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Crusted scabies is associated with increased IL-17 secretion by skin T cells

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Summary
Scabies is an ectoparasitic infestation by the mite Sarcoptes scabiei. Although commonly self-limiting, a fraction of patients develop severely debilitating crusted scabies. The immune mechanisms underlying the development of crusted scabies are unclear, and undertaking longitudinal infection studies in humans is difficult. We utilized a porcine model to compare cellular immune responses in peripheral blood and skin of pigs with different clinical manifestations of scabies (n=12), and in uninfected controls (n=6). Although clinical symptoms were not evident until at least 4 weeks post infestation, the numbers of peripheral IFNγ secreting CD4+ T cells and γδ T cells increased in infected pigs from week 1 post infestation. γδ T cells remained elevated in the blood at week 15 post infestation. At week 15, skin cell infiltrates from pigs with crusted scabies had significantly higher CD8+ T cell, γδ T cell, and IL-17+ cell numbers than those with ordinary scabies. Peripheral IL-17 levels were not increased, suggesting that localised skin IL-17 secreting T cells may play a critical role in the pathogenesis of crusted scabies development. Given the potential of anti-IL-17 immunotherapy demonstrated for other inflammatory skin diseases, this study may provide a novel therapeutic avenue for patients with recurrent crusted scabies.

Introduction
Scabies is an ectoparasite skin infection caused by the Sarcoptes scabiei variety hominis mite. The disease is endemic in impoverished communities of developing countries (1). In developed countries, outbreaks of scabies often occur in hospitals, child care and aged care centers (2, 3). Scabies commonly presents as a mild skin disease characterized by an intensely pruritic skin rash. In some patients however, crusted scabies develops, a condition
characterized by hyper-proliferation of mites, hyperkeratosis and depigmentation of skin. It is often complicated by secondary bacterial infection that may be life threatening (4).

Although crusted scabies is often associated with general immunosuppressive conditions such as HIV, HTLV-1 or iatrogenic immunosuppression, some patients with no apparent immune dysfunction develop crusted scabies for reasons that remain unclear (5). The underlying immunological mechanisms explaining why crusted scabies develops are largely unknown (6). T cell infiltrates in scabietic lesions are observed in humans and other animals (4, 7, 8). CD4+ T cells are found to dominate the lymphocytic infiltrate of inflammatory skin lesions in ordinary scabies (9, 10). In individuals with crusted scabies, the main skin infiltrating T cells have been reported to be CD8+ T cells (4). It has been suggested that a Th2 biased immune response in *S. scabiei* infestation may account for the development of crusted scabies (6, 11). Peripheral blood mononuclear cells (PBMCs) isolated from patients with crusted scabies secreted higher levels of IL-4, IL-5 and IL-13, and lower levels of IFNγ, compared to those of ordinary scabies patients; levels of total and antigen specific IgE are also higher in crusted scabies patients (12-15).

Scabies mites cannot be cultured *in vitro* and it is logistically and ethically difficult to study longitudinal immune responses in humans with scabies. Animal models provide the opportunity to investigate the immunopathologic mechanisms of scabies development, and a tractable porcine model of scabies has been recently developed (11, 16). When infested with *S. scabiei* var. *suis*, pigs show similar clinical and immunological changes to humans. In this porcine model the corticosteroid Dexamethasone is commonly utilized to induce crusted scabies and provide large numbers of mites for biological studies. However, of particular interest is the fact that some infected pigs develop a manifestation of scabies closely resembling human crusted scabies even in the absence of corticosteroid immunosuppression (11). Therefore the porcine model provides a unique opportunity to study immunologic
responses following scabies infection, and may help to understand differences in individual susceptibility and why crusted scabies develops after scabies mite infestation.

Using this porcine model, we investigated the cellular immune responses in *S. scabiei* infested pigs, with a focus on T cell responses. Peripheral blood responses were compared at early (one week) and late (15 weeks) stages of scabies mite infestation, with local skin responses also examined at 15 weeks post-infestation. It has been suggested that IL-17 secretion by CD3+γδ+ T cells plays a critical role in the pathogenesis of skin chronic inflammation, such as psoriasis (16-18). As some of the clinical manifestations of crusted scabies, such as epidermal hyperproliferation, are similar to psoriasis, we also investigated whether the levels of IL-17 were increased in mite infested pigs, especially those with crusted scabies. Our results show that IL-17 secreting T cells are present in skin lesions of pigs with crusted but not ordinary scabies. These results may provide a potential avenue for the management of crusted scabies.

