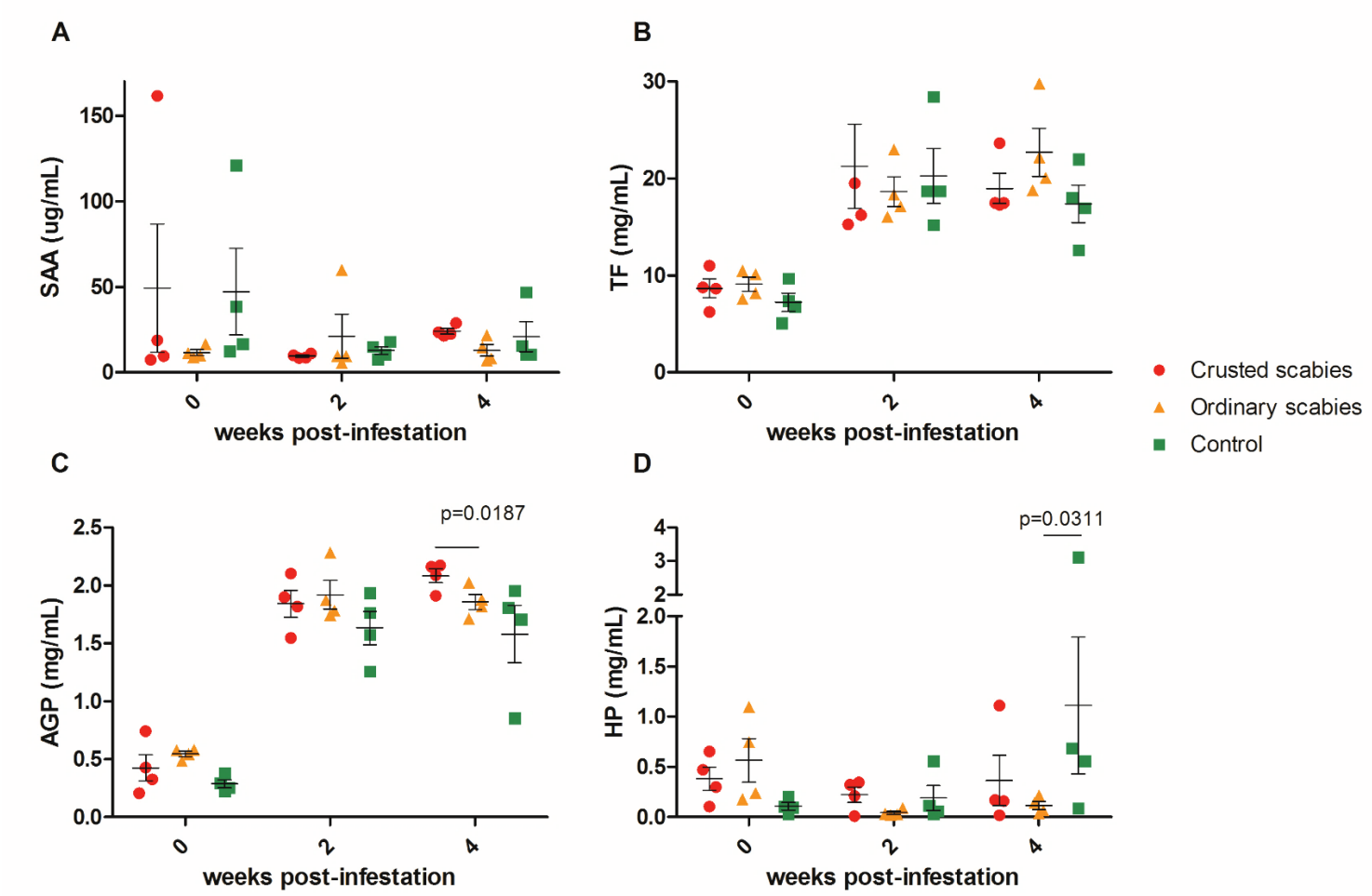

Figure 6. 2. Quantitative analysis of plasma SAA (A), TF (B), AGP (C) and HP (D) acute phase protein levels during *Sarcoptes scabiei* infestation in pigs. Concentrations are shown as mean (±SEM) at baseline (week 0 pre-infestation), 2 and 4 week post-infestation. Statistical significance was carried out using two-way ANOVA followed by Tukey's multiple comparisons test comparing CS vs OS, CS vs C and OS vs C samples, n = 4. Differences with $p < 0.05$ were considered as significant.

Table 6. 2. Serum TF, HP, AGP and SAA concentrations in pigs with (CS and OS) or without (C) sarcoptic mange. Plasma samples from CS (n = 4), OS (n = 4) and C (n = 4) animals at each time point were analyzed in duplicates by ELISA. The absorbance values were averaged and concentrations were obtained from standard curves. Mean (± SEM) of 4 samples at each time point was calculated and are indicated.

		SAA (µg/mL)		TF (mg/mL)		AGP (mg/mL)		HP (mg/mL)	
Group	**Time in weeks**	**Mean**	**± SEM**	**Mean**	**± SEM**	**Mean**	**± SEM**	**Mean**	**± SEM**
CS	**0**	49.3	37.5	8.66	0.97	0.42	0.22	0.38	0.11
	2	9.51	0.59	21.25	4.35	1.84	0.23	0.22	0.07
	4	24.09	1.63	18.97	1.55	2.08	0.12	0.36	0.25
OS	**0**	11.66	1.69	9.1	0.71	0.54	0.04	0.56	0.21
	2	21.17	12.95	18.6	1.52	1.92	0.24	0.04	0.01
	4	12.92	3.39	22.7	2.46	1.85	0.12	0.11	0.04
C	**0**	47.22	25.27	7.23	0.95	0.28	0.06	0.1	0.03
	2	12.79	2.26	20.27	2.84	1.63	0.29	0.19	0.12
	4	20.89	8.74	17.39	1.92	1.58	0.49	1.11	0.68

6. 4. Discussion

The APPs can easily be measured and their concentration in serum may act as an indicator of disease severity and the extent of tissue damage caused by inflammation in affected animals. APPs could thus be useful biomarkers and measurement of APPs levels in serum could provide diagnostic and prognostic information if proper timing of sampling is assured. This is the first study investigating, by transcriptional profiling (microarray) and quantitative immunoenzymatic (ELISA) techniques, of acute phase protein response to sarcoptic mange in pigs. Earlier studies in pigs have provided a picture of acute phase protein response in the course of various diseases (Heegaard et al., 2013; Pomorska-Mól et al., 2013; Pomorska-Mól et al., 2015; Skovgaard et al., 2010; Sorensen et al., 2006) and also a few papers reported APPs in ibexes with sarcoptic mange (Raez-Bravo et al., 2015; Rahman et al., 2010). In this

analysis, AGP levels were significantly increased with the infestation in CS compared to healthy animals and this is in line with the increased AGP reported in the sarcoptic mange-affected ibexes (Raez-Bravo et al., 2015; Rahman et al., 2010) and in inflammation in goats (Gonzalez et al., 2008). This change in AGP levels in pigs may be related to the appearance of pathological lesions and inflammation as is the case in ibexes and goats. To my knowledge, up till now, there have not been any reports published on the development and kinetics of the APP response in pigs infested with *S. scabiei*.

Expression of the pro-inflammatory mediators OSM and IFNγ revealed by microarray analysis indicates that there are immune signals available in the local skin environment which may regulate the expression of APPs and hence activate the systemic acute phase response. Furthermore, in the gene expression analysis, genes for SAA, HP and TF were up-regulated (Table 6. 1) and in the immunoassays HP, TF, SAA and AGP were detected pre-infestation at week 0. Acute phase proteins have been proposed to be indicators of stress in animals (Petersen et al., 2004) and several studies have reported APPs as stress markers (Francisco and Weigel, 1996; Grellner et al., 2002; Qiu et al., 2007; Sauerwein et al., 2005). This gene expression, high protein levels and variability at baseline may reflect the stress associated with age of piglets (3 weeks old used here), separation from the mother, early weaning, movement of the pigs from the nursery to experimental facility, and the stress of adjusting to new environment (Grellner et al., 2002; Itoh et al., 1993). Studies report various concentrations of AGP, HP and SAA in early weaned pigs before experimental challenge/inoculation, for AGP mean concentration of 0.77 mg/mL (Grellner et al., 2002) in 12-14 days old piglets, and 0.7 mg/mL (Itoh et al., 1993) and 1.1 mg/mL (Heegaard et al., 2013) at 4 weeks of age has been reported. Pomorska-Mól et al. (2013) determined pig HP mean concentration ranging from 0.56 to 0.87 mg/mL and pig SAA at 2.8 μg/mL in 6 week-old pigs before inoculation. Our results in 3 week old pigs at week 0 prior to mite infection

