MASTER OF SCIENCE

Preclinical evaluation of a vaccine against IL-17 mediated inflammatory diseases including psoriasis

West, Jonathan David

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PRECLINICAL EVALUATION OF A VACCINE AGAINST IL-17 MEDIATED INFLAMMATORY DISEASES INCLUDING PSORIASIS

Jonathan David West
Bachelor of Medical Sciences in Human Genetics and Experimental Medicine

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List of Abbreviations

ACR	American College of Rheumatology
ATP	Adenosine triphosphate
BCG	Bacillus Calmette–Guérin
BSA	Bovine serum albumin
CMV	Cucumber mosaic virus
DC	Dendritic cells
DDC	Dermal dendritic cells
DLQI	Dermatology life quality index
DPT	Diphtheria, pertussis and tetanus
ELISA	Enzyme-linked immunosorbent assay
H&E	Hematoxylin and eosin
HLA	Human leukocyte antigen
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
LC	Langerhans cells
MHC	Major histocompatibility complex
MS	Multiple sclerosis
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHP	Non-human primate
PASI	Psoriasis area and severity index
PBS	Phosphate-buffered saline
PSORS1	Psoriasis susceptibility locus 1
RA	Rheumatoid arthritis
SCID	Severe combined immunodeficiency
TC	Telocyte
Th	T helper cell
TLR	Toll like receptor
TNF	Tumour necrosis factor
VEGF	Vascular endothelial growth factor
VLP	Virus-like particles

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Declaration

I hereby declare that this dissertation entitled “Preclinical evaluation of a vaccine against IL-17 mediated inflammatory diseases including psoriasis” has been prepared by me under the direct guidance of Dr John Foerster and Dr Jonathan Berg as part of my study for the award of Master of Science by Research in Medicine Degree at the University of Dundee, Dundee, Scotland.

I have not submitted this dissertation previously for the award of any degree or diploma at any other institution.

Jonathan West May 2017

Supervisor's Declaration

I certify that Jonathan West has spent the equivalent of at least six terms in research work in the Centre for Clinical and Molecular Medicine, Ninewells Hospital, University of Dundee and that he has fulfilled the conditions of the Ordinance General No. 39 of the University of Dundee and is qualified to submit the accompanying thesis in the application for the degree of Master of Science.

Dr John Foerster May 2017

Senior Clinical Lecturer

College of Medicine, Dentistry and Nursing

University of Dundee

Abstract

Psoriasis is a chronic autoimmune skin condition. Interleukin 17A (IL-17A) is a pro-inflammatory cytokine with a key role in the pathogenesis of this disease. Antibodies targeting IL-17A have shown superior efficacy compared with other psoriasis treatments, and long-term studies have demonstrated these to be safe in humans. Nevertheless, despite the excellent safety and efficacy record of these monoclonal antibodies in psoriasis treatment, patient access to these new first line therapies is restricted for financial reasons. Replacing passive immunisation by therapeutic vaccination targeting IL-17A has the potential to overcome this serious impediment and address this currently unmet medical need. A series of experiments were performed in mice as proof of concept for the primary pharmacodynamic action of such a vaccine and in order to obtain informative data on the principle pharmacokinetic parameters. We used the most widely employed in vivo model for psoriasis, the topical imiquimod challenge. This elicits a psoriasis-like skin disease, which has been extensively characterised and, importantly, involves IL-17A signaling. A concern with auto-vaccines is safety. The data demonstrate that the vaccine-induced antibody titre is reversible, that it can be boosted by a variety of immunisation schedules, and that antibody titres are not affected by an imiquimod-induced psoriasis-like inflammatory flare. Furthermore, the antibody response generated was highly specific, as no significant antibodies reactive to the most closely related isoform, IL-17F, were detectable. Most importantly, we conducted a head-to-head comparison with a monoclonal antibody targeting IL-17A, which is known to be highly effective against psoriasis, to assess efficacy. The results show that in mice the vaccine effects are of equal efficacy to injected IL-17A monoclonal

antibodies. These findings support the rationale for clinical development of the vaccine.

Introduction

This introduction will cover the fundamental histology, genetics and immunology of psoriasis. The relationship between interleukin-17 and development of psoriasis will be covered in detail. The mechanism of action of biological agents, in particular those which target interleukin-17 and supporting pathways will be explored. Past approaches for IL-17 vaccination use in inflammatory disorders and vaccine development in psoriasis will supplement the above and be integrated as far as relevant for this thesis. This introduction will not cover other treatments available for psoriasis. It will not focus on the pathogenesis of psoriatic arthritis or other associated co-morbidities (1-5).

1.1 The Skin

The skin is the largest organ of the human body covering approximately 1-2m^2 surface area and accounts for 16% of an adult's body weight. The skin is in direct contact with the external environment and represents the primary defence layer against harmful pathogens and microbes. It also has essential functions in immune surveillance, thermoregulation, vitamin D synthesis, sensory function and retention of moisture (6, 7). There are significant differences in human skin based on its location. Skin found on the palms and soles is characterised by a thickened epidermis, compared to other areas of skin elsewhere which have both sebaceous glands and hair follicles. The many cell types form the complex architecture which can be considered as three distinct skin layers: epidermis, dermis and subcutaneous layer (8).

1.1.1 Layers of the skin

The epidermis is the uppermost of the skin layers and is completely avascular, relying on nourishment via diffusion from the dermis. It can be further categorised into the keratin layer, granular layer, prickle cell layer and basal layer, as shown in figure 1.1. The basal layer lies adjacent to the dermis and is composed primarily of keratinocytes, which divide and differentiate as they move closer to the surface. Melanocytes, which produce skin pigment melanin, and Merkel cells, which contribute to light touch sensation, are also resident in this layer (9). In the prickle cell layer, desmosomes link keratinocytes and give rise to the ‘prickle’ microscopic appearance the layer is named after. Langerhans cells are in their highest concentration in the prickle cell layer, and have important immunological function. By the time cells reach the granular layer they have flattened, lost their nuclei and their cytoplasm has a granular appearance. The keratin layer is the uppermost layer, composed of layers hexagonal corneocytes. The interior of corneocytes is primarily keratin filaments held together by filaggrin protein (10). Between corneocytes in the extracellular space are layers of lipid bilayers, which help provide the structure critical to epidermal function - water retention and a physical barrier pathogens, oxidative stress and chemical compounds. The different component layers of the epidermis undergo constant turnover to replace dead or damaged cells (8).

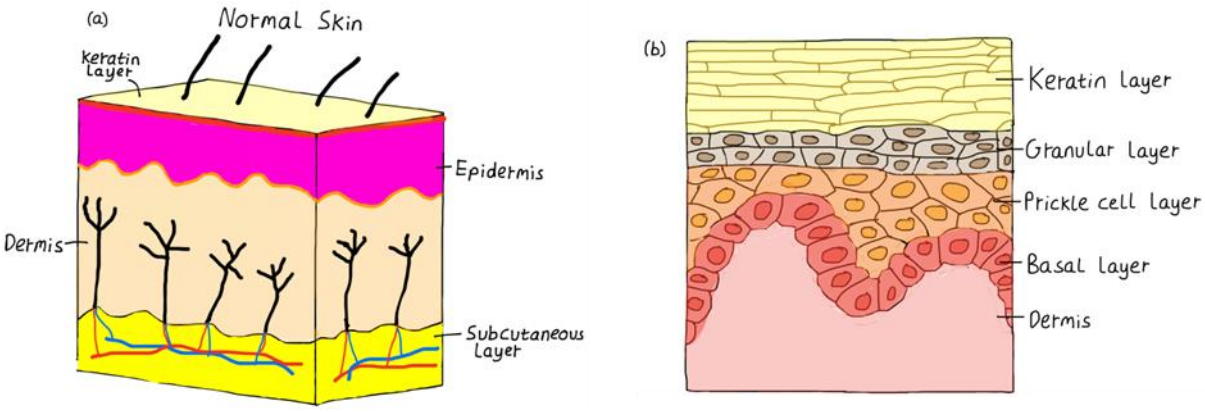

Figure 1.1. Histological representation of human skin layers. (a) Demonstrates the different layers of human skin. (b) Demonstrates in further detail the individual layers composing the epidermis.

The dermis is the connective tissue component of the skin and provides its pliability, elasticity and tensile strength. It is derived from the mesoderm germ layer and protects the body from injury, retains water and assists in temperature regulation. The dermis can be divided into two layers, the thinner superficial layer called the papillary dermis and the thicker layer adjacent to the subcutaneous tissue called the reticular dermis. The subpapillary plexus marks the boundary and the distinction is based on the different cell density and connective tissue organisation (11). The dermis is home to many of the derivative structures of the skin including hair follicles, sebaceous glands, sweat glands and lymphatic vessels. It is vastly thicker than the epidermis and its size ranges from 0.6mm on the eyelids to 3mm on the palms and soles. In spite of the rich blood supply, no vessels pass through the dermal epidermal junction. The most abundant cell population in the dermis are fibroblasts which are responsible for synthesis of connective tissue matrix proteins collagen and elastin. The collagen provides great tensile strength and between 80-90% of dermal collagen is type I collagen with 8-12% being type III collagen. The

elastin produces elastic fibres which allow the skin to return to its normal configuration after distortion (12).

The subcutaneous layer lies deep to the dermis and is not technically considered a skin layer. It is composed primarily of fat lobules and connective tissue. Cell types found in the subcutaneous layer include fibroblasts, adipocytes and macrophages. The deposition of fat protects internal organs and the skeleton from trauma, assists with the regulation of body temperature via insulation and acts an energy reservoir. The extensive connective tissue helps to connect the dermis to the underlying musculoskeletal system. Large blood vessels present in this layer supply the dermis with nutrients for homeostasis of the skin, which are able to pass into the epidermis by diffusion. Due to this high vascularity the tissue absorbs drugs quickly and is often used clinically as a route of drug administration. The thickness of this layer varies between considerably individuals and at different sites of the body dependent on nutritional status (13).

1.1.2 Immune function of the skin

A diverse immune cell population resides in both the epidermis and dermis of the skin. The primary cells involved in this complex system are phagocytes, antigen-presenting cells, mast cells and T cells. Together, despite the highly specialised functions of each of these cell types, they provide the host protection against pathogens whilst maintaining tolerance to innocuous substances (14).

Macrophages occupy the highest percentage of blood cells in the skin during steady-state conditions. They have important roles in maintaining skin function

and promoting wound closure and tissue repair. Macrophages have genetically encoded cell surface receptors and intracellular molecules which recognise damage associated and pathogen associated motifs. Based on the nature of the stimulus macrophages are able to produce a range of cytokines and chemokines to attract the relevant immune cells to the site of the stimulus. At the late stages of wound repair, they are able to switch to a growth promoting and less inflammatory phenotype, aiding the resolution of inflammation by phagocytosing apoptotic cells (15). There is huge functional diversity of macrophage subsets across different organ systems. Initially macrophages in the skin were viewed as a rather homogenous group, acting as defence against invading pathogens. It is possible that the different anatomical sites within the skin, for example perivascular macrophages as opposed to lymphatic macrophages, may be more heterogeneous in function than first thought (14).

Dendritic cells are a type of antigen-presenting cell that have the capacity to present antigens to naïve T cells in order to generate immunity against invading pathogens, in addition to tolerance to self-antigens. These functions are only possible in skin dendritic cells (DC) due to the capability to migrate via the lymphatics to local lymph nodes present in the skin, where they initiate and influence the adaptive immune response. Skin DCs can be divided into two groups based on their location: epidermal based Langerhans cells (LC) and dermal dendritic cells (DDC), which reside in the dermis. LCs form a network between keratinocytes of the epidermis and they are able to maintain their numbers in the absence of an inflammatory response by in-situ proliferation (16). DDCs are continuously renewed by bone marrow derived progenitor cells. In addition to their role in initiating pathogen specific immune responses, DDCs are also detectable within cutaneous lymph nodes during homeostatic

conditions, which suggest they may have a role in maintaining tolerance to innocuous agents (17). Further subtypes of DC's exist (for example, plasmacytoid versus myeloid) but are beyond the scope of this thesis.

Mast cells are abundant at all host-environmental interfaces and in the skin they are present in the dermis, close to the vascular supply. Mast cells are crucial in allergic responses where environmental allergens trigger mast cells to release histamine. This is mediated via cross-linking of immunoglobulin (Ig)E receptors on the mast cell surface by IgE bound allergens, promoting tissue inflammation (18). Beyond this response, mast cells have a role in pathogen defence, contact hypersensitivity responses and skin tumours (19).

In normal skin under homeostatic conditions B cells make up a small proportion of cells. In contrast, the number of T cells (2.0×10^{10}) is approximately twice that of the T cell population in the peripheral circulation (1.1×10^{10}) (20, 21). B cells function in the adaptive immune system and are responsible for the production of antibodies by differentiating into plasma cells, as shown in figure 1.2. B cells have important roles in both maintaining skin homeostasis and disease, with key roles in the cutaneous tumour microenvironment in melanoma. T cells also have a role in humoral immunity and are activated by antigen presenting cells. The migration of activated T cells into the skin is guided by cutaneous lymphocyte antigen. Regulatory T cells assist in reducing inflammation and maintaining self-tolerance through secretion of IL-10 and transforming growth factor β. These have been shown to have a role in the pathogenesis of psoriasis, in addition to other autoimmune skin diseases. The majority population of T cell subtypes varies with location, with a higher proportion of $CD8^+$ T cells in the epidermis and higher number of $CD4^+$ T cells in the dermis (21).