**Materials and Methods**

*Ethics Statement:* 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 and the Australian National Health and Medical Research Council’s Animal Code of Practice.
Animal Trial: This trial was conducted from March-June 2013 and the study presented here was undertaken as part of a larger project exploring scabies immune responses. Eighteen three week old piglets of the “large white” breed- a common meat producing breed in Australia, were obtained from the University of Queensland piggery, Gatton, QLD, and randomly allocated to infected (n=12) or non-infected control groups (n=6). Pigs were maintained at the adjacent Queensland Animal Science Precinct, Gatton, QLD, in indoor rooms with a constant temperature of 24°C. Infected and non-infected pigs were housed in separate rooms with strict isolation protocols to ensure that accidental transmission did not occur. These infected pigs were not exposed to Dexamethasone to avoid confounding interpretation of changes occurring due to mite infestation, and those due to corticosteroid immunosuppression alone. Based on previous trials (11), a proportion of the infected pigs were expected to develop hyperkeratotic mange with high mite burdens, akin to crusted scabies, reaching maximum clinical severity between 12 and 16 weeks, whereas others would maintain infestation with few clinical signs and low mite burden (akin to ordinary scabies).

Methods for collection of mites and experimental infection of pigs were as previously described (16). All pigs in the infected group were infested with approximately 200 mites per ear. Pigs were observed weekly for the development of skin lesions. As described previously (10), clinical manifestations were scored on a 1-8 scale (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). At the 15 week time point, pigs were euthanized and final samples collected.

Sample collection: Blood was collected from all pigs at three time points: one week prior to infestation (baseline), one week post infestation, and 15 weeks post infestation. Six to eight mL of blood was collected from the cephalic vein or anterior vena cava into Lithium Heparin
CPT tubes (BD Biosciences), which contain pre- aliquoted Ficoll-Hypaque for the simplified isolation of PBMCs. After separating plasma, PBMCs were isolated according to the manufacturer’s protocol.

Skin samples were collected from pig ears at week 15 using a 3.5mm punch biopsy (McFarlane Medical). Two adjacent biopsies from each pig were pooled, cut into small pieces and immediately placed in sterile phosphate buffered saline (PBS) containing 2 mg/mL collagenase, 100 µg/mL penicillin/streptomycin and 2.5 µg/mL Fungizone®, and kept on ice during transport. Upon return to the laboratory, skin was incubated for 2.5 hours at 37°C. The skin tissues were disrupted using the end of a plunger of a 5 ml syringe before passing through a 70µm cell strainer. After washing with PBS, lymphocytes were purified and isolated by discontinuous density gradients of Ficoll-Hypaque (GE Health Care) before surface and intracellular staining.

**Antibodies:** The following antibodies were used for defining cell surface markers and for intracellular staining: Anti-pig-CD3-FITC (clone PPT3, Abcam); anti-pig-CD3 PerCP Cy5.5 (clone BB23-8E6-8C8, BD Biosciences); anti-pig-CD3-PE (clone 76-2-11, Abcam); anti-pig-CD4-FITC (clone 76-2-11, Abcam); anti-pig-CD4-PE Cy7 (clone 74-12-4, Abcam); anti-pig-CD8α-PE (clone 76-2-11, Abcam); biotin-anti-pig-CD8 (clone PT8, BD Biosciences); anti-pig-γδ T-PE (clone MAC320, BD Biosciences); anti-human IL-17Alexa 647 (clone SCPL1362, BD Biosciences); and anti-pig-IFN-γ-PE (clone P2G10, BD Biosciences). Isotype control antibodies were utilized throughout and included avidin-APC (e-bioscience); anti-mouse-IgG-FITC (clone X40, BD Biosciences); anti-mouse IgG PE (clone X39, BD Biosciences); and anti-mouse-IgG1-Alexa 647 (MOPC-21, BD Biosciences);

**Cell surface staining:** 1x10⁶ cells were incubated for 30 minutes at 4°C with the selected antibodies. Cells were washed twice with cold PBS supplemented with with 2% of fetal calf
serum (FCS, Life technologies) and then analyzed by flow cytometry (Accuri C6, BD Biosciences). To assess cell viability for skin cell surface staining, 7-AAD (e-bioscience) was added to stained, washed cells immediately before flow cytometry. Flow cytometry data was analyzed using BD Accuri C6 Sampler or Flowjo software.