were slightly lower than these reports, as we detected SAA in the range of 11.6 to 49.3 μg/mL, AGP 0.28 to 0.42 mg/mL, HP 0.1 to 0.56 and TF in the range of 7.2 to 9 mg/mL. According to our analysis, AGP could be considered as a 'moderate APP' (Ceron et al., 2005; Raez-Bravo et al., 2015) whereas HP, SAA and TF were apparently not affected by sarcoptic mange in pigs early in the infestation. AGP was significantly higher in CS pigs compared to healthy animals at week-4 post-infestation with AGP levels in plasma increased by more than 5-fold compared to pre-infestation levels. The pigs in animal trial showed clinical signs of disease and lesion development at week 4 following infestation, could it be that disease severity has a role in APP response as previously significantly higher AGP levels have been reported in ibexes with clinical signs of mange (Raez-Bravo et al., 2015; Rahman et al., 2010) and with episodes of skin pathology during *Nippostrongylus brasiliensis* infection in rats (Stadnyk et al., 1990). Also, given that AGP is anti-inflammatory in function, its increased secretion may explain a way of dampening the immune and inflammatory responses by inhibiting lymphocyte differentiation (Itoh, 1989) and leukocyte activity and trafficking to sites of inflammation (Barroso-Sousa et al., 2013; Mestriner et al., 2007; Okumura et al., 1985) hence allowing the mites to establish and grow, explaining its positive correlation with disease severity.

In the microarray analysis, TF gene was expressed at week 2 but down-regulated at week 4 post-infestation in OS pigs compared to healthy pigs. Although no significant differences were observed in the circulating concentrations of pig TF protein when comparing CS with C, OS with C and CS with OS animals, an approximate 3-fold increase in TF levels at week 4 post-infestation from pre-infestation (week 0) levels in CS, OS and C pigs was detected. These results suggest that increase in TF levels in the CS, OS and C pigs might be age related or the stress associated with the movement of the pigs and adjusting to new environment (Grellner et al., 2002; Itoh et al., 1993; Thoren-Tolling, 1984). Analysis of mange positive

sera revealed anti-transferrin antibodies against antigens in skin scrapings from experimentally *S. scabiei* infected pigs (Zalunardo et al., 2006). Increased antibody (IgG and IgM) titers were detected by ELISA in sera from both naturally and experimentally *S. scabiei* infected pigs against transferrin (Toet et al., 2014). The study authors report that antibody reactivity increased between weeks 4 and 8 post-infestation in mange positive pigs (Toet et al., 2014). Similar increase in TF levels between infested and control pigs in this analysis reflects that the autoantibodies are likely a result of cross reactivity with mite transferrin. Transferrin is considered as a negative APP as there is evidence that TF levels decrease in cattle with infection (McNair et al., 1998; Moser et al., 1994) or infected by parasite *Trypanosoma vivax* (Sampaio et al., 2015) or pigs with experimental infection (Kramer et al., 1985). However, sheep (Almeida Kde et al., 2012) and rats infected with *Trypanosoma* sp. (da Silva et al., 2013) showed increased levels of TF protein. TF increase in response to inflammation has also been demonstrated in chicken (Tohjo et al., 1995; Xie et al., 2002), thus behaving typically as a positive APP (Murata et al., 2004).

Transferrin protein binds ferric ions and iron laden TF is an essential growth factor for the parasite *T. brucei* in cattle (Schell et al., 1991). The increase in TF protein levels which our results suggest occurring irrespective of the mite infestation, may be a source of nutrients providing iron for growth of the mites in CS and OS pigs and may play a role in the disease.

Haptoglobin gene transcription was down-regulated in CS compared to OS and C pigs at week 2. Although no statistical significant difference was detected, HP protein plasma levels were measured at very low levels in CS and OS pigs at week 2 post-infestation compared to pre-infestation levels at week 0. However, CS pigs showed up to 9-fold higher HP protein levels compared to OS and C pigs at week 2. Also, a significant difference in the HP levels in OS versus C samples was observed and this could be due to an outlier pig in the control group (Figure 6. 2D). In parasitic infections such as *N. brasiliensis* infection in rats (Stadnyk

et al., 1990) and *T. evansi* infection in camels (El-Bahr and El-Deeb, 2016) significant increases in HP levels was observed. Similarly elevated HP levels were reported in ibexes with sarcoptic mange (Rahman et al., 2010), however recently a study (Raez-Bravo et al., 2015) showed no statistically significant difference in HP levels between healthy and mange positive ibexes. Haptoglobin has various immunomodulatory effects - it inhibits granulocyte chemotaxis (Rossbacher et al., 1999), prevents spontaneous maturation of Langerhans cells (Xie et al., 2000) and suppresses T-cell differentiation (Arredouani et al., 2003). The increased circulating HP protein in CS pigs may be able to inhibit innate immune and T cell responses early in the infestation allowing the mites to establish and grow.

Serum amyloid A is non-specific in terms of infection, injury or inflammatory disease, but at the same time is highly sensitive and an effective marker of inflammation in animals (Eckersall and Bell, 2010; Petersen et al., 2004). In this analysis, no significant differences were seen in SAA levels between the infested and non-infested animals. However, there was an increasing trend in SAA protein levels at week 4 in the infestation in CS pigs. Similarly, our gene expression analysis also demonstrated up-regulation of SAA gene at 4wpi in CS compared to OS pigs. Significant increase in SAA have been reported in camels with *T. evansi* parasitic infection (El-Bahr and El-Deeb, 2016). Elevated SAA protein levels have been demonstrated in ibexes with sarcoptic mange (Raez-Bravo et al., 2015; Rahman et al., 2010), sheep with *P. ovis* infestation (Wells et al., 2013) and the authors reported higher levels after the onset of clinical disease. These later studies demonstrated approximately 1000-fold increase in the SAA levels to 211 μg/mL in sheep (Wells et al., 2013) and 130.7 ± 0.16 μg/mL in ibexes (Rahman et al., 2010) compared with pre-infestation levels. In pigs with experimental *Streptococcus suis* infection (Sorensen et al., 2006), SAA increased to peak levels of 30–40 times the day 0-level (6 μg/mL) on days 1–2 post-infection. In our analysis, the SAA protein levels showed around 2.5-fold difference from week 2 and

increased to 24 μg/mL at week 4. In ibexes infested with *S. scabiei* (Raez-Bravo et al., 2015; Rahman et al., 2010) and sheep with *P. ovis* (Wells et al., 2013), SAA increased in serum following experimental mite infestation and this trend of increase at week 4 in acute phase response in CS pigs may be due to the production of proinflammatory cytokines or inflammation related to the skin lesions (Raez-Bravo et al., 2015; Rahman et al., 2010). Also, this delay in SAA increase in scabies compared to psoroptes might be due to the delayed appearance of disease signs and inflammation in scabies. In the study with experimental infection of pigs with *S. suis,* APPs tested were increased significantly from day 1 post infection and some of them remained significantly elevated for up to 2 weeks (Sorensen et al., 2006). Here also a correlation between clinical signs and the APP response was observed (Sorensen et al., 2006). It is observed that sarcoptic mange disease initiation has an asymptomatic phase at the start of the infestation which lasts for around 3 - 4 weeks as the mites become established. After this period, the mite numbers increase and the host starts to exhibit clinical signs - development of skin lesions and inflammation. In this investigation, SAA gene expression and increase in its protein levels in CS pigs at week 4 post-infestation suggests this SAA response may be due to the inflammation related to the skin lesions as in these animals the clinical disease started to appear after week 4 post - infestation.

In addition, the significant increase observed in AGP at week 4 and the increasing trend of SAA also warrant further investigations in to these APPs. In preliminary studies by our group SAA was also detected at higher concentration (>500 μg/mL) in mite infested pigs at weeks 13 and 18 (Kate Mounsey, personal communication) and it has been demonstrated that AGP, SAA and HP increased in concentration as the mite infestation/disease progressed in mange positive ibexes and *P. ovis* infested sheep (Rahman et al., 2010; Wells et al., 2013).