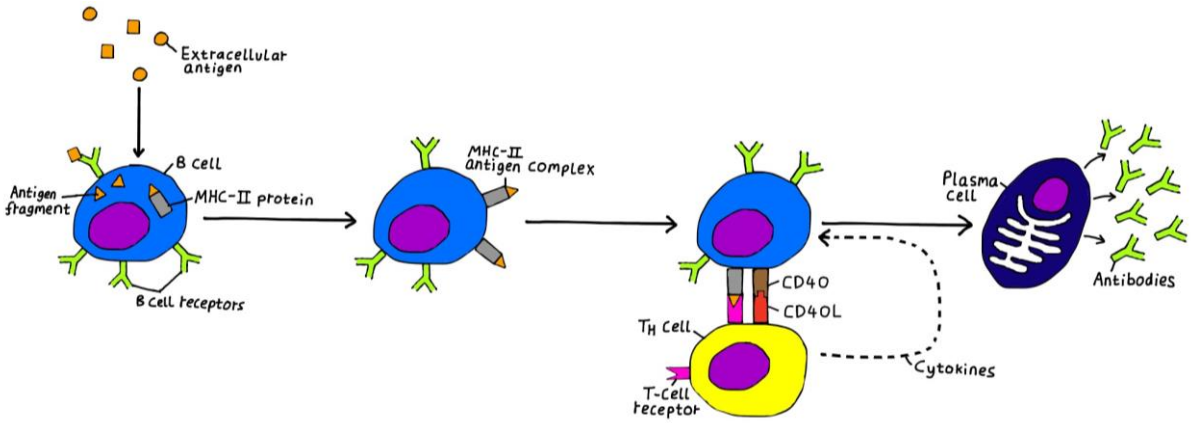

Figure 1.2. The role and activation of B cells. B cells recognise free antigen in the serum using a B cell receptor. The B cell then displays the antigen bound to MHC-II complex which attracts a helper T cell. CD40 ligand on the T cell acts as a co-stimulatory factor by binding to CD40 on the B cell. In addition the helper T cell releases cytokines which helps the B cell multiply and mature into plasma cells which produce antibodies. Adapted from http://classes.midlandstech.edu/carterp/courses/bio225/chap17/lecture3.htm

1.2 Overview of psoriasis

Psoriasis is a chronic, autoimmune condition resulting in characteristic skin lesions (22, 23). The disease commonly manifests as erythematous, well demarcated plaques covered with silvery scales interspersed among areas of unaffected skin. Chronic plaque psoriasis accounts for 85% of psoriasis cases. The typical distribution of lesions is symmetrical on the extensor surfaces of the knees and elbows, lumbosacral region and scalp (24), as shown in figure 1.3. The scalp is the most frequently affected area and associated with poor quality of life and extensive pruritus (25, 26).

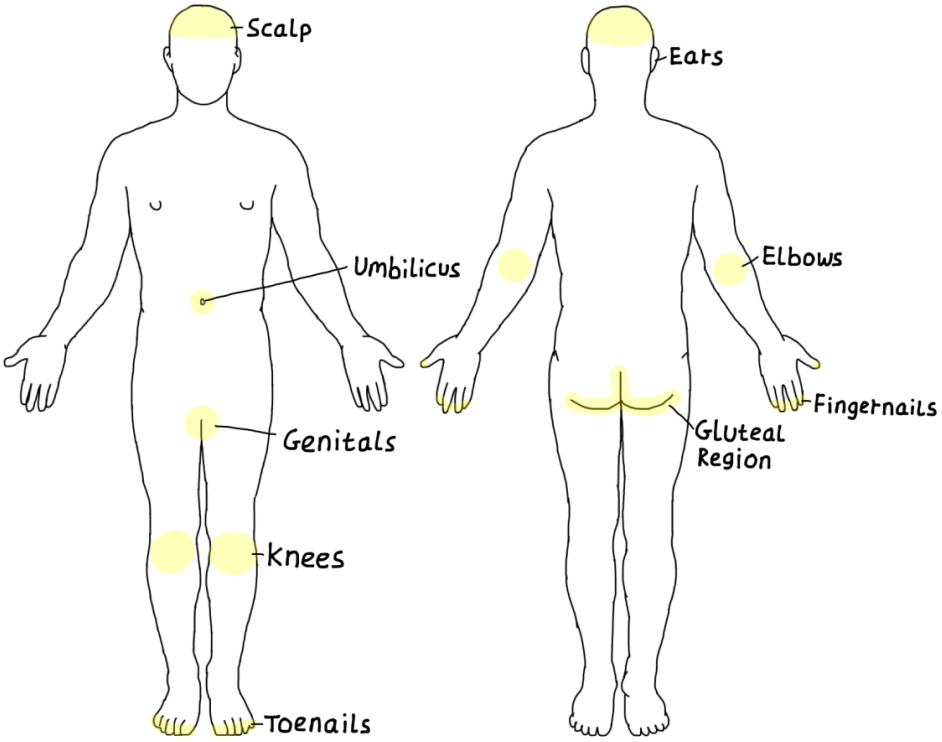

Figure 1.3. Clinical map of psoriasis lesions. Highlighted regions are the most frequent areas for chronic plaque psoriasis to manifest.

The exact causative mechanism of psoriasis is not fully understood, however studies have shown both strong genetic and environmental factors have key roles in the pathogenesis. Smoking (27) and obesity (28) have been associated with higher risk of psoriasis development. Psoriasis has a prevalence of between 0-11.8% worldwide (29). Higher incidence rates have been reported at higher latitudes and also in Caucasian ethnic groups relative to other populations (30).

In addition to chronic plaque, other forms of psoriasis include guttate, erythrodermic, inverse and pustular. Guttate psoriasis presents in younger individuals with sudden dissemination of erythematous papules covering the trunk and extremities. It is typically preceded by a streptococcal respiratory infection and has strong associations with human leukocyte antigen (HLA)-Cw6

allele. The clinical course shows more rapid involution and longer remission compared with other psoriatic subtypes (31, 32). Inverse psoriasis is a rare subtype difficult to diagnose and treat due its area of distribution in skin folds. It should be considered a diagnosis in any chronic inflammatory skin process affecting the skin folds which is unresponsive to fungal treatment (33, 34). The least common but potentially most harmful form is erythrodermic psoriasis accounting for up to 1.5% of psoriasis cases. This form can develop gradually or acutely from any pre-existing form of psoriasis and can affect up to 100% of total body skin, leading the patient susceptible to fluid loss and infection. Individual patients can exhibit different variants of psoriasis listed above at different points in time (35).

1.2.1 Clinical symptoms of psoriasis

Psoriasis can first present clinically in infancy, childhood or in adult years. Clinical presentation in children may differ from adults, with more frequent involvement of the face in children. Spontaneous remissions of varying length have been reported in 32% of childhood-onset psoriasis cases (36, 37).

Psoriasis can improve or worsen in pregnancy. A study by Murase *et al* (38), involving 47 pregnant psoriasis patients showed 55% had improved symptomatic relief during their pregnancy, 21% saw no effect with pregnancy and 23% found their disease was exacerbated. Disease improvement is thought to be attributable to increasing oestrogen levels in pregnancy. Oestrogen inhibits the secretion of cytokines which drive the inflammatory processes in psoriasis (39, 40).

The lifetime incidence of nail disease in psoriasis is estimated between 80-90% and isolated nail disease without skin symptom manifestation accounts for fewer than 5% of cases of psoriasis (41). A strong correlation has been shown in those patients with nail involvement and those with concurrent psoriatic arthritis (42). Clinically nail disease in psoriasis is highly heterogeneous and depends upon the area of the nail affected, as shown in figure 1.4. Nail pitting, leukonychia and nail dystrophy are due to nail matrix involvement. Splinter haemorrhages, subungal hyperkeratosis and onycholysis result from disease of the nail bed (43, 44).

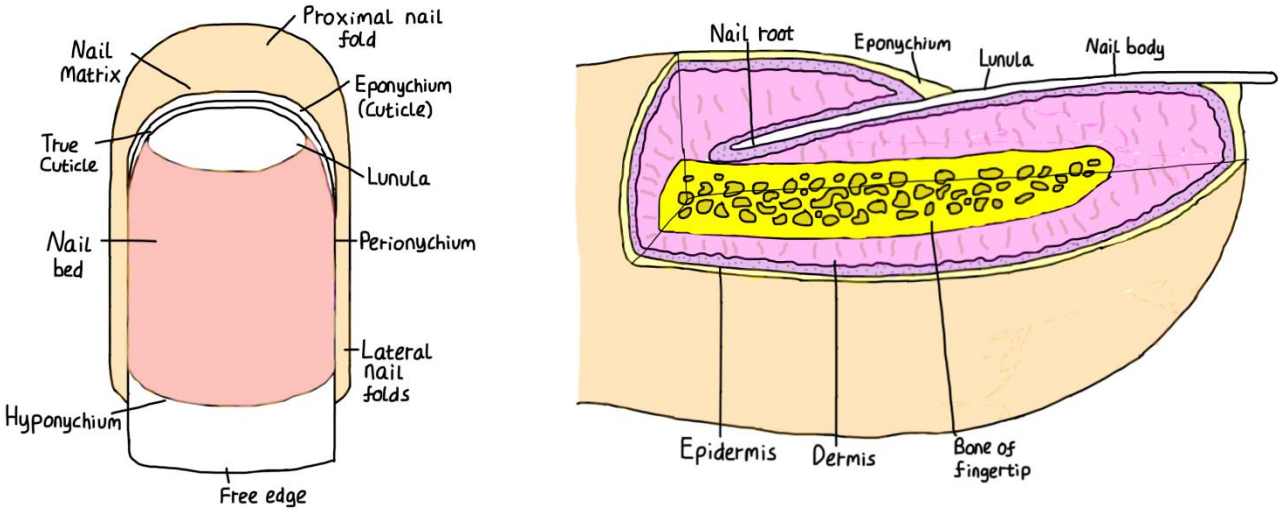

Figure 1.4. The external and internal anatomy of human nails. Psoriatic nail disease symptoms depend on whether the nail matrix or nail bed is primarily affected. Adapted from http://healthfavo.com/human-nail-diagram.html

The prevalence of psoriatic arthritis in psoriasis patients is variable affecting between 10-40% (45). This large range is attributable to the lack of definitive diagnostic criteria for psoriatic arthritis with most commonly employed being the CASPAR criteria (46). Clinically psoriatic arthritis can be divided into five subtypes and the clinical phenotype is highly variable but involves inflammatory

changes to joints, bones, ligaments and tendons (47). Disease affecting the joints commonly presents 10 years following the initial skin symptoms presentation (46, 48).

Quality of life (QoL) is severely affected in psoriasis. The impact on QoL appeared more significant on younger patients diagnosed with psoriasis than those diagnosed in older age brackets (49). Moreover higher levels of anxiety and depression have been seen in family members of those with the disease, close to the anxiety level of the patient (50). In particular increased severity of pruritus, scaling and pain with psoriatic lesions is associated with lower QoL scores (51). Moreover, those with concomitant psoriatic arthritis report significantly lower QoL than patients without joint involvement (52).

Psoriatic symptoms also result in considerable loss of productivity and absenteeism at work. A study by Graham *et al*, showed that on average those with moderate to severe psoriasis lost >8 hours of productivity at work per week due to their psoriasis symptoms (53). A separate study in the UK demonstrated that 45% changed employment completely, or role at their employment due to their psoriasis (54).

The gold standard for monitoring improvement in psoriasis skin lesions has been the psoriasis severity and area index (PASI) score. The majority of clinical trials consider a minimum improvement of 75% reduction in psoriatic skin lesions (PASI 75) for an intervention to be considered successful treatment (55). The PASI 75 is generally considered a more useful evaluation tool than the physicians global assessment (56). Despite this reduction in PASI score does not always correlate with dermatology life quality index (DLQI) scores,

suggesting patient scoring systems should be considered more highly when choosing treatment methods (57).

1.2.2 Genetics of psoriasis

Two types of chronic plaque psoriasis have been proposed based on bimodal age of onset and genetic factors. Type 1 psoriasis is associated with a positive family history, presence of HLA-Cw6 allele and age of onset below 40 years of age. Type 2 psoriasis is less common and is not associated with a family history of disease, population frequency of the HLA-Cw6 allele and age of onset above 40 year of age (58).

Twin studies have shown greater concordance between monozygotic twins than dizygotic twin pairs and estimate heritability of psoriasis between 66-90% (59). Paternal anticipation has been demonstrated and also a parent-of-origin effect with a significantly higher percentage of probands having an affected father as opposed to an affected mother (60, 61).