**Intracellular cytokine staining:** Following the above isolation of PBMCs and skin cells, samples were subjected to intracellular cytokine staining following mitogen stimulation. After extensive washing with PBS, single cell suspensions (1x 10^6) were cultured in complete RPMI (Life technologies) with 10% FCS containing 100 µg/mL of penicillin/streptomycin, and 2.5 µg/mL of Fungizone and stimulated with cell stimulation cocktail containing PMA and ionomycin (e-bioscience) at 37°C, 5% CO₂ for 10 hours in the presence of protein transport inhibitor containing monensin (BD Biosciences). After stimulation, cells were first surface stained with antibodies specific for surface markers, and then subject to intracellular staining for IL-17 or /and IFN-γ using an intracellular staining kit (BD Biosciences) following the manufacturer instructions.

Additionally, supernatants collected from PMA and ionomycin stimulated PBMCs in the absence of protein transport inhibitor were measured for IL-17 by ELISA (Swine IL-17 Vet Set, Kingfisher Biotech), following the manufacturer instructions.

**Immunohistochemical staining of γδ T cells:** Following the porcine trial, tissue sections from a pig with crusted scabies obtained from our routine “maintenance colony” (16) were collected from lesional and non-lesional areas using a 3.5mm punch biopsy. Sections were fixed in methacarn solution (60% methanol, 30% chloroform, 10% glacial acetic acid), processed into paraffin blocks, and cut into 4-7 µm serial sections. Sections were dewaxed and blocked with 5% skim milk powder for 30 minutes. Mouse anti-bovine WC1 antibody (clone CC101, AbD Serotec), known to cross-react with porcine γδ T cells (17), was applied.
undiluted, and sections incubated overnight at room temperature. Negative control sections were also included where staining with the primary antibody was omitted. After washing in Tris buffered saline (TBS), sections were then incubated with anti-mouse alkaline phosphatase (Mach 2, Biocare) for 30 minutes. Finally, sections were incubated with vector red alkaline phosphatase substrate (Vector labs), and lightly counterstained with haematoxylin and eosin.

Statistical analysis: Statistical analysis was performed by the two tailed Student’s test, using Prism 5.0 (Graphpad Software, USA). Results were considered significant if the p value was less than 0.05.

Results

Scabies infested pigs develop different clinical phenotypes

Signs of skin infestation began to appear in infected pigs from four weeks post infestation (Figure 1A). Based on clinical scores, at the end of the trial in week 15, seven infested pigs were classified as ordinary scabies (score <4) (Figure 1B), and five pigs were classified as crusted scabies (score ≥4) (Figure 1C). Pigs in the non-infested groups did not develop skin lesions at any time during the trial.

Peripheral blood T cells respond to skin infestation before skin lesions appear

PBMCs were collected one week prior to infestation (baseline), one week post infestation, and finally at 15 weeks post infestation, were stained with T cell surface markers and intracellularly stained with IFNγ. At baseline, no significant differences in peripheral CD4+, CD8+, or γδ+ T cell proportions or ratios were observed between groups. IFNγ+ T cells were also similar between the two groups (data not shown). At one week after infestation, total peripheral blood CD4+ and CD8+ T cell proportions were similar between the infected and non-infested pigs.
uninfected groups (Figure 2A, B), but CD3+γδ+ T cells and IFNγ+CD4+ T cells were higher in the infected group (p= 0.007 and p=0.004 respectively) (Figure 2C, 2D). Analysis of the week 1 subsets in pigs that went on to develop ordinary versus crusted scabies at 15 week did not show any differences.

**Peripheral γδ T cells remain increased at 15 weeks post infection**

At 15 weeks post infestation, there were no differences in the CD4+ and CD8+ T cell proportions in the peripheral blood between infested and non-infested pigs (Figure 3A, B). However, the level of CD3+γδ+ T cells was significantly higher in infested groups (Figure 3C) (p=0.017). When the CD3+γδ+ T cell proportions were compared between pigs with crusted and ordinary scabies phenotypes, no differences in were observed (Figure 3C).