To summarize, different patterns of responses could be identified within the APPs analyzed in pigs infested with mite *S. scabiei*. This difference in APP response affected the information

that can be derived from these APPs. AGP increased 5-fold during CS in pigs, and may be considered as ‘‘moderate acute phase protein’’ during sarcoptic mange in pigs. Higher AGP response was observed at the onset of clinical disease and seems to be due to inflammation related to skin lesions as has been demonstrated previously/earlier (Raez-Bravo et al., 2015; Rahman et al., 2010). However, this needs to be further confirmed by investigations in order it to be given significance and to establish if pig AGP might be useful as a biomarker in scabies with increased differential diagnostic capabilities as compared to classically behaving acute phase proteins.

As APP response is not specific and has been demonstrated in pigs with bacterial infections (Sorensen et al., 2006). The variations in the recorded measurements may be due to underlying bacterial infections associated with scabies, age, inherent variability between pigs or their ability to cope with stress and new environment. Additional plasma samples must be analyzed from pigs with such underlying conditions to confirm these APPs during these infections. Furthermore, APP levels increase in various other inflammatory conditions, vary with the degree of inflammation and also the individual protein response is different (Eckersall and Bell, 2010). This should be taken into consideration in future works and as suggested (Wells et al., 2013) these APP biomarker assays should be used along with specific antibody assays which will allow a sensitive and highly specific diagnosis of *S. scabiei* infestation. In conclusion, further studies of APP response are needed to clarify and determine whether these or other APPs are reliable predictors of disease status in scabies.

Chapter 7

Concluding remarks

Scabies is an infestation of skin by the mite *Sarcoptic scabiei* impacting humans all around the globe. The disease is endemic and a significant public health problem in developing regions. In 2010, over 100 million cases of scabies were reported with young children mostly affected. Scabies prevalence rates in different parts of the world ranged from 0.2% to 71.4% with Pacific and Latin American regions showing the highest burden of the disease (Hay et al., 2014; Romani et al., 2015a). In Australia, scabies prevalence is higher in remote Aboriginal communities compared to the general population. The burden of scabies is amplified by secondary implications – acute rheumatic fever, rheumatic heart disease, sepsis and acute post-streptococcal glomerulonephritis in developing countries which may cause serious downstream systemic and life-threatening conditions (Hay et al., 2012; Hoy et al., 2012). *Sarcoptes scabiei* mite infestation also affects and causes substantial morbidity in both domestic and wild animals (over 100 species in 10 different mammalian orders) globally, and can cause huge economic losses in livestock.

A characteristic feature of primary infestation is the lag phase of 4 to 6 weeks before the onset of clinical signs and inflammatory and adaptive immune responses (Liu et al., 2014b; McCarthy et al., 2004; Rampton et al., 2013). The disease then manifests in the form of cutaneous inflammation, skin lesions and intense itching mediated through allergic and inflammatory reactions (Walton, 2010). A wide range of clinical disease manifestations, from mild (ordinary scabies) to extremely debilitating (crusted scabies), occur in scabies. In ordinary scabies (OS), there is low parasite load (< 20 mites) and mite infestation results in skin lesions with an intense generalised pruritus (Mellanby, 1944; Wendel and Rompalo, 2002). In contrast, crusted scabies (CS) is a rare condition with individuals/animals carrying

thousands of mites and the development of thick scaly crusts on the epidermis (Walton et al., 2010). Crusted scabies has been seen in individuals with underlying immunosuppression (Einsiedel et al., 2014; Gregorini et al., 2012; Hulbert and Larsen, 1992), developmental disabilities such as Down's syndrome (Hay et al., 2012) and also in patients with overtly healthy immune systems (Roberts et al., 2005). In animals, experimental infestation using same and similar number of mites results in OS in some animals while others develop CS type phenotype (Liu et al., 2014b; Mounsey et al., 2015; Rampton et al., 2013).

From the current scabies knowledge, it is evident that the host immune responses in OS are divergent to that seen in CS with Th1 type and $CD4^+$ T cell dominated response in OS, and non-protective Th2 allergic type, $CD8^+$ T and γδ T cell prevalent response in CS. Also, CS cases show increased antibody (IgE) response and eosinophilia (Liu et al., 2014a; Mounsey et al., 2013; Walton, 2010).

In this PhD project, the aim was to elucidate the key factors and signalling events in the immune and inflammatory pathways involved in the disease development, progression and immunopathogenesis to better understand the host responses in scabies.

In vitro studies have shown that the mites are able to suppress early host responses in order to establish and grow and I wished to identify the key modulators and mechanisms involved in this suppression. Also, to identify differentially regulated genes and pathways responsible for the contrasting pathology and host responses observed in different disease manifestations (OS and CS). Furthermore, I wished to investigate the early events in the host response to *S. scabiei* infestation particularly those involved in disease susceptibility in CS, and to determine the later responses and associated severe pathology. Also the aim was to better understand the mechanisms and immune mediators behind the elevated Th2 allergic and IL-17 responses reported in CS.

DNA (porcine transcriptome) microarray was applied to study gene expression upon mite infestation and elucidate the disease-specific gene profiles of tissue inflammatory and immune responses and gain insights into the molecular mechanisms and signalling pathways associated with these responses. Microarray analysis was combined with network and pathway mapping approach (IPA) to identify the key regulators, pathways and signalling mechanisms involved in the disease development, progress and pathogenesis. Quantitative reverse-transcriptase PCR (qRT-PCR) was used to validate selected differentially expressed genes from the microarray results. Venn diagram comparative analysis was carried out to identify overlapping genes in CS and OS, and unique genes in CS. A porcine model was employed for this study as carrying out longitudinal studies in humans can be logistically and ethically a challenging process. Pigs have been found most suitable for immunology studies because they are natural hosts to *S. scabiei* var. *suis* and upon mite infestation develop clinical disease resembling both OS and CS phenotype. Previous works in scabies were mostly *in-vitro*, utilising mite extracts and cultured cells or skin equivalents and also mismatched parasite-host systems. Also, the interpretation of important findings from human studies is limited due to small sample numbers.

In the first microarray analysis (chapter 4), it was observed that *S. scabiei* mites affect gene expression as a large number of genes in the skin of pigs were differentially expressed in response to mites at different stages of the infestation illustrating transcriptional differences in scabietic and control animals. Early in the infestation, complement components, cell surface receptors and ligands, and transcription factors involved in innate immunity, cytokine secretion, T and B cell activation, differentiation and secretion of immunoglobulins were repressed. Up-regulation of cytokines (IL-27 and IFN-γ) associated with the immune regulation and suppression was observed. It seems this early gene suppression might inhibit the immune and inflammatory responses against the mites and be helpful in their

establishment and colonisation, and disease development. Later in the infestation, this analysis illustrated gene expression profiles of mixed Th1, Th2 and Th17 responses which might be associated with the exacerbated inflammation and lesion development at this time in infestation. Of considerable significance was the expression of genes associated with the inflammasome pathway which is involved in pro-inflammatory IL-1β secretion and this is the first report of *S. scabiei* mite infestation inducing inflammasomes. This analysis provides information into the distinct patterns of gene expression accounting for varied immune and inflammatory responses in scabies. It also provided important information about the immune pathways – complement system, IL-12 and IL-4 signalling, and Th1 and Th2 cell differentiation, and mediators C5, NLRP3, TLR4, MYD88, CD70, STAT4, CD8a and SOCS5 by which *S. scabiei* may repress the host responses early and induces such a complex inflammatory and allergic responses later in the infestation.

In the second transcriptomic analysis (chapter 5), mostly down-regulated gene expression in mite infestation of skin from CS pigs relative to pigs with OS was observed. It seems both the host and the mites are involved in this gene down-regulation. Gene expression patterns prior to infestation and the early observation of gene down-regulation in CS pigs indicates genetic susceptibility may responsible for the disease development and pathogenesis.

An interesting finding of this work was the detection of immunity and inflammation related gene expression prior to and early in the infestation which might have a role in disease susceptibility and development, and earlier studies have shown that early age is essential to facilitate successful mite infestation.

Down-regulation of MHC expression (RFX5) and Dendritic cell (DC) maturation (HLA-DOB, MYD88, TLR3, TLR9, JAK2, STAT2, IL1β, IL15 and NFκB2) associated molecules early in the infestation may be an essential mechanism in the regulation of APC function and antigen presentation and hence adaptive immune response in CS. Antigen presentation by

DCs contributes to the priming of T cell responses. This gene (RFX5, HLA-DOB, MYD88, TLR3, TLR9, JAK2, STAT2, IL1β, IL15 and NFκB2) down-regulation may decrease DC expression of MHC molecules and antigen presentation in order to down-modulate T cell responses during early stages of *S. scabiei* infestation in CS skin. This inhibition would/may allow the mites to grow in number and play a role in disease susceptibility. On contrary, late in the infestation increased expression of antigen presentation related genes (RFX5, HLA-3, HLA-DRA, HLA-DMB, HLA-DQA1 and HLA-DQB1) indicates that the antigen presentation is delayed in CS. This early down-regulation and then increased up-regulation of genes involved in antigen presentation during the infestation represents a mechanism which may be associated with the development of CS diseases, and increased humoral and cell mediated response late in the infestation in CS cases. In addition, given the differential expression of these gene especially HLA-3 and RFX5, and their importance in regulation of MHC transcription and antigen presentation may provide potential anti-T cell mediated response strategies in CS.