Epigenetic phenomena including DNA methylation, histone modifications and regulation by miRNAs also impacts on psoriasis pathogenesis and heritability (62, 63). Areas of CpG methylation differ between lesional and non-lesional skin in psoriasis patients. The promoter regions of p15 and p21 are hypo-methylated in psoriatic lesions. Moreover 30% of patients with psoriasis are shown to have methylation of the promoter of anti-apoptotic gene $p16^{INK4a}$. These mechanisms help psoriatic keratinocytes avoid apoptosis and remain in a state of senescence (64).

Genome wide association studies have mapped greater than 30 susceptibility loci, however up to 50% of genetic variance is accountable to an area on

chromosome 6p21.3 known as the psoriasis susceptibility locus 1 (PSORS1), as shown in figure 1.5. Within this area association with several genes has been shown with the strongest genetic linkage as seen with HLA-Cw6 allele (65, 66). Psoriasis patients who are HLA-Cw6 positive have earlier onset disease, more severe phenotype, koebnerization and higher remit rates in pregnancy compared to HLA-Cw6 negative patients. Presence of this disease allele has been used in clinical trials as a pharmacogenomic marker predicting response to ustekinumab and methotrexate treatment (67, 68).

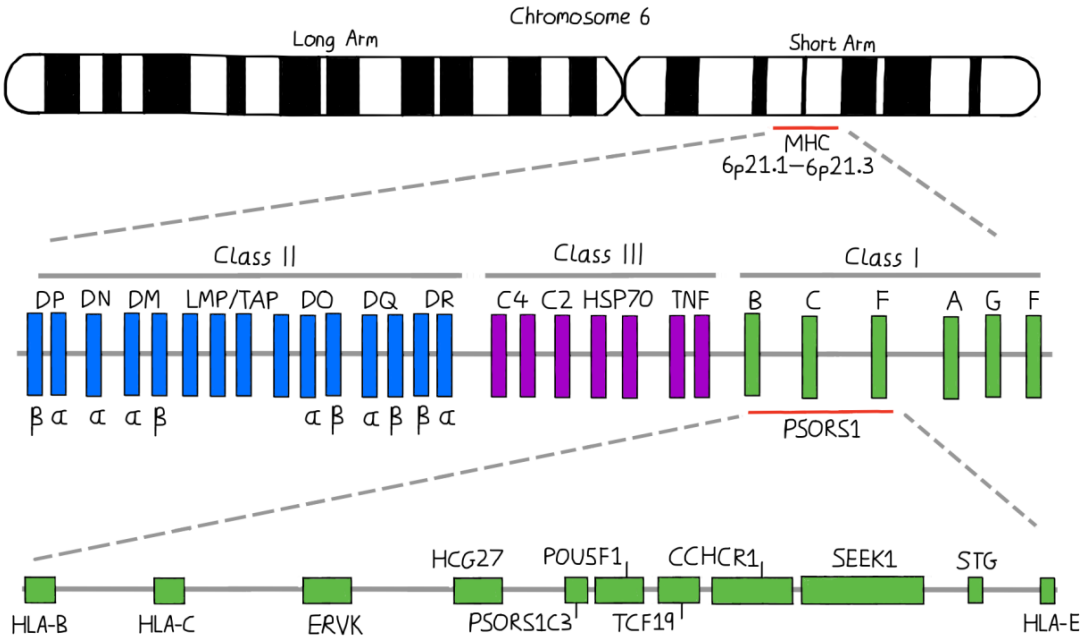

Figure 1.5. The MHC complex and PSORS1. Shows a map of chromosome 6 with a magnified area of the MHC region covering 6p21.1 - 6p21.3. HLA-C is found within class 1 of the HLA region. Adapted from http://journals.cambridge.org/fulltext_content/ERM/ERM5_07/S1462399403005957sup002.htm

1.2.3 Histology of psoriasis

Hyperproliferation of the epidermal keratinocytes is one of the histological hallmarks of psoriasis. Mitotic activity increases dramatically causing a

reduction in the cell cycle from 13 days in healthy skin to 36 hours in psoriatic keratinocytes. This reduction in maturation time is accompanied by altered differentiation, which is reflected in parakeratosis (persistence of nuclei in the keratin layer) and absence of the granular layer of the epidermis. Scales are formed on the skin surface as the stratum corneum thickens under the tiny abscess which is rejected by exfoliation (69). These histological hallmarks are shown in figure 1.6.

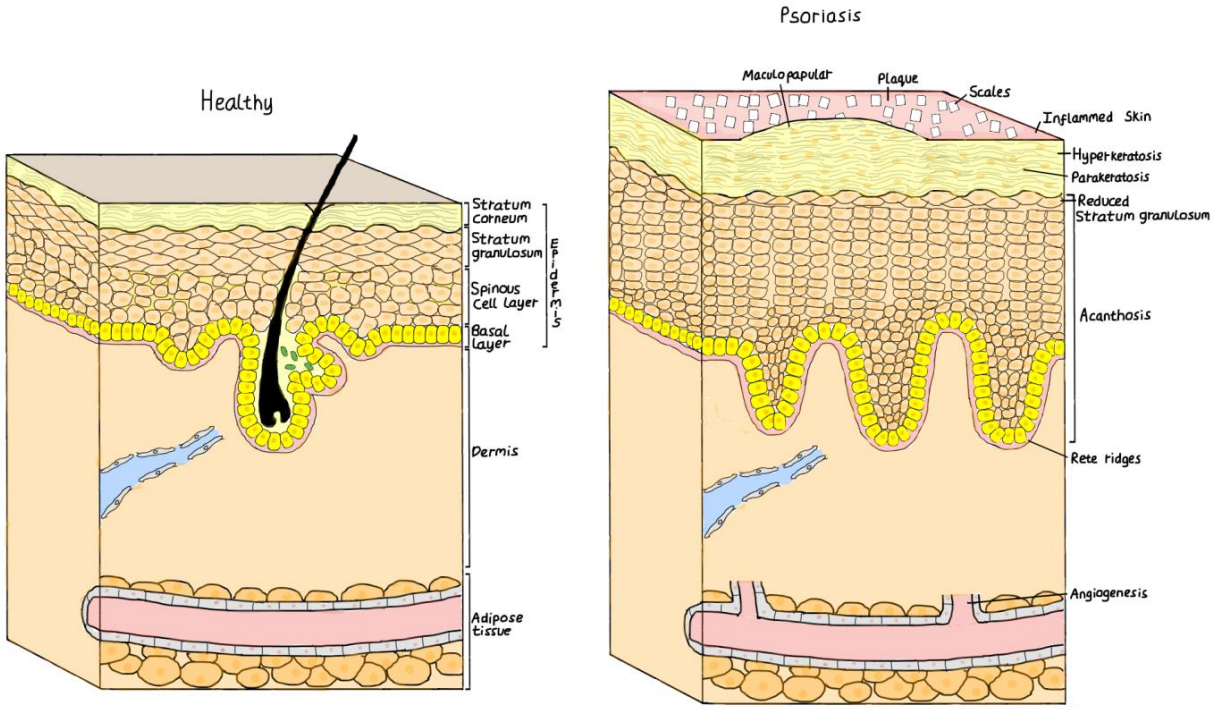

Figure 1.6. Comparison of healthy and psoriatic skin. Shows the different histological features between healthy skin and areas of skin affected by psoriasis. Adapted from (70).

Abnormalities of the wnt signalling pathway have been identified in a large range of skin disorders and processes, including malignancy (71), aging (72) and wound healing (73). A study showed that *W1F1* gene in the wnt signalling pathway was the most strongly downregulated gene compared with non-

psoriatic individuals (74). Wnt5a has a role in epithelial differentiation and stimulates proliferation of endothelial cells, in part due to induction of IL-12, and has been shown to be strongly induced by STAT3. Wnt5a and its putative receptors are localised primarily to the basal layer of the epidermis of normal skin, with a few cells in the granular layer. In contrast, Wnt5a is overexpressed throughout the whole of the spinous layer in psoriasis. In addition, activated endothelial cells, dermal fibroblasts and neutrophil aggregates of the keratin layer are all strongly positive for Wnt5a (75).

Pathological angiogenesis and lymphangiogenesis have been confirmed in psoriasis patients (76). Pro-angiogenic cytokines including vascular endothelial growth factor (VEGF), endothelial cell stimulating angiogenesis factor and tumour necrosis factor (TNF)-α are released from keratinocytes of psoriasis patients. Four times the volume of new blood vessel formation has been demonstrated in psoriatic skin compared with non-psoriatic comparisons (77). Telocytes (TC) are dermal interstitial cells distinct from fibroblasts which are involved in skin remodelling and repair. TCs distribute around blood vessels and contribute to the composition their extracellular membrane. Reduction in TC number and structural changes have been shown to be associated with other skin conditions, including systemic sclerosis (78). The loss of perivascular TCs has been hypothesised to potentially trigger the pathognomonic clinical sign of psoriatic lesions, Auspitz's sign (79).

The presence of Munro's microabscesses are highly specific of psoriasis lesions. These are infiltrating leukocytes which collect within small foci of the stratum corneum visible on histological analysis only (80, 81). Newer studies have implicated the matricellular protein CCN1, which promotes epidermal hyperplasia and inflammation (82).

1.3 The immune system and the role of IL-17

1.3.1 Immunology of psoriasis

The pro-inflammatory actions of cytokines are responsible for the epidermal changes seen in psoriasis and lead to a self-sustained feedback loop and chronification of the disease, as shown in figure 1.7. The production of interferon (IFN)-α drives autoimmunity. The dermis is infiltrated by high numbers of plasmacytoid dendritic cells, which become active to produce IFN-α. Once active, IFN-α induces activation and maturation of conventional dendritic cells, which stimulate T cell proliferation and polarise T cells to form T helper (Th)1 and Th17 cells. This process bridges the gap between innate and adaptive immunity. Following these processes autoreactive T cells proliferate and migrate into the epidermis, a process which is controlled by the expression of alpha 1 beta 1 integrin on effector T cells. This precedes the onset of psoriasis and is crucial for development of hallmark epidermal changes (83, 84).

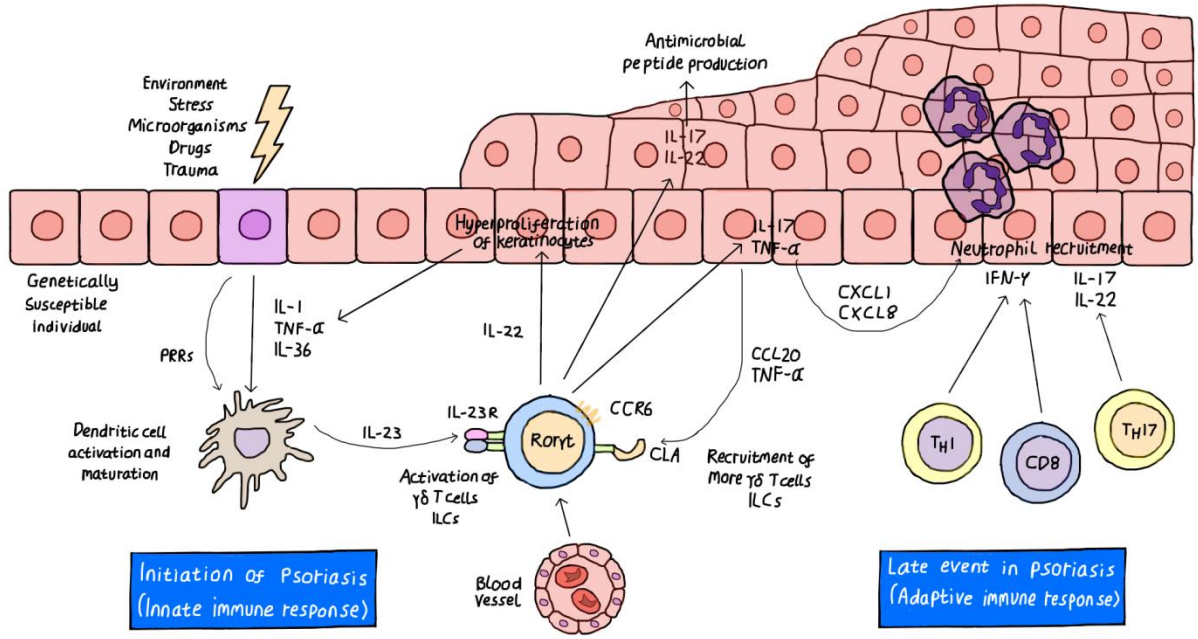

Figure 1.7. The immune response in psoriasis. Following an initial stress to cells a complex interplay of inflammatory markers leads to hyperproliferation of keratinocytes. Adapted from (85).