**IL-17 secretion by PBMCs are not increased in infected pigs at 15 weeks post infection**

The levels of IL-17 in the culture supernatants of mitogen-stimulated PBMCs were lower in the infested group compared with the non-infested group, in both the baseline samples and in the samples collected at 15 weeks post infestation. There was no difference in the level of IL-17 between crusted and ordinary scabies groups (Figure 4A).

As there were no suitable commercial antibodies against pig IL-17, an anti-human IL-17 antibody was used to detect intracellular pig IL-17 by flow cytometry. This antibody (Clone SCPL1362) had been shown previously to cross react with pig IL-17 (18). As shown (Figure 4B), we could detect pig IL-17 within T cells using this antibody to stain mitogen stimulated PBMCs from a pig with crusted scabies, and minimal staining with the isotype control antibody (MOPC-21). However, IL-17 secretion by mitogen stimulated peripheral blood CD3+T cells was significantly lower in infested pigs compared with non-infested pigs (Figure 4C) (p=0.024). Moreover the percentage of IL17-secreting γδ+ T cells were similar
between pigs with the crusted and ordinary scabies phenotype, and similar to those from uninfested pigs (Figure 4D).

**Skin infiltrating CD8+, CD3+γδ+ T and IL-17 secreting CD3+ T cells are increased in crusted scabies**

Reduced levels of PBMC secreting IL-17 in infested pigs may indicate migration of IL-17 secreting T cells to infested skin lesions. We therefore investigated skin infiltration with T cells among infested and non-infested pigs at the 15 week time point. The proportion of skin CD4+ T cells in infested pigs were significantly lower compared to non-infested pigs at week 15 (p=0.008). However, there were no differences between crusted and ordinary scabies phenotypes (Figure 5A). The proportion of skin CD8+ T cells and CD3+γδ+ T cells were slightly higher in infested pigs compared with non-infested pigs, but the differences were not statistically significant (Figure 5B, C). When infested pigs were compared between ordinary and crusted scabies phenotypes, the skin CD8+ T cell and CD3+γδ+ T cell proportions in crusted scabies were significantly higher than those of ordinary scabies (Figure 5B, C) (p=0.014 and p=0.002 respectively).

To confirm the increased CD3+γδ+ T cell infiltration in crusted scabies lesions observed by flow cytometry, skin sections from lesional and non-lesional areas in the same pig were stained with anti-γδ T cell antibodies and visualised by immunohistochemistry. Positively immunolabelled γδ T cells in lesional sections were localised in perivascular clusters of lymphocytes in the reticular and perivascular dermis. There were also significant numbers of these cells in the stratum basale and spinosum of the epidermis. In non-lesional sections, positive cells were predominantly located perivascularly in the papillary dermis and only occasionally in the reticular dermis, and in the stratum basale or stratum spinosum of the epidermis (Figure 5D).
Next, we investigated IFNγ and IL-17 secretion by skin infiltrating T cells following mitogen stimulation and culture. Levels of IFNγ secretion by T cells were slightly increased in infested pigs but without statistical significance, with no differences between ordinary scabies and crusted scabies (Figure 6A). Of note, the proportions of skin CD3+IL-17+ T cells were significantly higher in infested pigs compared to non-infested pigs (Figure 6B) (p=0.028). Furthermore, skin IL-17 secreting CD3+ T cells from pigs with the crusted scabies phenotype were significantly higher compared to pigs with the ordinary scabies phenotype or non-infested pigs (p=0.001). Skin IL-17 secreting T cells were similar between pigs with ordinary scabies or non-infected pigs (Figure 6B).

Discussion

Crusted scabies is a severe clinical manifestation of *S. scabiei* infestation. While ordinary scabies is associated with a relatively low mite burden (<15 mites per patient, reducing with repeat infestation), crusted scabies patients appear unable to control the infestation, resulting in hyper-proliferation of mites and considerable damage to the skin. Overall, the immunopathogenesis of both ordinary and crusted scabies is poorly understood. Patients most commonly present at an advanced stage of infection. It is not possible to prospectively monitor the immunologic progression of untreated disease, particularly when progression to development of crusted or ordinary scabies is likely established early in infection. Consequently, in this study, we have utilized a tractable porcine model of human scabies (16) to investigate scabies immunopathology.