In addition, it seems complement system plays a role in CS disease as we found down-regulation of genes (C3, C4A, C6 and CFD) associated with complement system in CS skin early in the infestation. Previously studies have demonstrated scabies mite proteases inhibit all three pathways of the complement system (Bergström et al., 2009; Mika et al., 2012a). In CS, it seems the activation of complement system is inhibited and hence the innate defense against the mites enabling them to survive and replicate within the host, which might also lead to susceptibility to CS disease.

Furthermore, early gene down-regulation seem to affect/inhibit another important aspect of innate immune system - pathogen recognition in CS. Toll-like receptors (TLRs) are essential components of the innate immune system engaged in pathogen recognition which triggers both immediate defences against the invading pathogens and multiple mechanisms to regulate

the adaptive immune responses (Pasare and Medzhitov, 2004). In CS, gene down-regulation of TLR2, TLR3 and TLR9 may affect mite recognition by the host leading to impaired activation of the signal transduction pathways that trigger protective inflammatory and adaptive immune responses.

In this analysis, JAK/STAT, STAT3 and NF-κB signaling pathway associated gene (JAK2, STAT3, STAT4, IL12B, IL12Rβ2, IFNγ, NFκB2, etc.) down-regulation early in the infestation may impact granulocyte, T and B cell differentiation, and hence innate and adaptive immune responses in CS (Cai et al., 2015; Hayden and Ghosh, 2012; Hillmer et al., 2016). Inhibition of these important signalling pathways might contribute to CS susceptibility.

Another major finding of this analysis was gene expression profile associated with pathophysiologic pathways involved in the pathogenesis of various inflammatory disorders including Th2 allergic (by inducing OSM, IL17RB, IL5, IL13, IL19, iCOS and CD40L) and Th17 (by inducing IL17A, CCL20, TGFβ, IL1β and ARG1) pronounced responses. Expression of these large number of molecules at this point of the disease might be due to the large antigenic material available provided by the high number of the mites.
IL17A mediates pro-inflammatory responses and its increased expression can result in excessive pro-inflammatory cytokine secretion and severe inflammation, which can cause tissue damage. IL-17A has been linked to disease progression and pathogenesis in several inflammatory diseases such as asthma, RA and psoriasis (Jin and Dong, 2013). In this study, IL-17A was expressed in CS pigs late in the infestation and may be responsible for promoting and extending inflammation by inducing pro-inflammatory cytokines and chemokine secretion. Also, Th17 cytokines IL-17 and IL-23 have been reported previously by our group in CS skin (Liu et al., 2014b; Mounsey et al., 2015) and in this analysis, IL-17A expression and of other essential factors (TGFβ and IL1β) responsible for Th17 cell differentiation

points towards the role of Th17 cells and IL-17A in CS cases. Also, due to its broad involvement in an array of immune-mediated diseases IL-17A is an ideal drug target for treatment against CS cases.

In CS skin, IL13 expression seems to/may drive the Th2 component in the immune responses and may play a role in disease progression and contribute to tissue damage by inducing IgE antibodies and eosinophilia. Increased IL-13 response has been associated with CS cases previously (Mounsey et al., 2015) and IL-13 expression has been correlated with tissue damage and inflammation in immune-inflammatory diseases such as allergy, asthma, AD and SLE (Seyfizadeh et al., 2015).

This analysis also showed late in the infestation strong up-regulation of chemokines CCL20, IL8, IL8R, CXCL6, CCL27 and CCL17 which mediate and enhance development and migration of immune effector cells – DCs, neutrophils, macrophages and T cells into the skin leading to and amplifying inflammation and tissue damage in CS. CCL17 may be useful biomarker for the diagnosis of scabies and evaluation of disease activity. Given its key role in the pathogenesis of various skin inflammatory diseases it may be a possible future target for therapy of scabies (Kataoka, 2014; Yoshie and Matsushima, 2015). In addition, CCL27 – CCR10 and CCL17 – CCR4 interactions are essential in T cell recruitment to the skin lesions during inflammation and can be potential targets for therapies against CS.

Another important finding of this analysis was that the mites induced IL20, S1000A7, S100A8 and S1000A9 which are involved in epidermal barrier disruption and keratinocyte hyper-proliferation and abnormal differentiation in psoriasis (Benoit et al., 2006; Martinsson et al., 2005; Sabat et al., 2007) and demonstrated in severe crusted lesions in sheep scab (Burgess et al., 2010; Burgess et al., 2012). Psoriasis is a recurrent chronic inflammatory skin disease and shares clinical similarities with scabies. Similarly, as in psoriasis, the induction of S100s and IL20 in CS skin may play a similar role in the development of thickened epidermis

- hyperproliferation and abnormal differentiation of keratinocytes, scales and crusts and be responsible for a rich immune cell infiltrate and complete the histological aspect of CS.
In this analysis, another interesting finding was Arginase (ARG) 1 gene expression late in the infestation in CS skin. Arginase 1 promotes Th17 cell differentiation (Wu et al., 2016) and type 2 inflammation (Monticelli et al., 2016) and has been implicated in the pathogenesis of allergic asthma, schistosomiasis and SLE (Getaneh et al., 2015; Rotondo et al., 2011; Wu et al., 2016; Zimmermann et al., 2003). Th2 specific cytokines IL-4, IL-5 and IL-13 promote ARG1 production and transcripts of IL5 and IL13 were up-regulated here and previously these cytokines have been associated with CS (Mounsey et al., 2015; Walton et al., 2010). Our findings suggest that Th2 cytokines induced ARG1 may modulate arginine metabolism and be an additional inflammatory mediator in the pathogenic process by promoting proliferative capacity and proinflammatory functions of Th17 and Th2 cells in CS cases. Therapeutically targeting ARG1 can be way to inhibit arginase activity and may offer potential therapeutic strategies for the treatment of CS disease.

Gene polymorphisms/mutations have been associated with susceptibility to various immune mediated diseases. IL13, ARG1, TLR8, STAT1 and RFX5 were expressed during mite infestation in CS and polymorphisms/mutations in these genes have been linked with psoriasis, allergy, asthma, SLE and AD (Bustamante et al., 2014; Granada et al., 2012; Hanna and Etzioni, 2014; Moller-Larsen et al., 2008; Nilsson et al., 2012; Paternoster et al., 2011; Zimmermann et al., 2003). These molecules could be of future interest to look into their potential role in dysregulated immune and inflammatory responses, and in the development of disease in CS cases. Polymorphisms in STAT6, high affinity receptor for IgE (FCER1A) and HLA-DRB1 genes could also be investigated as potential predisposing factors as they confer susceptibility to allergy, infectious and inflammatory diseases (Backes et al., 2013;

Okada et al., 2014; Qian et al., 2014) which share clinical and immunological similarities with CS.

Furthermore, in future, gene expression analysis at later time points in infestation may reveal immune and inflammatory molecules involved in CS disease prolongation.

This analysis has refined our understanding of CS pathogenesis and development, and provided molecular insights of various immune pathways and the inflammatory mediators associated which may be useful markers for disease diagnosis and severity and also therapeutically targeted for patients with CS. It also provides information about cytokines and chemokines in CS playing a role in enhancing leukocyte trafficking, cell differentiation and vascular permeability. This may exacerbate further the ongoing inflammation and worsen the disease symptoms of inflamed skin resulting in the crusted pathology. In addition, the analysis has revealed certain genes (TLR8 and STAT1) which might be expressed especially early in the infestation and whose genotypic variations might be predisposing the individuals/animals to CS disease.