There was surprise amongst clinicians and researchers when the primary source of IL-17 was identified as γδ T cells, distinct invading immune cells, and not Th17 cells as previously thought. Studies of murine psoriasis models have shown that approximately 80% of IL-17A, in addition to 60% of IL-22 were produced by γδ T cells. The remaining IL-17A and IL-22 producers were RORγt^{+}CD3–ILCs, giving rise to 10% of IL-17A and 40% of IL-22. In human skin, dissimilar to mice skin (86), γδ T cells do not exist in the epidermis, with CD3^{+}γδ TCR^{+} T cells accounting for 4% of human dermal leukocytes. γδ T cells express chemokine receptor CCR6 which provides them an opportunity to enter into the skin by activated keratinocytes and DCs (85). A study by Cai *et al (87)*, demonstrated the significance of these cells in psoriasis by two methods. Analysis of confocal microscopy from human skin showed the dermis of psoriatic skin lesions was readily infiltrated by γδ T cells, whereas there were significantly fewer of this cell type in healthy patients. Moreover the function of these cells to produce IL-17 was assessed, with and without IL-23 stimulation. Even when stimulated with IL-23 minimal IL-17 was produced from control patient γδ T cells, whereas 15% of psoriasis patient γδ T cells produced IL-17 upon IL-23 stimulation. Thus, both the absolute number of IL-17-secreting γδ T cells and percentage of γδ T cells secreting IL-17 were significantly higher compared to control samples.

Genetic associations with genes important in T cell differentiation and polarisation have been shown in psoriasis. A key gene is *RUNX3*, a transcription factor important for CD8^{+} T cell development and promotion of Th1 polarisation, and possibly polarisation of Th17 also. Moreover another transcription factor within the same Runt-domain containing family *RUNX1*, has been associated with psoriasis. RUNX1 is expressed in naïve CD4^{+} T cells and

is downregulated upon T cell receptor stimulation. The exact mechanism of how these genes contribute to psoriasis pathogenesis is uncertain, however it is possible that gain of function mutations in *RUNX3*, and loss of functions in *RUNX1* may be responsible (88). Cytokines derived from Th2 cells have not been shown to play a role in psoriasis pathogenesis and are not increased in psoriatic lesions. Nevertheless, epigenetic changes including DNA methylation and chromatin modification of *IFNG* and *Foxp3* genes for Th2 cells and Th2 regulatory cells have been demonstrated when T cells are pushed more towards Th1 fate. It has been proposed that fewer Th2 cell associated SNPs in psoriasis patient genomes may be associated with T cells being more easily able to differentiate into pathogenic Th1 and Th17 cells, as opposed to Th2 variants (89).

T cells can adopt Th1 fate via secretion of IL-12 by dendritic cells. IL-12 is composed of two subunits, p35 and p40. The regulation and expansion of IL-17 producing Th17 cells is controlled by IL-23, which shares the common p40 subunit with IL-12. Expression of both p40 and p19 are significantly increased in psoriasis. The key role of these cytokines has been demonstrated by the clinical efficacy of ustekinumab monoclonal antibody, targeting the p40 subunit (90). TNF-α has long been associated with having a key role in psoriasis pathogenesis and has also been a target of biologic treatments. TNF-α is produced from activated T cells and antigen presenting cells. Independently, TNF-α is incapable of evoking significant response from cultured keratinocytes. However in combination with interleukins and other cytokines it forms strong synergies, amplifying responses and playing a key role in the formation of cytokine storms. TNF-α stabilises IL-17A, which potentiates the effects of IL-17A (91, 92).

The failure of psoriasis inflammation resolution and the role of negative regulators have been studied far less than the role of initial pro-inflammatory molecules. Mutations in multiple negative regulator genes, including *TNIP1*, *TNFAIP3* and *ZC3H12C*, involved in downregulation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway are associated with psoriasis. TNIP1 and TNFAIP3 interact with each other to inhibit NF-κB signalling by preventing NEMO poly-ubiquitination with subsequent degradation of the NF-κB inhibitor, IκB (93). ZC3H12C supresses NF-κB signalling and pro-inflammatory gene expression in endothelial cells. Loss of function psoriasis associated variants in *ZC3H12C* gene have been associated with increased dermal and epidermal inflammatory molecules through overactive NF-κB signalling. Given ZC3H12C inhibits vascular inflammation it has been hypothesised this could be a contributing factors towards cardiovascular comorbidities in psoriasis patients (94). One gene with highly penetrant mutations leading to psoriasis is *IL36RN*, which encodes for anti-inflammatory protein IL-36RA, a natural antagonist for IL-1F9. Mutations in *IL36RN* lead to unopposed effects of the inflammatory cytokine IL-1F9 where NF-κB regulated cytokines are highly increased (95).

1.3.2 The role of IL-17 in psoriasis

IL-17A is a pro-inflammatory cytokine and part of a six-member interleukin family including IL-17B, IL-17C, IL-17D, IL-17E and IL-17F, and five receptors (IL-17RA-RE), as shown in figure 1.8. This family of cytokines are unique in bearing minimal resemblance to other mammalian proteins. Despite structural similarity these six members have different roles in adaptive and innate immune system responses. Of these members the role of IL-17A is the best understood

and researched (96). IL-17A exists as a homodimer or a heterodimer with IL-17F. IL-17A, IL-17F and 17A/F heterodimer have their effect via binding to a receptor with IL-17RA and IL-17RC subunits (97).

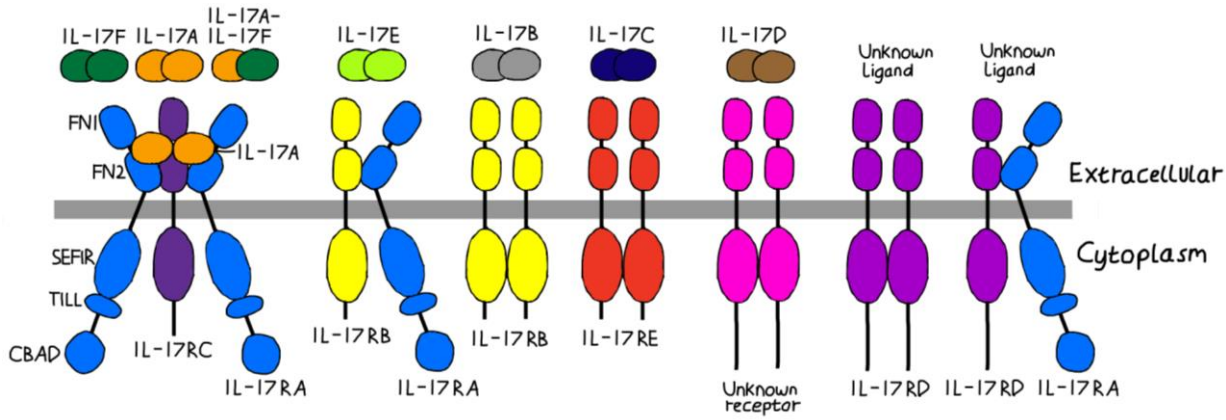

Figure 1.8. IL-17 molecules and IL-17 receptors. Adapted from (98).

From this point in the thesis IL-17A will be referred to IL-17. A major source of IL-17 are Th17 cells which are distinct from Th1 and Th2 cells (99). When IL-17 was first identified in 1993 helper T cells were categorised into Th1 and Th2 cells dependent on the cytokines they produced. IL-17 challenged this prior binary classification as the amino acid sequence differed from other cytokines previously described, and the receptor structure was not the same as that of other cytokine receptor families. Subsequent research led to the discovery of Th17 as a separate $CD4^+$ T cell population responsible for production of IL-17 (100). The main source of IL-17 are γδ T cells, as discussed in detail above in section 1.3.1. Other sources of IL-17 include mast cells, neutrophils, fibroblasts and natural killer cells. Studies have demonstrated the predominant cell type in psoriatic lesions staining positive for IL-17 are mast cells and neutrophils, with a higher density of these cells than IL-17 associated T cells (101).

Increased levels of both IL-17 and Th17 cells have been identified in the skin and peripheral circulation of psoriasis patients, and correlate with disease severity (102). One study showed that patients with PASI scores of 10 or higher, suggestive of moderate to severe disease, had serum IL-17A levels three times greater than those with PASI scores of fewer than 10. In addition, IL-17A mRNA expression was elevated in lesional skin compared to healthy skin (103). IL-17 decreases expression of cell adhesion factors resulting in disruption of the skin barrier, increases expression of keratin 17, reduces expression of filaggrin and contributes to epidermal hyperproliferation, seen clinically as the production of scales on the skin surface (104). IL-17A stimulates the innate immune system by increasing the expression of antimicrobial peptides, primarily members of the β-defensin and S100A families (105). On its own, IL-17 has been shown to have only a modest effect on inflammation however it acts in synergy when combined with other cytokines, particularly TNF-α and IL-1 (106). There is local production of Th17 cytokines within plaques which contribute to increased CCL20 production. This is key for migration of Th17 cells and dendritic cells into the psoriatic skin and helps maintain the inflammatory process (107). By stimulating fibroblast to produce VEGF IL-17 may promote angiogenesis in psoriasis, by the same process it does for many malignancies (108).

1.3.3 The role of IL-17 in other autoimmune diseases

IL-17 has a role in many different cellular process and diseases due to the variety of cell types it has an effect on, as shown in figure 1.9. IL-17 has a key role in rheumatoid arthritis (RA) pathogenesis through the production of other pro-inflammatory cytokines, including IL-6, IL-8, PGE2, and G-CSF, and

increasing osteoclastogenesis through activation of the RANKL pathway. The level of precursor γδ T cells in the synovium of RA patients is higher compared to healthy controls. Similar to psoriasis, there is also elevated CCL20 levels, which act as chemoattractant for Th17 cells (100). Response to IL-17 targeting therapies has been variable, with both secukinumab and ixekizumab showing clinical benefit with improved American College of Rheumatology (ACR)20 responses in patients unresponsive to disease-modifying antirheumatic drugs therapy. Phase II clinical studies for brodalumab, which targets IL-17A receptors, show it appears to be ineffective in RA (109).

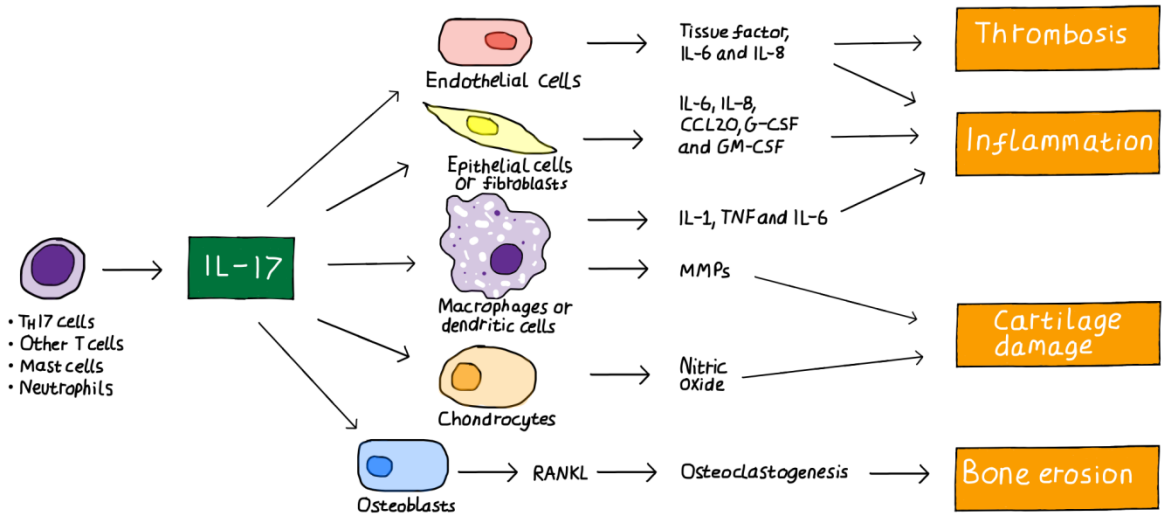

Figure 1.9. The role of IL-17 across various cells types and the end effect of altering these pathways. Adapted from (99).

Studies have shown that IL-17A and IL-17F levels are significantly higher in patients with multiple sclerosis (MS) than healthy controls. The higher IL-17 levels have been shown to be associated with pathogenesis of the disease with neutrophil expansion in cerebrospinal fluid and disruption of the blood-brain barrier. Moreover, a positive correlation has been shown between increased IL-17F levels and the number of relapses (110). It has been hypothesised that

Th17 cells might enhance glutamate excitotoxicity in the disease course. Studies have shown the IL-17A level and the number of neutrophils decreased with disease duration, suggesting the role of Th17 cells in MS pathology could be more significant in onset of the disease compared to disease progression (111). IL-17A targeting therapies have proven successful at reducing severity of the disease but this finding may explain why they fail to provide the same clinical benefit in later stages of the disease (112).

In addition to autoimmune diseases IL-17 has a role in protecting the host from bacterial, fungal and viral infections, in particular at mucosal sites such as the oral cavity and lungs. Th17 cells are enriched at mucosal surfaces, and produce cytokine receptors which target them to mucosal areas. IL-17 is considered to primarily have a larger role in protection from extracellular bacterial pathogens. Nevertheless, IL-17 has been shown to have a role in intracellular bacterial infections caused by *Listeria monocytogenes*, *Mycobacterium tuberculosis* and *Francisella tularensis* (113). The most studied fungal infection for IL-17 involvement has been *Candida albicans*, where together with IL-23, it has been shown to have a strong protective role against oral candidiasis (114). IL-17 can also have a negative effect on host response, as is the case in certain viral infections where it can contribute to cytokine storms. In an influenza infection model the survival rate of IL-17RA$^{-/-}$ mice was higher than wild type, with these mice showing fewer inflammatory cytokines (115).