As observed in previous studies (11), pigs developed different clinical outcomes after infestation following inoculation with similar numbers of mites. Of 12 infested pigs, five had the crusted scabies phenotype at the conclusion of the trial, while the other seven had the
ordinary scabies phenotype. This reinforces the value of this model in the comparative study of host immune responses in scabies.

Peripheral blood $\gamma\delta$ T cell numbers and IFN$\gamma$ secreting CD4$^+$ T cells were higher in infested pigs as early as one week following infestation, weeks before the development of clinical manifestations. These $\gamma\delta$ T cells remained elevated in infested pigs at the 15 week time point. Interestingly, the numbers of CD8$^+$ and $\gamma\delta^+$ T were specifically increased in the skin of pigs with the crusted scabies phenotype; IL-17 secreting T cells, but not IFN$\gamma$ secreting T cells were also increased in the skin of pigs with the crusted but not ordinary phenotype.

As observed in previous studies of human crusted scabies (4-6), peripheral blood CD4/CD8 ratios were not altered in scabies infested pigs. Overall, individual pigs were variable in T cell proportions, particularly at early infestation, which is consistent with previous observations for outbred domestic pigs of similar ages (19). In our study all infected pigs had reduced CD4$^+$ cells in the skin, and pigs with crusted scabies had significantly increased skin CD8$^+$ cell infiltrate. These results also validate previous studies of human crusted scabies, where altered CD4/CD8 ratios in the skin are described, with lymphocyte infiltrates found to be predominantly CD8$^+$ (4). High numbers of skin infiltrating CD8$^+$ T cells are also found in psoriasis, most notably in the epidermis (20-22). Why increased CD8$^+$ T cells infiltrate the lesional areas is not clear, and the role of CD8$^+$ T cells in the pathogenesis of crusted scabies warrants further investigation.

The finding that the numbers of $\gamma\delta$ T cells are increased and the numbers of IL-17 secreting T cells are also increased in skin lesions of pigs with crusted scabies is of relevance to the understanding the pathogenesis of crusted scabies and possibly new approaches to treatment. The marked increase in $\gamma\delta$ T cells was shown by immunohistochemistry to be localised to lesional areas of pigs with crusted scabies. This is the first documented association of $\gamma\delta$ T
cells with the immunopathology of crusted scabies. However, as γδ T cells are known to be highly enriched in the peripheral blood of juvenile pigs relative to humans (23), it will be of importance to investigate the application of these findings in human scabies.

Th-17 cells are a recently described class of helper T cells that secrete IL-17A, IL-17F, IL-21 and IL-22 (24). Differentiation of naïve T cells towards a Th-17 phenotype is supported by several cytokines including transforming growth factor-β, IL1β and IL-23 in mice and humans (25). IL-17 is a potent pro-inflammatory cytokine that amplifies ongoing inflammation by inducing expression of tumour necrosis factor-α, IL-1β, and IL-6 in epithelial as well as keratinocytes and fibroblasts. Th-17 cells play an important role in host defense against infection. In parasitic infection however, IL-17 has been described in both protective (26) and pathologic contexts (27, 28). For example, IL-27R-deficient mice infected with *Toxoplasma gondii* and *Leishmania major* develop severe disease that is characterized by a prominent IL-17 response (24). Therefore inappropriate development of Th-17 cells in some parasite infections could lead to increased disease progression and chronic infection rather than remission and microbe clearance. As the levels of skin IL-17 positive cells in ordinary scabies were at similar low levels to those non-infected controls, and high IL-17 was associated with mite proliferation and crusted scabies, IL-17 does not appear to be part of a protective immune response to *S. scabiei*.

Recently, it has been demonstrated that IL-17 secretion by skin γδ T cells plays a critical role in the pathogenesis of psoriasis (29). Although crusted scabies and psoriasis have differing aetiologies (psoriasis autoimmune, crusted scabies infectious), they share marked clinical and histological similarities including chronic skin inflammation, acanthosis and hyperkeratosis. IL-17 is now understood to be a key player in keratinocyte proliferation and epidermal hyperplasia observed in psoriasis, and therefore our observation of increased skin local IL-17 secreting T cells may be relevant to understanding the development of crusted scabies.