In comparison to CS, in OS skin protective responses seem to be occurring late in the infestation as greater number of genes were up-regulated at week 8. Moderate expression of molecules IL6, IL17F and IL5 may be involved in the generation of essential inflammatory response in OS cases (Cheung et al., 2008; Read et al., 2016). Given their roles in antigen presentation, B cell activation and Ig production, CD70 and CD207 up-regulation seem to be involved in immune response generation (Borst et al., 2005; Hunger et al., 2004). Also, it seems in OS skin the inflammatory responses late in the infestation are regulated/controlled by suppression of potent pro-inflammatory cytokine OSM (Boniface et al., 2007; Richards, 2013). At the same time, down-regulation of STAT3, AGP, IFNγ and C6 (Hochepied et al., 2003; O'Shea and Plenge, 2012; Schoenborn and Wilson, 2007; Varela and Tomlinson, 2015)

may be associated with mite infestation and clinical signs and inflammation observed late in the infestation in OS cases.

Early in infestation in OS, expression of immune mediators including HLA-DOB, ICOS, FADD, CD180, LY96, CCL27 and SELP (Akashi-Takamura and Miyake, 2008; Diacovich and Gorvel, 2010; Dziarski et al., 2001; Neefjes et al., 2011; Simpson et al., 2010; Tourneur and Chiocchia, 2010; Xiong et al., 2012; Zarbock et al., 2009) may control the mite spread and growth, and be the reason for low mite burden observed in OS cases. In addition, by inhibition of inflammatory T cell responses, it seems the tissue damage in OS cases is kept at minimal by the expression of immunosuppressive genes (STAT5A/B, TGFβ1) (Hadaschik and Enk, 2015; O'Shea and Plenge, 2012).

Furthermore, in this transcriptional profiling (Chapter 4 and 5), expression of acute phase proteins (HP, TF and SAA) and molecules (OSM, IFNγ, STAT3, NFκB2, and MYD88) associated with acute phase signalling pathways was detected. ELISA analysis (Chapter 6), the first such analysis of APPs in pigs with mange, revealed different patterns of responses in infested pigs. Important finding of this analysis was identification of AGP as “moderate acute phase protein” in pigs with CS and AGP response has been reported in Ibexes with sarcoptic mange (Raez-Bravo et al., 2015; Rahman et al., 2010). AGP could be used as a potential biomarker to diagnose the CS early and also to predict the disease status/severity. The identification of early affected individual/animal could be helpful in managing effectively and facilitate treatment of this parasitic disease prior to disease outcomes.

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Appendices

Appendix A

Supplementary table 1. The RIN values, yield and OD $_{A260/A280}$ ratios of total RNA samples.

Sample ID (Week)	Group (Phenotype)	NanoDrop		Bioanalyser
		Conc. (ng/µL)	260/280	RIN
517 (0)	B (OS)	126	2.03	7.40
509 (0)	B (OS)	390	2.06	8.20
584 (0)	B (OS)	214	2.04	8
479 (0)	B (OS)	108	2.05	7.50
543 (0)	B (CS)	200	2.07	7.70
598 (0)	B (CS)	148	2.04	7.80
547 (0)	B (CS)	338	2.07	7.90
550 (0)	B (CS)	192	2.00	8.50
574 (0)	D (C)	109	2.02	7.20
506 (0)	D (C)	186	2.02	8.10
481 (0)	D (C)	119	2.04	7.10
459 (0)	D (C)	188	2.10	8
517 (1)	B (OS)	165	2.06	8
509 (1)	B (OS)	130	2.11	8
584 (1)	B (OS)	236	2.02	8.20
479 (1)	B (OS)	125	2.07	8.80
543 (1)	B (CS)	95.2	2.04	N/A
598 (1)	B (CS)	93.2	2.06	8.30
547 (1)	B (CS)	108	2.02	8.10
550 (1)	B (CS)	108	2.02	8.40
574 (1)	D (C)	126	2.08	8.10
506 (1)	D (C)	153	2.08	6.70
481 (1)	D (C)	133	2.03	8.20
459 (1)	D (C)	173	1.99	8.40
517 (2)	B (OS)	412	2.03	7.70
509 (2)	B (OS)	336	2.02	7.60
584 (2)	B (OS)	402	2.03	7.30
479 (2)	B (OS)	334	2.04	7.60
543 (2)	B (CS)	332	2.00	8.20
598 (2)	B (CS)	326	2.00	7.80
547 (2)	B (CS)	193	2.00	7.80
550 (2)	B (CS)	236	1.99	7.60
574 (2)	D (C)	436	2.01	7.40
506 (2)	D (C)	312	2.02	7.50

481 (2)	D (C)	256	1.99	8.10
459 (2)	D (C)	310	2.02	7.80
517 (4)	B (OS)	182	1.98	N/A
509 (4)	B (OS)	406	2.02	7.50
584 (4)	B (OS)	338	2.01	7.60
479 (4)	B (OS)	234	2.00	7.80
543 (4)	B (CS)	240	1.99	7.70
598 (4)	B (CS)	156	1.97	7.50
547 (4)	B (CS)	139	1.95	6.80
550 (4)	B (CS)	176	1.92	7.20
574 (4)	D (C)	250	2.02	7.50
506 (4)	D (C)	292	2.00	7.60
481 (4)	D (C)	222	1.98	7.50
459 (4)	D (C)	306	2.00	7.20
517 (8)	B (OS)	142	1.95	7.30
509 (8)	B (OS)	187	1.98	7.50
584 (8)	B (OS)	210	1.95	8.20
479 (8)	B (OS)	289	1.94	7.90
543 (8)	B (CS)	192	1.99	8.60
598 (8)	B (CS)	330	2.01	8.20
547 (8)	B (CS)	195	2.00	8.40
550 (8)	B (CS)	308	2.00	8
574 (8)	D (C)	394	2.00	2.50
506 (8)	D (C)	494	2.00	8.10
481 (8)	D (C)	286	1.98	8.10
459 (8)	D (C)	306	1.97	7.80

*OS = ordinary scabies; CS = crusted scabies and C = control.
*Group B = mite infested (n=4 OS and n=4 CS); *Group D = non-infested controls (n = 4 C).
*n=12 (4 OS, 4 CS and 4 C) at each time point (Week 0 pre-infestation, 1, 2, 4 and 8 post-infestation); 60 samples in total.

Appendix B

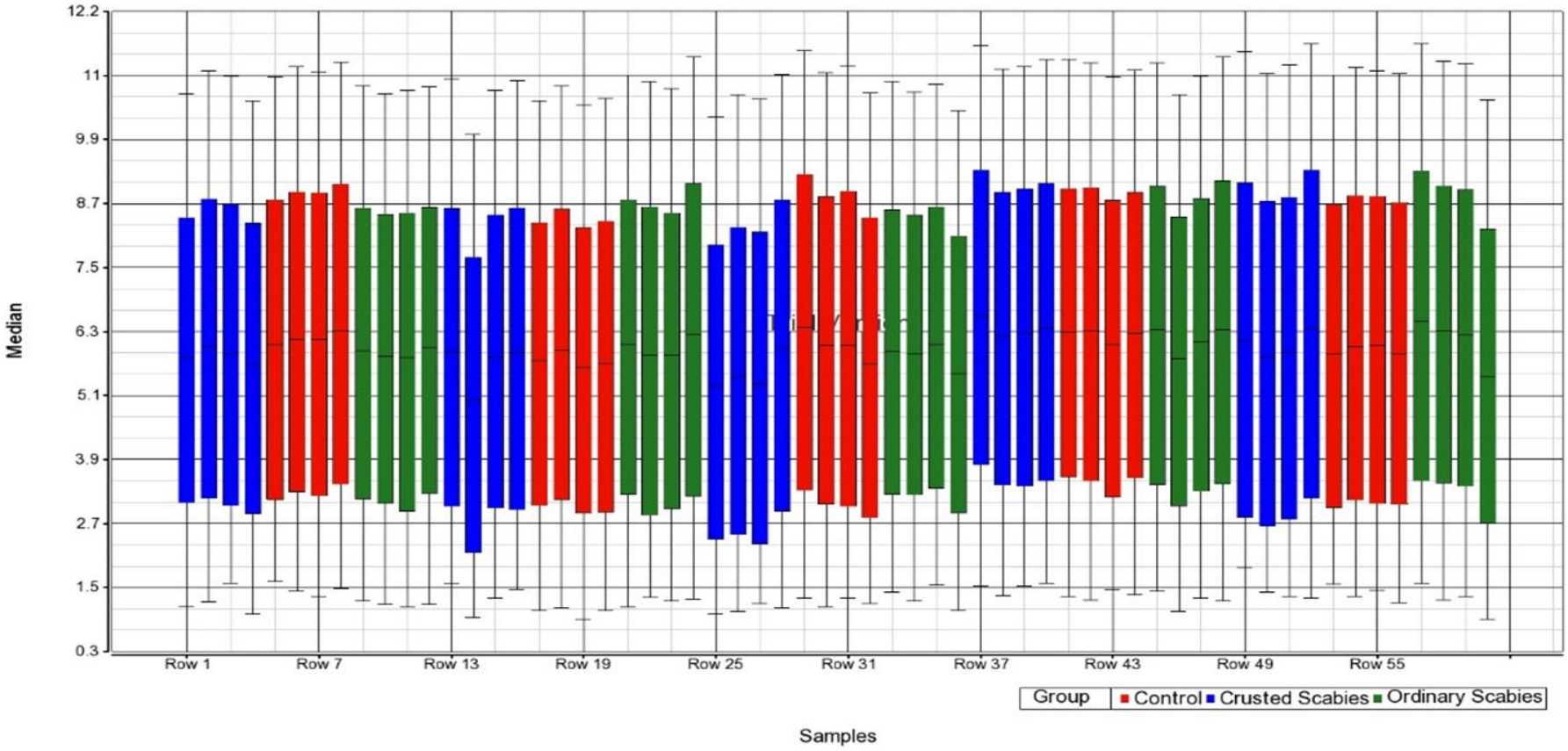
Supplementary table 2. The yield, specific activity and OD A260/A280 ratios of cRNA samples generated from total RNA.