1.4 Therapeutic development in psoriasis

At present there is no curative therapy for psoriasis and treatment is aimed at symptom resolution and remission. Mild cases of psoriasis are often managed

with topical therapies such as emollients only (116). Moderate to severe cases of psoriasis are treated with a range of options from systemic therapies (117), including methotrexate (118, 119), and phototherapy (120).

1.4.1 Biologic agents in psoriasis treatment

Biologics are reserved for treatment resistant cases in the NHS and target specific molecules. Biologics for psoriasis target key inflammatory molecules including p40, TNF-α and IL-22, as shown in figure 1.10. The cost of such treatments is increasing and significantly higher than non-biologic treatments. The annual cost of maintenance treatment with etanercept in the USA is $46,395 and $53,909 with ustekinumab (121). In comparison treatment with the first line systemic agent methotrexate is only between $794 - 1,503 per year (122). This is an important consideration for healthcare providers when considering treatment.

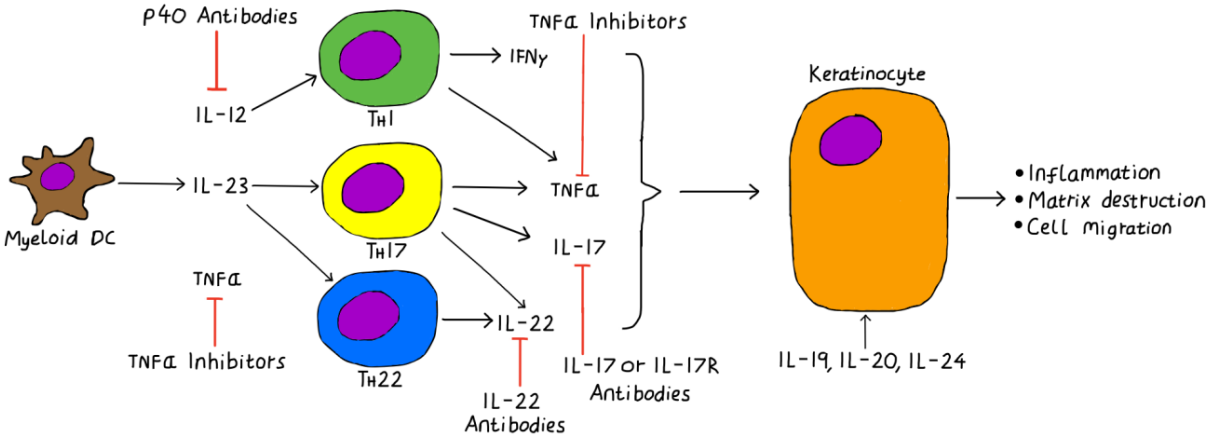

Figure 1.10. Cytokines targeted by biologics used in psoriasis treatment. Adapted from (123).

One disadvantage at present is the restriction of biologic therapies to injectable routes of administration, which is unsuitable for some patients. However oral methods of administration are being investigated for use including tofacinitib and apremilast (124). Tocafinitib is a JAK1/3 inhibitor developed with intent to treat psoriasis. The JAK family has a role in signal transduction from cytokine receptors to STAT in lymphocytes (125). Apremilast is a PDE4 inhibitor which results in anti-inflammatory effects by modulating the release and synthesis of cytokines and chemokines from immune cells (126, 127).

Three biologics which target IL-17 have recently been licenced: secukinumab, ixekizumab, and brodalumab, as shown in figure 1.11 (128). Secukinumab, as well as ixekizumab, directly bind and neutralise IL-17A, whereas brodalumab blocks the receptor IL-17RA. All show excellent results with regard to PASI scores with Phase II and Phase III clinical trials showing greater than 80% of patients achieving PASI 75 (129, 130). Ixekizumab has shown significant improvement in symptom resolution for those with scalp and nail lesions, which are historically resistant to treatment (131). The potential role of these IL-17 antagonists has also been explored for treatment of psoriatic arthritis (132). Short term safety data has been reassuring for all three biologics however concern has been raised over long-term safety. Blocking of IL-17A and IL-17F may increase risk of infection due to their role in mucocutaneous immunity. However due to inherent redundancy blocking of only one cytokine is unlikely to lead to serious comprise of host immune system (133).

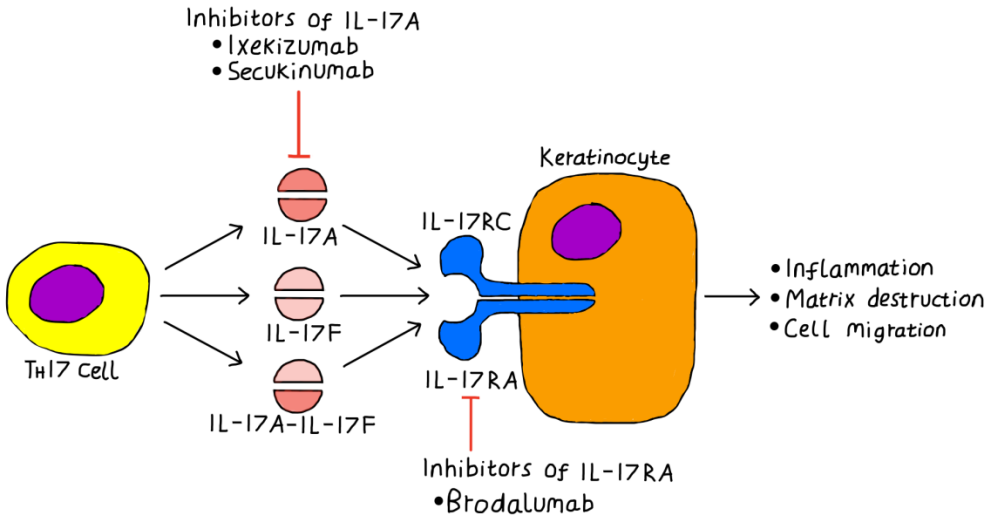

Figure 1.11. The precise mode of action of anti-IL-17 antibodies. Ixekizumab and seckunimab are direct IL-17A inhibitors, brodalumab inhibits IL-17RA. Adapted from (134).

1.4.2 Animal models of research in psoriasis

To assist with greater understanding of the disease and therapeutic development, animal models should successfully mirror human psoriasis phenotype, be predictive, inexpensive and reproducible. As not one model at present fulfils all of the criteria, different approaches exist and can be used relative to what outcome is being tested.

Animal models simulating psoriasis have mostly been hindered by the lack of such naturally occurring complex phenotype as in human psoriasis (135). Humans appear to be the only species where psoriasis naturally develops. The most commonly studied animals for psoriasis are rodents, which suffer from the above problem. Moreover there are several distinct differences between mouse skin and human skin including thickness and architecture of the epidermis, the density and thickness of hair follicles and the epidermal turnover rate, as shown in figure 1.12 (136). Nevertheless, animal models have allowed greater

understanding into the innate and adaptive immune systems involvement in psoriasis pathogenesis (137).

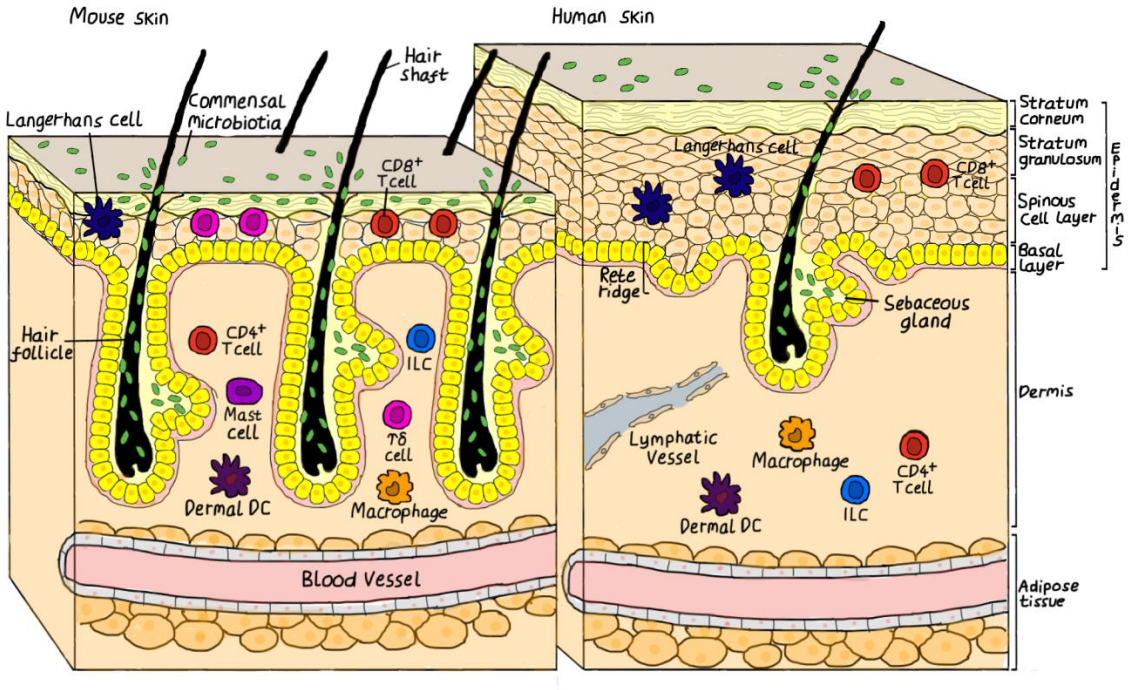

Figure 1.12. Histological differences between mouse and human skin. Adapted from (70).

To generate psoriasiform phenotypes in mice several approaches have been used, including genetically engineered animals and immunological approaches. Topical imiquimod application in mouse models can produce human-like psoriasis models which simulate histological changes, inflammation and phenotypic changes similar to human disease (138). Imiquimod generates a potent immunomodulatory effect by stimulation of Toll like receptor (TLR) 7 on plasmacytoid dendritic cells (pDCs). The chemical formula for imiquimod is shown in figure 1.13. Through this activation production of IFN-α is stimulated which results in broad immune system activation. Mouse studies have shown application of imiquimod increases the number of pDCs in the skin. Additionally

the imiquimod also has effect on Langerhans cells, B cells, macrophages and monocytes (139).

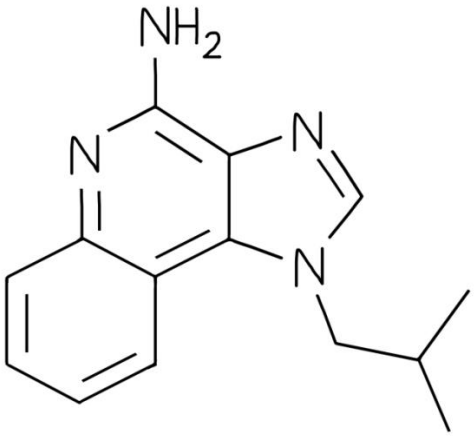

Figure 1.13. The chemical formula for imiquimod.

Due to the limitations of psoriasis like models in rodents, the best mouse model for study of the disease involves xenotransplantation of human skin onto immunodeficient mice. Mutations in forkhead box transcription factor N1 of nude mice results in defective thymus development with subsequent lack of T cells, and mice with *Prkdc*^{scid} mutations develop severe combined immune deficiency (SCID) (140). Studies in these groups of mice have shown psoriatic skin retains its phenotype for more than 2 months following transplantation and grafted non-lesional skin adopts psoriatic skin features in these mice. Interestingly, transplantation of non-lesional skin triggered the development of psoriasis in the formerly unaffected skin from psoriasis patients, but not in healthy donor tissue (141). Humanised mouse models could offer great value for studying targets which are only expressed in human tissues and for testing antibodies or biologic therapies targeted at human antigens (142).

The overwhelming majority of biomedical research models use rodents. However non-human primates (NHP) are still used due to their greater similarity to humans in physiology, development and neuroanatomy with respect to any other animal. In particular, their similar social interactions and complexities have made them particularly useful in studying disorders of dysfunctional social behaviour that could not be assessed as easily with rodent models (143).

An Aldara-induced skin inflammation model was developed in baboons for the first time in 2016. Reversibility of erythema and skin thickening was demonstrated on cessation of Aldara application and similar to mouse models, the histopathological analysis of the baboon skin showed acanthosis, hyperkeratosis, epidermal hyperproliferation and hypergranulosis. Moreover, mRNA analysis of skin biopsies showed overexpression of the IL-17 pathway (144). This suggests such a model could be of interest for testing efficacy, as well as safety, of anti-IL-17 agents on NHPs in the future.