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Further studies are now required to fully define the phenotype and functional relevance of this CD3+/IL-17+ cell population, including more targeted studies involving T cell stimulation with mite specific antigens such S. scabiei whole mite antigen extract or recombinant antigens. Based on the low numbers of CD4+ cells observed in crusted scabies lesions, Th17 cells are unlikely to be the predominant cell population, whereas γδ+ IL-17 or CD8+ IL-17 (Tc17) cells are more likely to be major populations based on our results. It is also important to acknowledge that increased IL-17 in the skin may also relate to secretion from other cell types, such as mast cells, which are also increased in crusted scabies (30, 31).

It has been suggested that a Th2 biased immune response against S. scabiei infection may account for the development of crusted scabies. Extremely high levels of IgE antibody have been reported in patients with crusted scabies, indicative of a Th2 biased immune response (5, 6, 15, 32). However studies in goat and sheep models suggested that high IgE levels may indicate protective immune responses (33, 34), and thus high levels of IgE may therefore not be directly related to a dysregulated immune response leading to the development of crusted scabies. PBMCs isolated from patients with crusted scabies secreted higher levels of IL-4, IL-5 and IL-13 and lower levels of IFNγ compared with those of ordinary scabies patients (15). Therefore, it is possible that both increased Th2 and increased IL-17 responses contribute to the pathogenesis of crusted scabies. Notably, a novel subset of CD4+Th2 T cells has been identified that secrete IL-17 and promotes the exacerbation of chronic allergic asthma, in which IgE levels are usually increased (35).

Treatment of crusted scabies is very difficult. Crust and scale removal is necessary and an intensive treatment regimen including both oral and topical acaricides is recommended (36). Clinical trials targeting IL-17 by administration of anti-IL-17 or anti-IL-17 receptor...
antibodies significantly improve the symptoms and skin damage of psoriasis. In a Phase II trial with 142 patients affected by moderate to severe plaque type psoriasis, 76% of patients received a substantial (>75%) reduction in disease severity after 12 weeks of treatment with ≥25mg anti-IL-17 antibodies (37). In a phase I trial with AMG827, a monoclonal antibody targeting the IL-17 receptor, all patients receiving a single 700mg dose achieved a minimum of 50% improvement, 88% had 75% improvement, and 38% had 90% improvement (38). An alternative strategy, targeting the Th17 transcription factor RORγT with small molecule inhibitors may also hold promise at inhibiting IL-17 production from gamma delta T cells and CD8+ Tc17 T cells in psoriasis (39). In order to confirm that increased IL-17 is involved in the pathogenesis of crusted scabies, similar in-vivo studies utilising anti-IL-17 antibodies in our porcine model would be necessary.

The underlying mechanisms responsible for the increased local IL-17 secretion by T cells in crusted scabies are unknown. We found from our porcine trial that peripheral blood CD4+Foxp3+ T cells were increased in both crusted and ordinary scabies pigs at all the time points investigated, from one week to 15 weeks post infestation (unpublished data), suggesting regulatory T cells are involved in the control or development of the disease. It has been shown previously that IL-10 secretion by T cells in crusted scabies is reduced, compared with normal control or ordinary scabies (4, 15, 40). It will be interesting to investigate whether IL-10 secretion by both peripheral and local skin regulatory T cells in pigs with crusted scabies is reduced, and/or whether the trafficking of IL-10 secreting regulatory T cells is impaired, therefore leading to uncontrolled expansion of IL-17 secreting T cells in crusted scabies. This has recently been described in a mouse model of mucocutaneous leishmaniasis, where blocking of IL-10R was associated with increased IL-17 and severe skin pathology. This pathology could be reversed by treatment with anti-IL-17 neutralising antibodies (28). On the other hand, the local skin environment of crusted scabies...
may foster the generation of IL-17 secreting T cells; innate immune cells, such as dendritic
cells from crusted scabies pigs may secrete more IL-23 when responding to scabies antigen,
or Toll like receptor stimulation when subsequent infections exist. Indeed, microarray studies
demonstrate that burrowing mites are capable of inducing IL-23 expression in human skin
equivalents (41). Future studies related to this trial will concentrate on further elucidation of
factors driving clinical progression to crusted or ordinary scabies, in particular transcriptomic
analysis of skin immune responses in early infection.