Sample ID (Week)	Group (Phenotype)	NanoDrop		
		Conc. (ng/µL)	260/280	Sp. Activity (pmol Cy3/ µg of cRNA)
517 (0)	B (OS)	49.4	2.12	12.14
509 (0)	B (OS)	40.2	2.09	17.41
584 (0)	B (OS)	70.5	2.04	11.34
479 (0)	B (OS)	36.5	2.10	16.43
543 (0)	B (CS)	108.8	2.03	12.86
598 (0)	B (CS)	71.8	2.14	9.74
547 (0)	B (CS)	42.2	2.21	7.10
550 (0)	B (CS)	46.0	2.11	13.04
574 (0)	D (C)	55.4	2.14	12.63
506 (0)	D (C)	73.8	2.16	9.48
481 (0)	D (C)	47.2	2.09	12.71
459 (0)	D (C)	43.9	2.20	9.11
517 (1)	B (OS)	98.6	2.10	11.51
509 (1)	B (OS)	107.1	2.11	11.20
584 (1)	B (OS)	92.9	2.11	10.76
479 (1)	B (OS)	107.7	2.10	12.07
543 (1)	B (CS)	71.2	2.07	11.23
598 (1)	B (CS)	64.7	2.09	12.36
547 (1)	B (CS)	98.2	2.15	12.21
550 (1)	B (CS)	74.6	2.08	9.38
574 (1)	D (C)	87.1	2.12	10.33
506 (1)	D (C)	113.3	2.10	11.47
481 (1)	D (C)	78.9	2.00	10.13
459 (1)	D (C)	110.5	2.14	11.76
517 (2)	B (OS)	56.9	2.08	12.30
509 (2)	B (OS)	60.7	1.88	9.88
584 (2)	B (OS)	65.5	2.11	9.16
479 (2)	B (OS)	47.0	2.14	12.76
543 (2)	B (CS)	105.9	2.11	13.22
598 (2)	B (CS)	70.2	2.13	9.97
547 (2)	B (CS)	39.2	2.16	7.65
550 (2)	B (CS)	62.2	2.11	6.43
574 (2)	D (C)	74.3	1.81	8.07
506 (2)	D (C)	92.0	2.10	11.95
481 (2)	D (C)	92.6	2.10	12.95
459 (2)	D (C)	42.4	2.05	11.79
517 (4)	B (OS)	55.4	2.34	10.83

509 (4)	B (OS)	95.4	2.23	12.57
584 (4)	B (OS)	79.0	2.21	11.39
479 (4)	B (OS)	80.6	2.19	12.40
543 (4)	B (CS)	65.1	2.20	10.75
598 (4)	B (CS)	61.2	2.26	9.80
547 (4)	B (CS)	40.6	2.32	9.85
550 (4)	B (CS)	37.7	2.18	10.61
574 (4)	D (C)	63.7	2.25	10.98
506 (4)	D (C)	63.8	2.21	10.97
481 (4)	D (C)	59.3	2.24	11.80
459 (4)	D (C)	76.0	2.23	13.15
517 (8)	B (OS)	45.4	1.78	8.81
509 (8)	B (OS)	53.6	1.99	11.19
584 (8)	B (OS)	33.4	1.97	14.97
479 (8)	B (OS)	38.8	1.99	12.88
543 (8)	B (CS)	47.7	2.00	13.53
598 (8)	B (CS)	51.7	2.05	13.53
547 (8)	B (CS)	64.5	2.09	13.95
550 (8)	B (CS)	67.4	2.00	16.32
574 (8)	D (C)	55.7	1.99	12.56
506 (8)	D (C)	61.1	2.11	13.09
481 (8)	D (C)	64.1	2.10	12.48
459 (8)	D (C)	50.9	2.10	15.71

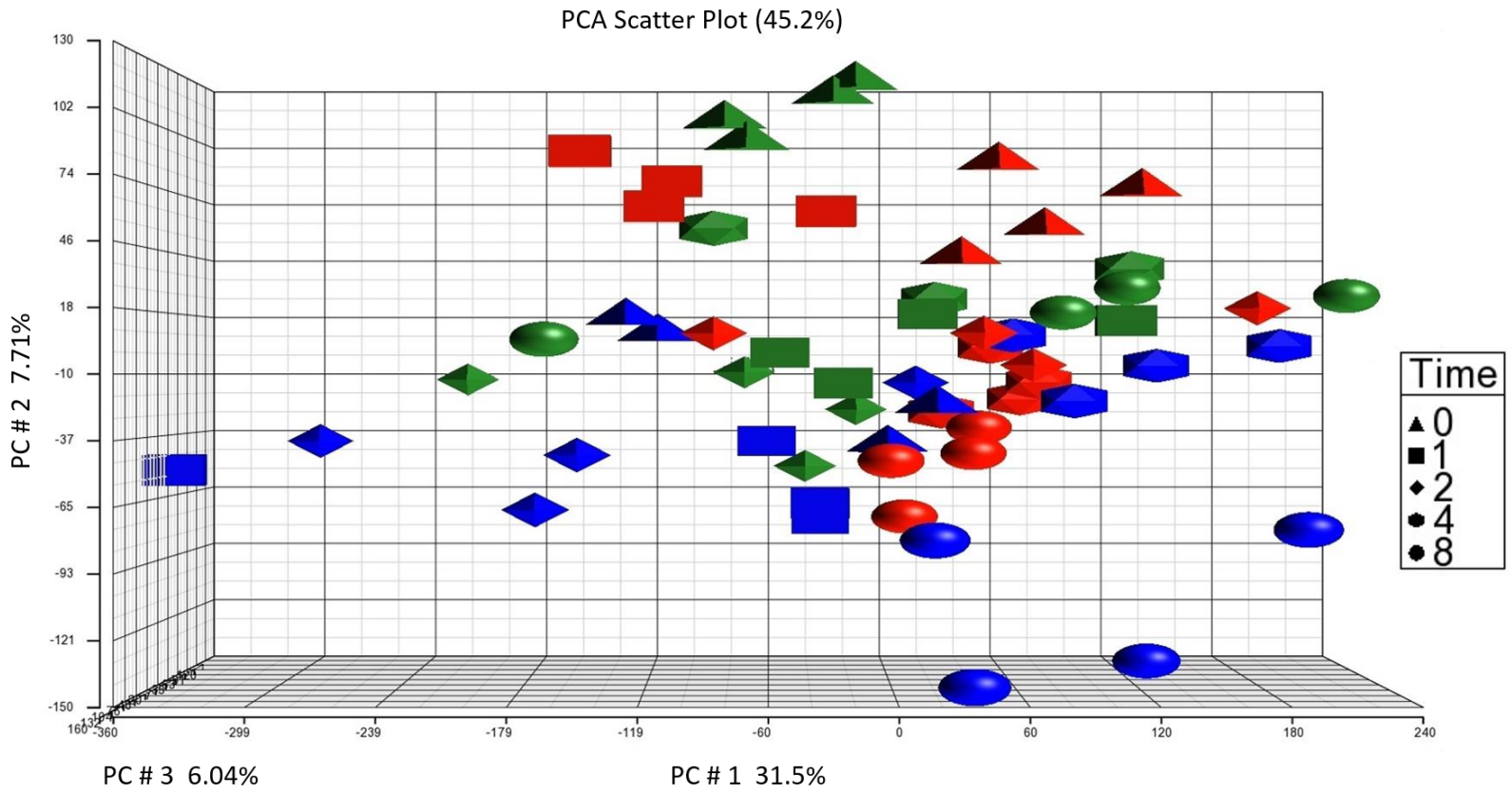
*OS = ordinary scabies; CS = crusted scabies and C = control.
*Group B = mite infested (n=4 OS and n=4 CS); *Group D = non-infested controls (n = 4 C).
*n=12 (4 OS, 4 CS and 4 C) at each time point (Week 0 pre-infestation, 1, 2, 4 and 8 post-infestation); 60 samples in total.