1.4.3 Development of a vaccine towards psoriasis treatment

The therapeutic use of vaccines has been demonstrated in other autoimmune disorders such as myasthenia gravis. Similar to treatment with antibodies, the use of an antigen specific vaccine could avoid the nonspecific effects of current immunosuppressive drugs, such as infection. The results suggest the vaccine is a safe, rapidly acting and effective treatment for myasthenia gravis (145). Autovaccination aims to induce self-specific antibodies with the assistance of a small number of T cells. This is preferentially B-cell targeted with VLPs. Thus these vaccines are designed to induce antibodies as opposed to T cells. The vaccine used in this thesis employs use of virus like particles (VLP) for this

purpose, which has two advantages. First, when arranged in repetitive structures antigens on the surface of VLPs can directly activate B cells. Second, VLPs bypass Th cell tolerance as they are not innate cells, thus they produce a strong B cell response without self-specific Th cell responses (146). Clinical studies have been undertaken where VLP-based vaccines have targeted angiotensin for arterial hypertension and interleukin-1β to treat type I diabetes. In these trials no safety issues have been raised. Moreover, these studies have shown the vaccines to be highly immunogenic and achieve clinical efficacy (147, 148).

Many potential vaccines fail to make it beyond clinical trials. The reasons for this include lack of immunogenicity in human trials and the costs associated with proving vaccine efficacy (149). Another potential problem facing vaccine development for late onset disorders is the aging population. Immunosenescence in the elderly population makes these individuals increasingly susceptible to disease, and although vaccination is a protective factor, the failing immune system as we age makes targeting vaccines at this age group more difficult (150). However vaccines targeted at elderly populations have been successful, such as vaccination against Herpes Zoster and post-herpetic neuralgia in elderly patients, which has shown to dramatically reduce morbidity from these conditions and is cost effective (151).

IL-17 has been extensively studied as a target of biologic therapies with promising safety and efficacy data (146). In terms of safety this has already been demonstrated in patients who lack genes responsible for making IL-17A and those who cannot make its receptor molecule IL-17RA, in addition to those with naturally occurring high levels of antibody against IL-17A and IL-17F. In

these patients tuberculosis, fungal and microbial infections did not occur nor did the patients develop cancer (152, 153).

Development of a vaccine allows for several advantages against current biologic treatments. Firstly, vaccine production costs could dramatically undercut current prices for biologic drugs offering a cheaper alternative for patients and healthcare providers. Secondly, vaccines promote active immunity whereby the body naturally produces an immune response, as shown in figure 1.14, compared to passive immunity provided by biologic medications. This mechanism of generating an adaptive immune response offers long-term - potentially lifelong - immunity whereas biologics require regular doses to be administered to patients (154).

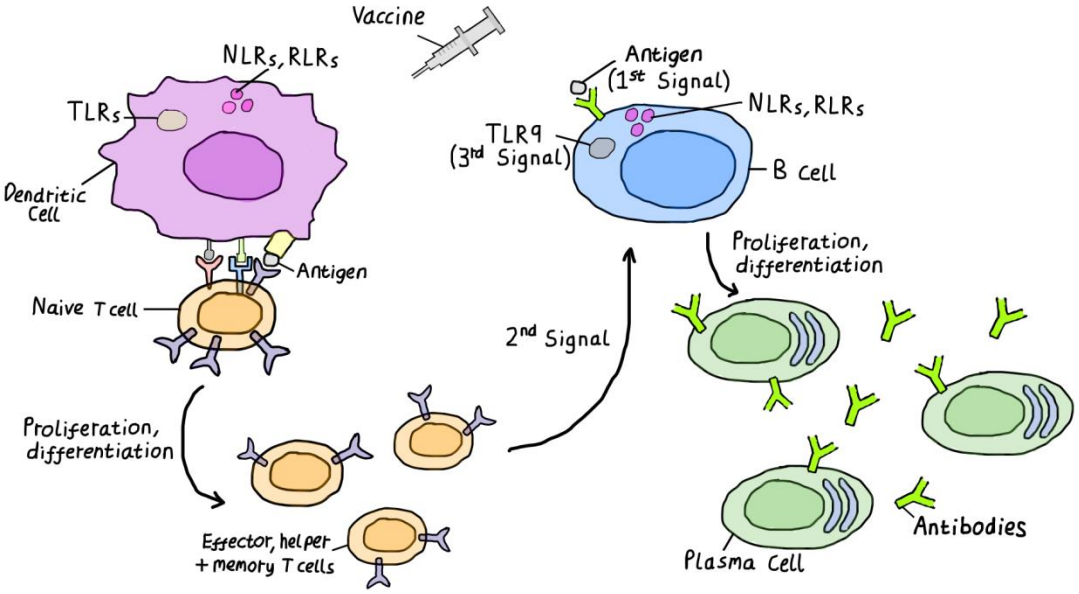

Figure 1.14. The mechanism of action of vaccines. Vaccines promote active immunity where the body creates memory T cells which, when exposed to the antigen, are able to stimulate B cells to differentiate into plasma cells and produce targeted antibodies. Adapted from (155).

1.4.4 Description of the vaccine

The complete IL-17A heterodimer, as opposed to specific domains, is used as the antigen. Despite increased production complexity the entire protein was used as studies of VLP based vaccines have shown greater efficacy using entire proteins rather than selected epitopes. The most likely reasons for this are because using the entire protein will capture not only the greatest number of potentially immunogenic linear epitopes but also, in difference to single domains, all conformational epitopes. Multiple copies of the IL-17A are covalently attached to the VLP using a hetero-functional chemical cross-linker succinimidyl-6-[(β-maleimidopropionamido) hexanoate]. The VLP is derived from the plant restricted cucumber mosaic virus (CMV). This is a linear single-stranded RNA icosahedral plant virus. Both the IL-17 and VLP intermediates are recombinantly expressed in E. coli and purified. The vaccine is designed to overcome immunological self-tolerance and to elicit autoantibodies that are able to bind specifically to IL-17A.

The amino acid sequence of the CMV coat protein differs from natural CMV sequence by way of a tetanus toxoid T cell epitope inserted at the N terminus. This modification is designed to improve vaccine immunogenicity in those who have previously been exposed to tetanus vaccination, which is close to the whole population in developed countries.

1.5 Thesis Aim and Rationale

Antibodies targeting IL-17A to treat psoriasis have confirmed that at present up to 5 years continuous inactivation of IL-17A in humans is safe. Between the three IL-17A targeting antibodies several thousands of years of patient safety

data show they do not result in systemic immuno-suppression, increase incidence of malignancies or other treatment associated side effects (156). Moreover, similar data is observed in studies of patients with inborn mutations in the IL-17A signalling pathway, with the main increased risk being of mucocutaneous limited candidiasis (157). Combined, these provide independent clinical evidence that long-term IL-17A inactivation is not associated with increased risk of severe toxicity or mortality.

Prolonged clinical studies for the IL-17A targeting monoclonal antibodies have also confirmed that neutralisation of IL-17A is a highly effective treatment option in psoriasis patients, with superior efficacy compared to other treatment modalities, and achieving measurable improvement in disease control for most patients taking these therapies (158, 159). Their superiority is to such an extent that all three monoclonal antibodies (secukinumab, ixekizumab, brodalumab) have been licensed as first-line treatment for psoriasis in the USA, Japan and the European Union.

The data generated for IL-17A neutralising monoclonal antibodies demonstrates a comparatively safe and highly effective treatment modality, which makes this highly sought after by patients and clinicians. However, the cost of placing patients on monoclonal antibody treatment is a major limitation to their use in clinical practice at present. Despite production of biosimilars for monoclonal antibodies and market competition reducing prices, the production process required to generate these complex molecules on large scales translates into significant per-patient cost. As such this makes it difficult for health care providers, including the NHS, to offer this readily to patients before much cheaper, but often less effective, treatment modalities.

The advent of IL-17A active vaccination offers the opportunity to replace the limitation of high costs associated with passive immunisation by monoclonal antibodies. In addition to the financial advantage, active vaccination could offer advantages including ease of dosing and avoidance of anti-drug antibodies. Should the vaccine demonstrate consistent effectiveness it has the potential of being used in treatment of other common diseases with underlying IL-17 associated aetiology, including rheumatoid arthritis and multiple sclerosis.

VLP based vaccine technology has been extensively studied (160), offering a validated platform for clinical development of an vaccine targeting IL-17A. At present, IL-17A is the cytokine with the most supporting evidence to validate a potential therapeutic auto-vaccine in psoriasis patients.

Given the above, the current thesis explores whether IL-17A-targeted vaccination could provide a feasible treatment approach for psoriasis. To do so, a prototype of such a vaccine was characterised with respect to its immunogenicity, the specificity of antibodies generated, the clinical in vivo efficacy in a mouse model of psoriasis, and the long-term development of antibody titres, including reversibility and ability to re-boost titres. By undertaking this pre-clinical trial our aim was to understand whether this treatment approach had potential applicability for human psoriasis patient use in the future.

Materials and Methods

2.1 Ethics statement

All regulated procedures performed on the mice as part of this study were conducted by personal licence holders in concordance with The Animals (Scientific Procedures) Act 1986. Study design and rationale for each individual experiment was approved by the Welfare and Ethical Use of Animals Committee at the University of Dundee.

2.2 Home Office Licence

In order to carry out experiments involving animals, individuals must have a personal licence from the Home Office and the procedures only authorised if carried out as part of a project for which there is a project licence granted. The project licence number used for this work is 64/2950 and the personal licence number used is ID74393FD.

2.3 Chemicals, reagents and buffers

All general chemicals were of molecular biology grade and obtained from Sigma-Aldrich (Gillingham, Dorset, UK), Fisher Scientific (Loughborough, Leicestershire, UK), eBioscience (Altrincham, Cheshire, UK), R&D (Abingdon, Oxfordshire, UK), Promega (Southampton, Hampshire, UK), BioLegend (London, UK) unless otherwise stated.

The IL-17 vaccine was obtained through academic collaboration with the Jenner institute at Oxford University.

2.4 Mice information

2.4.1 Species of mice and breeding

Wild type female C57Bl/6j mice were used across all the experiments. All mice were aged 6-10 weeks at the beginning the experiments, with the exception of the older group of mice involved in the diphtheria, pertussis and tetanus (DPT) pre-immunisation experiment, who were aged 7-8 months. All mice were purchased from a commercial breeding unit. No mice used in these experiments were bred in the resource unit.

2.4.2 Animal husbandry

Mice were checked everyday by members of the resource unit staff to ensure their health and wellbeing, and provided adequate food and water supply. Up to 5 mice were stored in M2 cages. Environmental enrichment was provided in each cage and changed over time to provide a suitable living environment for the mice.

2.4.3 Mouse identification

All mice in the experiments underwent ear tagging so that they could be easily identified for hematoxylin and eosin (H&E) analysis and ear thickness measurements. The designation for ear tagging is shown below in figure 2.1.

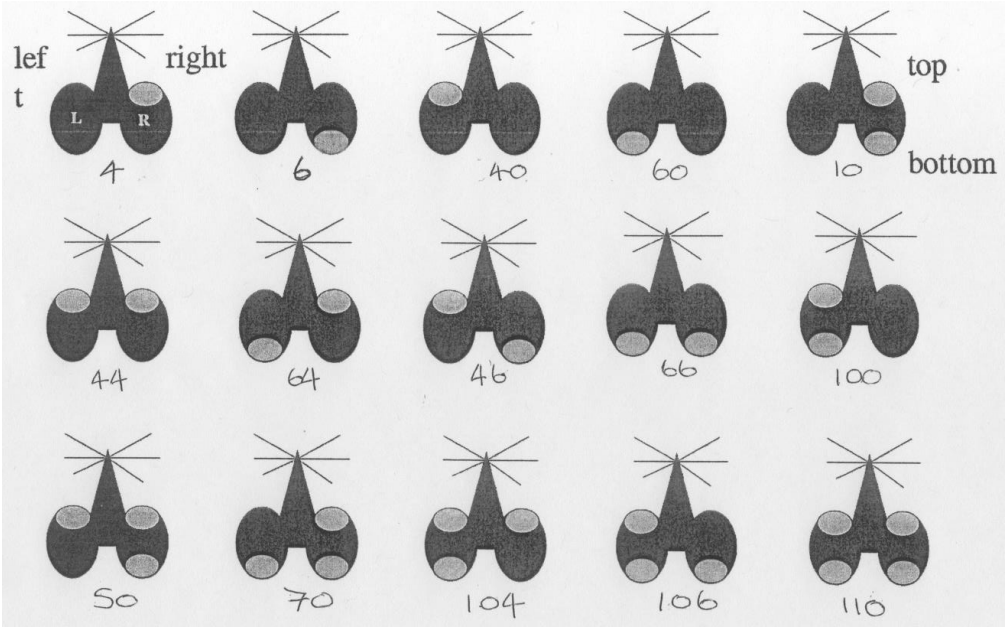

Figure 2.1. Mouse identification scheme. Shows the ear tagging system used for mouse identification as part of this study.

2.5 Procedures

2.5.1 Blood Sampling

The procedure used for tail vein blood sampling was as follows. The cages were placed on heat pads for 15 minutes prior to tail vein blood sampling to allow adequate dilation of the tail veins. A small incision over the vein was made and a capillary tube used to collect the tail vein blood. No restraint device was used during the procedure. Up to 200μl of blood was collected at each occasion.

Cardiac puncture was used in place of tail vein blood sampling when blood samples were to be acquired at the time of scheduling. The procedure used for cardiac puncture was as follows. The mice were placed in dorsal recumbency and the xiphisternum palpated. The 21G needle was inserted inferior to the xiphisternum and gentle suction applied to the syringe. The needle was

advanced until blood was aspirated. Up to 200μl of blood was collected at each occasion.