Taken together these data suggest that local uncontrolled expansion of IL-17 T cells may in
part account for the development and pathogenesis of crusted scabies. These results may lead
to novel therapeutic treatment methods for crusted scabies by targeting IL-17.

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Author contributions
Conceived and designed the study: KM, XSL, JMC, SW, DH, BC. Performed the research:
XSL, KM, AK, MK, HM. Analyzed and interpreted the data: XSL, KM, SW. Wrote the
paper: XSL, KM. Edited the paper: XSL, KM, SW, JMC. All authors have read and approved
the final version of the manuscript.
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Figure Legends

Figure 1: Scabies infested pigs develop different clinical phenotypes

A: Representative clinical progression of individual pigs with ordinary (B2) and crusted (B3) phenotype. B: clinical appearance of ordinary scabies. C: clinical appearance of crusted scabies. Pigs were either infected with approximately 200 mites or left uninfected. Ear lesions of all pigs were scored weekly. Score of 1-4: generalized rash and papular lesions of increasing density; >4: development of increasing encrustment; 8: extensive encrustment spreading external to ears.
Figure 2: Peripheral blood T cells respond to skin infestation before skin lesions appear

Peripheral Blood Mononuclear cells (PBMCs) isolated from infested and non-infested pigs at one week post infestation were surface stained with anti-CD3, anti-CD4, anti-γδ, anti-CD8 and intracellularly stained with IFNγ. Lymphocytes were gated A: CD3+CD4+ T cells, B: CD3+CD8+ T cells, C: CD3+γδ+ T cells, D: CD4+IFNγ+ T cells and FACS profile.

Figure 3: γδ T cells remain increased 15 weeks post infestation

PBMCs from infested and non-infested pigs at 15 weeks post infestation were surface stained with anti-CD3, anti-CD4, anti-CD8 and anti-γδ. Lymphocytes were gated. A: CD3+CD4+ T cells, B: CD3+CD8+ T cells and C: CD3+γδ+ T cells and FACS profile.

Figure 4: Peripheral IL-17 levels are not increased in infected pigs 15 weeks post infestation

A: PBMCs isolated at baseline and 15 weeks post infection were stimulated with ionomycin and PMA overnight; supernatants collected were examined for IL-17 by ELISA. B: PBMCs isolated from a pig with crusted scabies were stimulated with ionomycin and PMA for different time points in the presence of protein transport inhibitor. The cells were than stained with anti-CD4 and intracellularly stained with anti-human IL-17 or isotype control antibody. Lymphocytes were gated and CD4+IL-17+ T cells were shown. C & D: PBMCs isolated from 15 weeks post infection were stimulated with ionomycin and PMA overnight in the presence of protein transport inhibitor. PBMCs were then stained with anti-CD3, anti-γδ and intracellularly stained for IL-17. CD3+ cells were gated. CD3+IL-17+ T cells (C) CD3+δγ+IL-17+ T cells (D).
Figure 5: Skin infiltrating CD3+γδ+ T and CD3+CD8+ T cells are increased in crusted scabies 15 weeks post infestation

Skin biopsy samples collected at 15 weeks post infestation were digested with collagenase, and single skin cells suspensions prepared by passing through 70µm cell strainer followed by enrichment by ficoll separation. Cells were stained with anti-CD3, anti-γδ, anti-CD4 or anti-CD8. 7-AAD was added to exclude non-viable cells. A: CD3+CD4+ T cells, B: CD3+CD8+ T cells, C: CD3+γδ+ T cells and FACS profile of skin CD3+δγ+ T cells (A). D: Lesional and non-lesional skin sections stained with anti-γδ T cell antibody. Extreme epidermal thickening and extensive staining for γδ-T cells (red) are observed in the lesional section. Scale bar = 100µM.

Figure 6: Skin infiltrating CD3+IL-17+ T cells are increased in crusted scabies 15 weeks post infestation

Skin biopsy samples collected 15 weeks post infestation were digested with collagenase, and single skin cells suspensions prepared by passing through 70µm cell strainer followed by enrichment by ficoll separation. Cells were stimulated with ionomycin and PMA overnight and stained with anti-CD3, IFNγ and IL-17. Lymphocytes were gated. A: CD3+IFNγ+ T cells; B: CD3+IL-17+ T cells and FACS profile of CD3+IL-17+ T cells.