Appendix C



Supplementary Figure 1. Box and whisker profile plot to examine the feature intensity distributions of microarray data over the time course of infestation with *S. scabiei*. Each row represents a single sample.

Appendix D



Supplementary Figure 2. Principal component analysis (PCA) of infested (CS and OS) versus non-infested (C) samples pre (at week 0) and post (at weeks 1, 2, 4 and 8) mite infestation with *Sarcoptes scabiei*. PCA demonstrated that the infested samples grouped separately to non-infested samples. Red circles indicate C samples, blue indicate CS samples and green indicate OS samples.

Appendix E

Additional File 1. Gene lists of the comparisons CS and OS vs C and CS vs OS at each time point of the study. The file is provided on a separate USB drive due to its large size. It contains the list of genes described in the text with gene symbol annotation where available and fold change data for each gene at each time point of the study.

Appendix F

Supplementary Table 3. qPCR validation of microarray data. Nine genes were identified in the 2-way ANNOVA analysis with a p-value of ≤ 0.05 and FC of > 2.0. Relative quantification of gene expression levels was determined by normalising to the HPRT1 control using the comparative Ct method with the formula $2^{-\Delta\Delta CT}$ and expressed as fold change. Duplicates of each cDNA sample from infested (OS, n = 4; CS, n = 4) and non-infested controls (C, n = 4) were maintained. Total RNA extracted from pig skin biopsies was used. Using reverse transcriptase, cDNA was synthesized from 1µg total RNA.

	IFNγ	**HPRT1**	**Mean Difference (Mean IFNγ - Mean HPRT1**		**IL1β**	**HPRT1**	**Mean Difference (Mean IL1β - Mean HPRT1**
CS				**OS**			
Mean	33.5 SEM ± 0.39 SD ± 0.78	29.69 SEM ± 2.21 SD ± 4.43	3.81 SD ± 2.25		26.78 SEM ± 0.95 SD ± 1.90	19.77 SEM ± 0.12 SD ± 0.25	7.01 SD ± 0.96
C				**C**			
Mean	29.43 SEM ± 0.36 SD ± 0.72	22.47 SEM ± 0.52 SD ± 1.05	6.96 SD ± 0.64		27.53 SEM ± 1.09 SD ± 2.18	20.56 SEM ± 0.23 SD ± 0.47	6.96 SD ± 1.11
	FOXP3	**HPRT1**	**Mean Difference (Mean FOXP3 - Mean HPRT1**		**IL17F**	**HPRT1**	**Mean Difference (Mean IL-17F - Mean HPRT1**
CS				**OS**			
Mean	29.71 SEM ± 0.44 SD ± 0.89	26.62 SEM ± 1.78 SD ± 3.56	3.09 SD ± 1.84		20.28 SEM ± 10.33 SD ± 14.61	20.83 SEM ± 0.24 SD ± 0.49	-0.55 SD ± 10.33
C				**C**			
Mean	28.45 SEM ± 0.32 SD ± 0.65	21.34 SEM ± 0.26 SD ± 0.53	7.10 SD ± 0.42		24.41 SEM ± 3.21 SD ± 6.43	24.55 SEM ± 3.14 SD ± 6.28	-0.13 SD ± 4.497
	GLO1	**HPRT1**	**Mean Difference (Mean GLO1 - Mean HPRT1**		**TGFβ**	**HPRT1**	**Mean Difference (Mean TGFβ - Mean HPRT1**
CS				**CS**			
Mean	23.49 SEM ± 1.70 SD ± 3.4	26.67 SEM ± 1.80 SD ± 3.60	-3.18 SD ± 2.48		25.35 SEM ± 1.62 SD ± 3.25	27.48 SEM ± 2.07 SD ± 4.14	2.99 SD ± 3.29
OS				**OS**			
Mean	20.51 SEM ± 0.73 SD ± 1.46	21.27 SEM ± 0.26 SD ± 0.52	-0.75 SD ± 0.77		22.07 SEM ± 0.25 SD ± 0.50	21.54 SEM ± 0.26 SD ± 0.52	0.52 SD ± 0.36

	CD274	HPRT1	Mean Difference (Mean CD274 - Mean HPRT1		NLRP3	HPRT1	Mean Difference (Mean NLRP3 - Mean HPRT1
OS				**OS**			
Mean	25.07 SEM ± 1.37 SD ± 2.75	16.47 SEM ± 0.36 SD ± 0.73	8.6 SD ± 1.42		21.19 SEM ± 0.74 SD ± 1.48	21.35 SEM ± 0.36 SD ± 0.73	-0.16 SD ± 0.82
C				**C**			
Mean	26.16 SEM ± 0.42 SD ± 0.85	15.79 SEM ± 0.48 SD ± 0.97	10.36 SD ± 0.65		23.98 SEM ± 0.13 SD ± 0.27	21.37 SEM ± 0.29 SD ± 0.58	2.60 SD ± 0.32
	TNF	**HPRT1**	**Mean Difference (Mean TNF - Mean HPRT1**				
OS							
Mean	25.73 SEM ± 0.70 SD ± 1.41	21.97 SEM ± 0.53 SD ± 1.06	3.76 SD ± 0.88				
C							
Mean	27.66 SEM ± 0.62 SD ± 1.24	20.66 SEM ± 0.40 SD ± 0.80	7.00 SD ± 0.74				

Appendix G

Supplementary Table 4. Immunity related genes in response to *S. scabiei* infestation in skin of CS compared to OS pigs. Genes that differed significantly in expression (either up or down regulated) levels upon mite infestation over the time course of first 8 weeks. Gene expression was carried out with a 2- way ANOVA combined with a Fisher's Least Significant Difference (LSD) post-hoc test in Partek. These genes had a FDR corrected p-value of ≤ 0.05 and fold change of ≥ ± 2.0. The gene function was verified with online databases IPA, UniProt and GeneCards. In IPA, gene networks and signalling pathway analysis was carried out on DEGs from each time point. The notable genes associated with signalling pathways and gene networks in the processes of immune, inflammatory and allergic responses are indicated.

Genes	Gene Description	Baseline week 0 (FC)	1wpi (FC)	2wpi (FC)	4wpi (FC)	8wpi (FC)
	Pro-inflammatory cytokines and receptors					
IFNα4	Interferon alpha 4			-2.98		
IFNγ	Interferon gamma	7.46		-4.86		2.19
IL12B	Interleukin 12 subunit beta	-2.65		-2.43		
IL12Rβ1	Interleukin 12 receptor subunit beta 1					2.3
IL12Rβ2	Interleukin 12 receptor subunit beta 2	3.4		-2.75		
IL13	Interleukin 13					2.35
IL17A	Interleukin 17A					4.67
IL17F	Interleukin 17F	-4.26				
IL17RB	Interleukin 17 receptor B					4.89
IL1β	Interleukin 1 beta		-6.01			
IL20	Interleukin 20					9.05
IL6R	Interleukin 6 receptor			-3.35		
IL8/CXCL8	Interleukin 8/C-X-C motif chemokine ligand 8					13.89
IL8R/CXCR2	Interleukin 8 receptor/C-X-C motif chemokine receptor 2					4.32
OSM	Oncostatin M	13.78		-6.67		3.42
TNF	Tumour necrosis factor	-3.36				
	Pathogen recognition receptors					
TLR2	Toll-like receptor 2	2.2		-4.17		
TLR3	Toll-like receptor 3		-2.09			