Once blood samples were acquired these were inverted 5 times each and then centrifuged at 10,000rpm for 90 seconds. Serum, ranging from 30 to 120μl, from each sample was aliquoted into safe-lock tubes. The serum was stored at -80^0C.

2.5.2 Injections

Subcutaneous injections were carried out with the mouse restrained with the scruff grasped between the thumb and forefinger and the tail gripped between the third and fourth finger.

2.5.3 Application of imiquimod

Dorsal skin was shaved prior to application of imiquimod cream. The mechanism by which imiquimod induces a psoriasis like reaction is discussed in the results section. Approximately 50mg of Aldara cream (one sachet) was applied to the dorsal skin and ear for each individual mouse at every application.

2.5.4 Scheduling

All mice in this study were humanely killed by dislocation of the neck or inert gas asphyxiation in accordance with Schedule 1 methods under The Animals (Scientific Procedures) Act 1986. Inert gas asphyxiation was used where blood samples were to be acquired at the time of scheduling. Where blood samples

were not required at time of scheduling dislocation of the neck was used. The death of each animal was confirmed before appropriate disposal.

2.6 Monitoring of mice

2.6.1 Ear thickness and body weight measurements

Ear thickness measurements and body weight measurements were recorded at different time intervals during the experiments using a micrometre and a scale respectively. Daily checks of the health of the mice and provision of food and water were made by staff in the animal resource unit.

2.6.2 Clinical photographs

For taking of photographs to document the dorsal skin phenotype after imiquimod application mice had their dorsal skin shaved to remove overlying hair obscuring the underlying view. The mice were photographed individually at the time of scheduling, against a plain background with the cage card for the corresponding cage photographed to help identify the mice in the experiment.

2.7 H&E based histology

2.7.1 Tissue acquisition

Ear samples and dorsal skin samples were taken, roughly 2cm by 2cm, and placed in formalin pots. Dorsal skin was shaved prior to sample analysis. Samples were taken from central areas with the least overlying hair, as shown in figure 2.2.

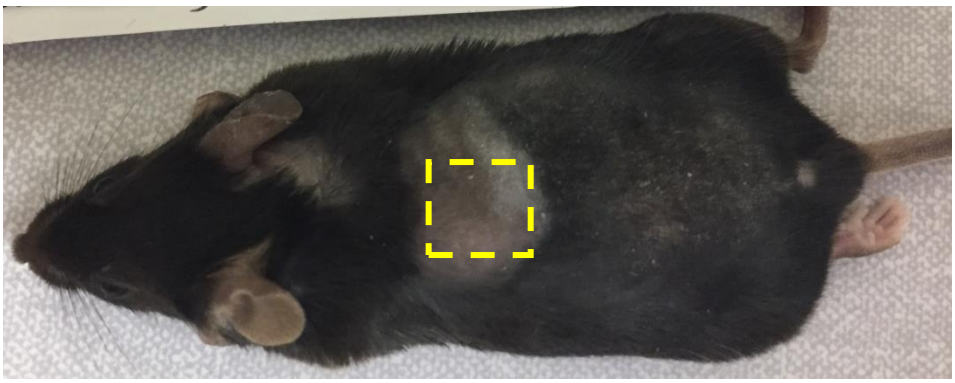

Figure 2.2. Areas of tissue acquisition for histology analysis. Outlined in yellow the area of dorsal skin removed for H&E analysis, as opposed to the shaved area with more overlying hair posterior to this outlined area.

Tissue fixation, embedding and staining were carried out by staff at the Tayside Tissue Bank Dundee. The procedures involved are described below.

2.7.2 Tissue fixation

The tissue was fixed in neutral buffered 10% formalin, which prevents the conversion of formaldehyde to formic acid. 2ml of formalin was used per 100mg of tissue. Tissues were fixed for a minimum of 48 hours at room temperature. Tissue was then dehydrated through a series of graded ethanol immersions to displace water. Once fixed, the tissue was processed as follows on a Leica Peloris tissue processor as per the standard NHS Tayside Pathology laboratory 8 hour protocol: formalin x2, 95% ethanol x4, 99% ethanol x4, xylene (first cleaning agent), 99% ethanol (second clearing agent), first paraffin wax and second paraffin wax.

2.7.3 Embedding process

Wax was melted by placing tissue cassettes in a 58^0C paraffin bath for 15 minutes. Mould was selected that left 2mm margins of paraffin wax around the tissue. Molten paraffin was dispensed in mould and warm forceps were used to transfer tissue into the mould, placing the cut side down. The mould was transferred to a cold plate and gently the tissue was pressed flat. With the tissue in the desired orientation the labelled tissue cassette was added on top of the mould as a backing. After 30 minutes the wax was cooled and hardened, and the paraffin block was then popped out of the mould. Tissues were then sectioned using a Leica microtome to 4µm thickness. The sections were dried in an oven at 37^0C. The tissue and paraffin attached to the cassette formed a block, which were stored at room temperature.

2.7.4 Staining procedure

Haematoxylin and Eosin staining was performed using Leica Autostainer XL. The procedure was as follows: tissue was placed in an oven at 60^0C for 15 minutes. Sections were deparaffinised by using 3 changes of xylene for 30 seconds each. These sections were re-hydrated in 2 changes of absolute alcohol for 30 seconds each. This was followed by 99% alcohol for 2 minutes and 95% alcohol for 2 minutes and washed in water for 30 seconds. Sections were stained in Harris haematoxylin solution for 4 minutes and washed in running tap water for 1 minute. Sections were differentiated in 0.1% acid alcohol for 1 minute and then washed in water for 1 minute. Bluing in saturated lithium carbonate solution for 1 minute followed this. Eosin solution was used to counterstain for 20 seconds and washed in tap water for 30 seconds. These were rinsed in 95% alcohol for 30 seconds two times and rinsed in 99% alcohol

for 30 seconds two times before final rinsing with Isopropyl alcohol for 30 seconds. Sections were cleared in 3 changes of xylene for 30 seconds each. Sections were coverslipped by Leica Coverslipper CV5030 using DPX mountant.

2.8 ELISA experiments

There are many different types of enzyme-linked immunosorbent assay (ELISA) tests, as shown in figure 2.3. The experiments carried out as part of this thesis used sandwich ELISAs involving secondary antibody conjugates.

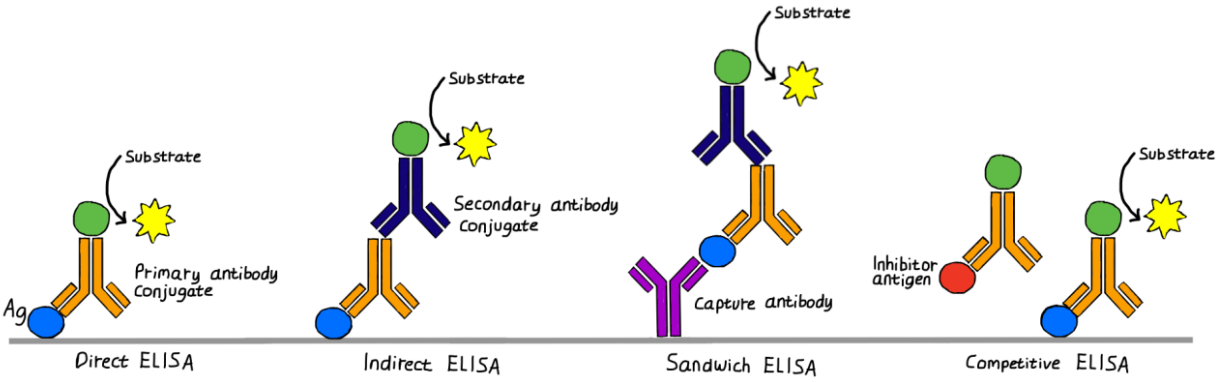

Figure 2.3. The different types of ELISA experiments. Adapted from http://www.abnova.com/support/resources.asp?switchfunctionid=%7B70196CA1-59B1-40D0-8394-19F533EB108F%7D

First Nunc-Immuno 96 clear polystyrene MicroWell solid plates were coated as follows. Recombinant murine IL-17 was diluted to a concentration of 2μg/ml in phosphate-buffered saline (PBS) and vortexed to allow adequate mixing. 100μl of this mixture was added to each well of the plate using a multi-channel pipette. The plate was covered with parafilm and stored at 4^0C for up to 72 hours.

Wash buffer was made using PBS with 0.05% Tween20 and blocking buffer made from the washing buffer composed of 2% bovine serum albumin (BSA) in PBS with 0.05% Tween20. The plate was removed from the fridge, the liquid expelled and the plates washed 3 times with 200μl wash buffer per well. 250μl blocking buffer was added to each well and the plates left for 150 minutes.

Serum was serial diluted in wash buffer on 96-well plates to the following concentrations: 1:30, 1:100, 1:300, 1:1000, 1:3,000, 1:10,000 and 1:100,000. Control serum, taken before any antibody or vaccination injections, was used in each experiment and diluted to a concentration of 1:100. This high concentration relative to the test samples was used to allow for enough control serum to be used for all the experiments. Liquid was discarded from the blocked plate and the plate was washed 3 times as above. 50μl of diluted serum was transferred onto the coated plate and with each sample a duplicate of each sample used. Empty wells were filled with 50μl sterile water for standardisation purposes and the plate incubated for 90 minutes on the shaker.

Detection antibody was made using Anti-mouse IgG labelled to alkaline phosphatase, diluted in blocking buffer to a concentration of 1:5000. Liquid was discarded from the blocked plate and the plate was washed 3 times as above. 100μl of detection antibody was added to each well and the plates covered with parafilm and left to incubate on the shaker for 45 minutes. Liquid was discarded from the blocked plate and the plate was washed 3 times as above. 50μl of alkaline phosphatase yellow (pNPP) liquid substrate was added to each well and the wells incubated on the shaker for 20 minutes. 5μl of stop solution (3M NaOH) were added to each well and the plate read in the SpectraMaxM3

spectrophotometer at 405 nanometres. This whole process is summarised in figure 2.4.

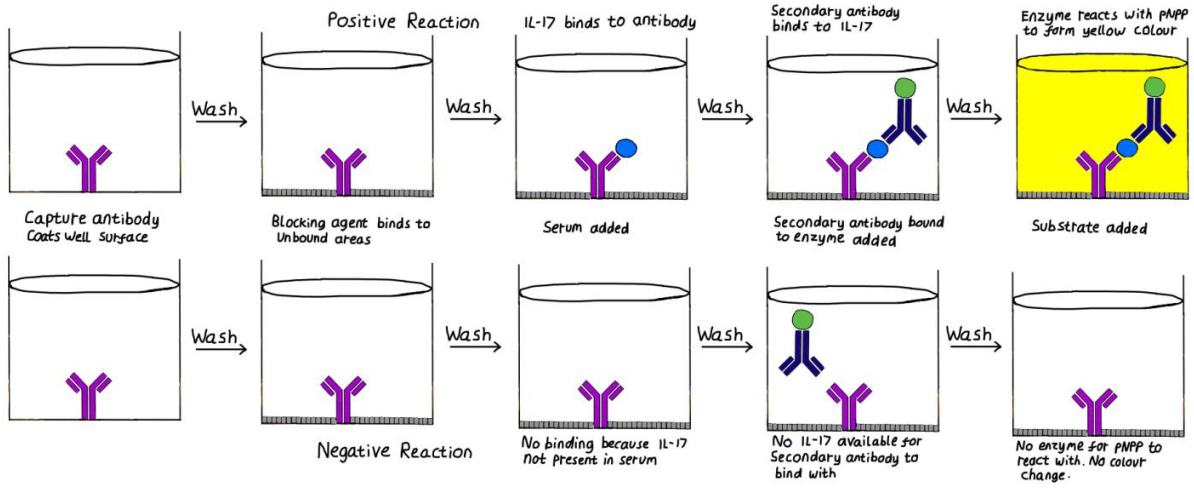

Figure 2.4. The sequence of events in the ELISA experiments. The top half shows positive results whereas the bottom half a negative result. Adapted from https://cellularphysiology.wikispaces.com/Enzymelinked+immunosorbent+assay+(ELISA)

SoftMax Pro6.3 software was used to read the plates. Absorbance instruments with endpoint settings of 405nm wavelength were used. ELISA data were analysed using a 3-parameter logistic fit according to standard sigmoid curve equation, Y = MAX/(1+(x/EC50)^(SLOPE)) where the minimum was set to zero after subtracting the sterile water optical densities from test values. Curve fitting was done using the ‘solver’ add-in using the GRC-nonlinear fitting algorithm.

2.9 Protocols for individual vaccine experiments

2.9.1 Immunogenicity of the immune response generated by the vaccine procedure

Mice were subjected to tail vein blood sampling, as described above in section 2.5.1. Tail vein blood (up to 200μl) was ascertained prior to the first vaccination and two weeks post final vaccination. Vaccination was carried out on day 1, day 15 and day 28.