TLR4	Toll-like receptor 4	2.42				
TLR5	Toll-like receptor 5			-3.11		
TLR8	Toll-like receptor 8		44.47		2.84	
TLR9	Toll-like receptor 9		-3.18			
	Cell surface receptors					
CD247	CD247 molecule		-3.22			
CD274	CD274 molecule		-5.02	4.08		
CD3δ	CD3 molecule, delta					
CD3ε	CD3 molecule, epsilon					3.43
CD3γ	CD3 molecule, gamma		20.56		8.86	3.57
CD4	CD4 molecule		-2.11			2.02
CD36	CD36 molecule	7.86		-4.09		3.47
CD40L	CD40 ligand					3.58
CD5L	CD5 ligand		-6.92	-4.97		
CD70/TNFSF7	CD70 Molecule/Tumour Necrosis Factor Ligand Superfamily Member 7	-5.04	-8.21	3.24		
CD82	CD82 molecule		-3.1			
CD86	CD86 molecule			-2.89		-3.31
CD8α	CD8 alpha molecule	-3.41	-3.04	2.93		
	Chemokine receptors and ligands					
CCL17/TARC	C-C motif chemokine ligand 17/Thymus and activation-regulated chemokine					2.38
CCL20/MIP3α	Chemokine (C-C motif) ligand 20/Macrophage inflammatory protein 3-alpha					9.05
CCL25	C-C motif chemokine ligand 25	3.46				
CCL27/CTACK	Chemokine (C-C motif) ligand 27/Cutaneous T-cell activating chemokine					3.24
CCL28	C-C motif chemokine ligand 28	-2.47				
CCL3L1	C-C motif chemokine ligand 3 like 1					2.14
CCL4	C-C motif chemokine ligand 4					2.36
CCL5	C-C motif chemokine ligand 5			-2.73		
CCR3	C-C motif chemokine receptor 3	10.13		-6.73		
CCR7	C-C motif chemokine receptor 7					4.59

CCR10/GPR2	C-C motif chemokine receptor 10/ G protein-coupled receptor-2	4.03		-2.26		
CXCL11	C-X-C motif chemokine ligand 11			-2.24		-2.24
CXCL16	C-X-C motif chemokine ligand 16	-7.42				-2.86
CXCL2/GROβ	C-X-C motif chemokine ligand 2					6.14
CXCL6	C-X-C motif chemokine ligand 6					64.32
ICAM3	Intercellular adhesion molecule 3	2.92		-6.19		3.31
SELPLG	Selectin P ligand	8.6		-4.08		3.9
	Complement factors					
C3	Complement C3		-2.08	-2.36		-2.63
C4A	Complement component 4A	12.35		-6.47		2.38
C6	Complement C6	6.78	5.01	-9.12		
CFD	Complement factor D			-2.43		
	Immunoregulatory molecules					
FOXP3	Forkhead box P3	3.25	3.11	-3.85		
IL27	Interleukin 27			-5.91		
TGFβ1	Transforming growth factor beta 1		-4.31			2.17
	Cell growth factors, cell signalling molecules and transcription factors/regulators,					
CSF3	Colony stimulating factor 3					-2.8
CSF3R	Colony stimulating factor 3 receptor					-2.73
iCOS	Inducible T-cell costimulator					4.33
IRF5	Interferon Regulatory Factor 5				3.14	
IRF3	Interferon regulatory factor 3		-2.06			
JAK2	Janus kinase 2		-2.52	-2.03		
LEP	Leptin		-4.5		10.10	
MTOR	Mechanistic target of rapamycin		-2.79			
MYD88	Myeloid differentiation primary response 88	-6.65	-9.71	3.92		
NFAT5	Nuclear factor of activated T-cells 5		-5.96			-3.74
NFATC4	Nuclear factor of activated T-cells 4		-8.44			-3.63

NFκB2	Nuclear factor kappa B subunit 2	2.54	-2.61	-2.93		-2.19
SOCS1	Suppressor of cytokine signalling 1			-2.13		-2.12
STAT1	Signal transducer and activator of transcription 1				3.77	
STAT2	Signal transducer and activator of transcription 2		-2.56			
STAT3	Signal transducer and activator of transcription 3	4.58		-3.55		
STAT4	Signal transducer and activator of transcription 4	-4.1		2.68		
STAT6	Signal transducer and activator of transcription 6		-2.58			
TNFAIP6	TNF alpha induced protein 6			-2.03		
TNFS4	Tumour necrosis factor superfamily member 4		-2.42			
TNFSF10	Tumour necrosis factor superfamily member 10		-3.11			
TRAF3	TNF receptor associated factor 3					
	Metallopeptidases					
MMP7	Matrix metallopeptidase 7		-2.73			
MMP9	Matrix metallopeptidase 9 (Gelatinase B)					9.12
MMP11	Matrix metallopeptidase 11	3.03	-2.44			3.35
MMP12	Matrix metallopeptidase 12 (Macrophage elastase)					11.67
	S100s					
S100A7	S100 calcium binding protein A7 (Psoriasin 1)					21.52
S100A8	S100 calcium binding protein A8 (Calgranulin A)					19.85
S100A9	S100 calcium binding protein A9 (Calgranulin B)					21.07
	Claudins					
CLDN2	Claudin 2	-6.78	-3.25			-4.17
CLDN5	Claudin 5		-2.06			
CLDN6	Claudin 6					-2.47
CLDN7	Claudin 7	2.2				
CLDN9	Claudin 9	2.87				
CLDN11	Claudin 11	-5.23				-2.86
CLDN12	Claudin 12	3.95				
CLDN18	Claudin 18					-4.43

CLDN22	Claudin 22	2.18				
	Miscellaneous – other important genes					
ARG1	Arginase 1	8.05				30.79
ARG2	Arginase 2		-2.39			4.03
CASP10	Caspase 10		-4.11			
CASP3	Caspase 3	2.72		-2.03	2.24	
CDH1	Cadherin 1			-2.44		-2.18
CTSB	Cathepsin B		-4.21			
DCT	Dopachrome tautomerase	-12.32	-33.75	18.1		-6.4
EGF	Epithelial growth factor	2.86		-2.42		
FAS/TNFRSF6	Fas cell surface death receptor/Tumor necrosis factor receptor superfamily member 6		-4.39			
FCGR2B	Fc fragment of IgG receptor IIb		-2.49		2.4	2.03
FOXH1	Forkhead box HA			-11.46		
FOXO3	Forkhead box O3		-2.25			
IL15	Interleukin 15	3.34	-2.73			
IL19	Interleukin 19	-2.71	-7.51			7.89
IL33	Interleukin 33			-3.05		
LY96	Lymphocyte antigen 96	2.67			3.81	-2.05
MAP2K1	Mitogen-activated protein kinase kinase 1	-5.58	-9.53	6.24		-4.16
MAP2K6	Mitogen-activated protein kinase kinase 6			-3.9		
MAP3K12	Mitogen-activated protein kinase kinase kinase 12	-2.51	-2.84			-2.17
MAP3K2	Mitogen-activated protein kinase kinase kinase 2	-5.48				
MAP3K6	Mitogen-activated protein kinase kinase kinase 6		-2.17		3	
MAP3K9	Mitogen-activated protein kinase kinase kinase 9	-7	-3.46	2.73		-2.06
MAP4K4	Mitogen-activated protein kinase kinase kinase kinase 4		-7.29			
MAPK8	Mitogen-activated protein kinase 8	5.38		-3.47		2.1
NCF2	Neutrophil cytosolic factor 2	2.24		-2.23		2.14

NLRC3/NLRP3	NLR Family CARD Domain Containing 3/ NLR Family Pyrin Domain Containing 3			-2.77		
NOS2	Nitric oxide synthase 2			-2.75		
RFX5	Regulatory factor X5			-3.52	11.70	
SERPINA3	Serpin family A member 3	2.08	-2.75		2.45	
SERPINB9	Serpin family B member 9			-2.77		
SERPING1	Serpin family G member 1		-3.68			
SLA-3	Major histocompatibility complex class I, antigen 3					2.6
SLA-5	Major histocompatibility complex class I, antigen 5	-4.58	-6.1			
SLA-A	Major histocompatibility complex class I, A				5.13	
SLA-DOB	Major histocompatibility complex, class II, DO beta		-2.81			
SOD1	Superoxide dismutase 1, soluble			-4.05		
SOD2	Superoxide dismutase 2, mitochondrial	6.64				4.38
TGFα	Transforming growth factor alpha		-2.03			
TGFβ1I1	Transforming growth factor beta 1 induced transcript 1		-5.18			
TGFβ3	Transforming growth factor beta 3				2.98	-2.26
TGFβR3	Transforming growth factor beta receptor 3		-2.42			
TIMP1	TIMP metallopeptidase inhibitor 1			3.08		
TYK2	Tyrosine kinase 2				2.51	
VNN1	Vanin1				3.19	
IRAK1	Interleukin 1 receptor associated kinase 1	3.04		-2.94		
RELA	RELA proto-oncogene, NF-kB subunit	-2.36				-2.05