The mice were divided into groups as documented in table 2.1

Table 2.1. Group allocation for immunogenicity experiment.

	Number of Mice	**Vaccination dose (micrograms)**
Group 1	4	1.25
Group 2	4	2.5
Group 3	4	5.0
Group 4	5	0.5

2.9.2 Assessment of vaccine efficacy procedure

The first phase of the experiment involved vaccination of the mice in group 1 only as follows. 5 female wild type C57Bl/6j mice, aged 6-8 weeks were chosen. Tail vein blood (up to 200μl) was ascertained on day 1, day 15 and day 29 of phase I. Mice were injected subcutaneously with a single dose of 50μl IL-17 vaccine on day 1 of phase I.

The second phase of the experiment involved IL-17 dosing as follows. The mice were divided into groups as documented in table 2.2

Table 2.2. Group allocation for efficacy experiment.

	Number of mice	**Imiquimod Application**	**Therapy**
Group 1	5	Yes	Vaccinated in phase 1
Group 2	5	Yes	None (positive control)
Group 3	5	Yes	Antibody injection
Group 4	3	No	None (negative control)

The below procedures were performed as part of the experiment.

Cream treatment: Aldara cream was applied to the one ear of all animals daily and to shaved dorsal skin (area approximately 2 x 2 cm) for up to 8 days of phase II to groups 1-3 mice only.

Antibody injection: 100μg of anti-IL-17 was injected in 100μl volume subcutaneously on day 1 and day 3 of phase II into group 3 mice only.

The mice ear thickness and body weight were monitored throughout. Ear thickness was recorded using a micrometer on day 1 and day 4 of phase II and the day of scheduling. Body weight was determined on day 1 of phase II and at scheduling.

2.9.3 Long-term kinetics experimental procedure

Three groups of C57Bl/6j females (n = 4 per group), aged 8-10 weeks at baseline. All groups were subjected to 50μl of vaccine subcutaneously on day 1, day 15 and day 29.

Tail vein blood (up to 200μl) was ascertained on the days of vaccination, 2 weeks after the last injection, then once monthly until scheduling of mice at week 58.

The mice were divided into groups as documented in table 2.3. The mice used for this experiment were used to evaluate several questions in line with the 3Rs of animal research - replacement, reduction and refinement. Therefore table 2.3 also documents the questions addressed by altering procedures between these three groups.

Table 2.3. Group allocation for long-term kinetics experiment.

	Number of Mice	Procedure	Question addressed
Group 1	4	1) Induced psoriasis flare (by imiquimod cream applied once daily for 7 days to shaved dorsal skin 3 months from baseline). 2) Repeated complete vaccine scheme as at baseline (3 injections) to be administered once antibody titre has decreased by >50% from maximal (group average).	1) Is there 'endogenous boosting' of titre? 2) Does repeat vaccination lead to overall higher titres?
Group 2	4	Single injections of vaccine administered at 3, 6, 9 months from baseline	Does repeated boosting prolong overall titre?
Group 3	4	Repeated complete vaccine scheme as at baseline (3 injections) to be administered once antibody titre has decreased by >50% from maximal (group average) or at nine months from baseline	How long does titre after single vaccination induction last? Is it higher after second boosting?

2.9.4 Enhancing vaccine responses by pre-DPT injection procedure

C57Bl/6j female mice, of ages specified below (n = 5 per group) were used. DPT vaccine was administered as single 50μl subcutaneous dose. IL-17 vaccine was administered in all groups as triple-induction (subcutaneous injections of 10μl of vaccine on day 1, day 15 and day 29). The first dose of IL-17 vaccine was given 4 weeks after DPT vaccination.

The mice were divided into groups as documented in table 2.4

Table 2.4. Group allocation for DPT pre-vaccine assessment experiment.

	Number of Mice	**DPT pre-vaccine**	**Mouse age**
Group 1	5	Yes	8 - 10 weeks
Group 2	5	No	8 - 10 weeks
Group 3	5	Yes	7 - 8 months
Group 4	5	No	7 - 8 months

Tail vein blood (up to 200μl) was ascertained at baseline, at each vaccination time point, 2 weeks after final IL-17 vaccination, and every 4 weeks thereafter up to 26 weeks when the mice were scheduled.

2.9.5 Enhancing vaccine responses by blocking IL-22 procedure

The first phase of the experiment involved vaccination of the mice in group 1 only as follows. 5 female wild type C57B/6j mice, aged 6-8 weeks were chosen.

Mice were injected subcutaneously with 50μl of IL-17 vaccine on day 1, day 15 and day 29 of phase 1.

Tail vein blood (up to 200μl) was ascertained prior to vaccination, on day 29 and at the beginning of dosing (phase II) on day 43.

The second phase of the experiment involved IL-17 dosing as follows. The mice were divided into groups as documented in table 2.5

Table 2.5. Group allocation for blocking of IL-22 experiment.

	Number of Mice	**Imiquimod Application**	**Therapy**
Group 1	5	Yes	Vaccinated in phase 1
Group 2	5	Yes	None (positive control)
Group 3	5	Yes	Antibody injection
Group 4	3	No	None (negative control)

The below procedures were performed as part of the experiment.

Cream treatment: Aldara cream was be applied to one ear of all animals daily and to shaved dorsal skin (area approximately 2 x 2 cm) for 11 days of phase II to groups 1-3 mice only

IL-22 antibody injection: 100μg of anti-IL-22 antibody was injected as 100μl subcutaneously on day 1 and day 3 of phase II into groups 1-3 mice only.

Antibody injection: 100μg of anti-IL17 was injected in 100μl volume subcutaneously on day 1 of phase II into group 3 only.

The mice ear thickness and body weight were monitored throughout. Ear thickness was recorded using a micrometer on day 1, day 4, day 8 and the day of scheduling. Body weight was determined on day 1 and at scheduling.

2.9.6 Enhancing vaccine responses by formulation with aluminum gel procedure

The first phase of the experiment involved vaccination of the mice in groups 1 and 2 only as follows.10 mice (n = 5 per group) were vaccinated on day 1, day 15, and day 28 of phase I with standard anti-IL-17 vaccine (group 1), or anti-IL-17 vaccine in alum-hydrogel (group 2). Vaccine dose was 25μg of vaccine in each group, the alum-hydrogel vaccine was composed of 25μg of vaccine and 25μg of alum-hydrogel.

Tail vein blood (up to 200μl) was ascertained at each vaccination, 2 weeks after the final vaccination and 4 weeks after the final vaccination.

The second phase of the experiment involved IL-17 dosing as follows. The mice were divided into groups as documented in table 2.6

Table 2.6. Group allocation for formulation of IL-17 vaccine experiment.

	Number of Mice	Imiquimod Application	Therapy
Group 1	5	Yes	Standard vaccine
Group 2	5	Yes	Vaccine in aluminum
Group 3	5	Yes	Antibody injection
Group 4	5	Yes	None (positive control)
Group 5	3	No	None (negative control)

The below procedures were performed as part of the experiment.

Cream treatment: Aldara cream was be applied to one ear of all animals daily and to shaved dorsal skin (area approximately 2 x 2 cm) for 11 days of phase II to groups 1-4 mice only.

Antibody injection: 100μg of anti-IL17 was injected in 100μl volume subcutaneously on day 1 and day 3 of phase II into group 3 mice only.

Groups 4 and 5 received no treatment in phase I. Group 4 received Aldara cream as above to induce inflammatory disease with no modifier, to act as positive controls for treatment groups, whilst group 5 did not have skin disease induced to act as the negative control. Groups 3 to 5 did not have blood ascertained.

The mice ear thickness and body weight were monitored throughout. Ear thickness was recorded using a micrometer on day 1 and day 5 of phase II and

the day of scheduling. Body weight was determined on day 1 of phase II and at scheduling.

2.9.7 Specificity of the immune response generated by the vaccine procedure

Residual tail vein blood samples known to be highly immunogenic for IL-17A were taken from week 10 mice of the long-term kinetics study. ELISA plates were coated for IL-17A and IL-17F. The same blood samples were tested for both IL-17A and IL-17F and the ELISA was run for both at the same time to minimise variables.

Results

A series of experiments were performed in mice as proof of concept for the primary pharmacodynamic action of the vaccine, that is, the production of antibodies and the ability to block a psoriasis-like disease model in vivo. In addition, in order to obtain informative data on the principle pharmacokinetic parameter, the duration of antibody response was also assessed. The results of these experiments are presented below.

3.1 Immunogenicity of the immune response generated by the vaccine

Mice were immunised with increasing doses of the murine IL-17 vaccine 0.5, 1.25, 2.5 and 5.0 micrograms respectively, via a regimen composed of three subcutaneous injections given two weeks apart. Serum was ascertained from the mice two weeks following the last injection and antibody titres determined by ELISA. As shown in figure 3.1, a dose of 2.5µg of VLP-coupled IL-17A vaccine substance elicited uniform anti-IL-17A Ig titres in excess of 1:1000 in all vaccinated mice. Moreover, figure 3.1 shows a vaccine dose as low as 0.5 micrograms still produces detectable titre with an average in excess of 1:1000 in this group.

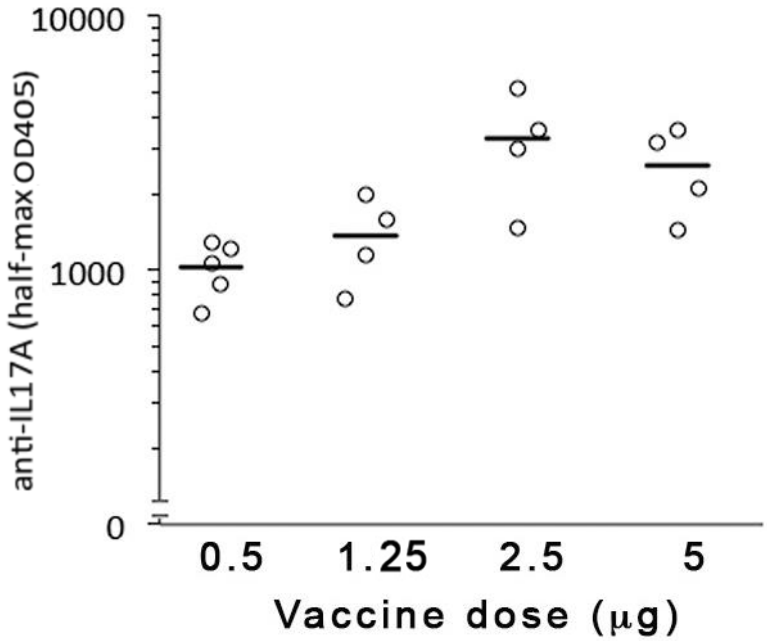

Figure 3.1. Immunogenicity of the immune response generated by vaccination with an anti-IL-17A vaccine in mice. Figure shows the anti-IL-17A Ig titres determined based on half-maximal OD405 values with altered vaccine dose. Horizontal black lines represent the average in each group. Pre-immunised mice yielded anti-IL-17A Ig titre levels between 0 and 50, which was comparable to sterile water.

3.2 Specificity of the immune response generated by the vaccine

Blood samples with high anti-IL-17A titre levels were used to see what effect this had on IL-17A's most closely related isoform, IL-17F. Figure 3.2 shows the antibody response generated was highly specific, as no significant antibodies reactive to IL-17F were detectable across all dilutions.

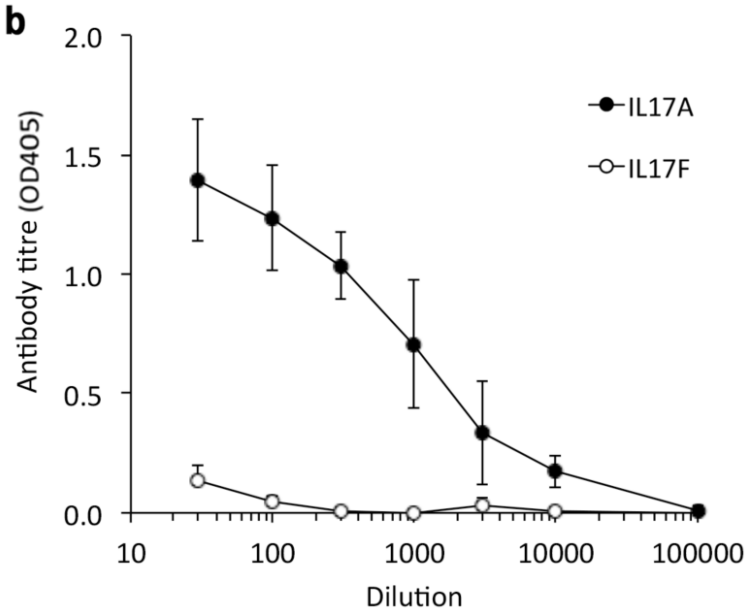

Figure 3.2. Specificity of the immune response generated by vaccination with an anti-IL-17A vaccine in mice. Demonstrates ELISA assays of n = 4 mice were performed with plating either recombinant murine IL-17A (black circles) or IL-17F (open circles). Data shown represent average and standard deviation.

3.3 Efficacy in an in vivo model of IL-17 mediated skin disease

As described in the methods section, imiquimod cream was used to induce psoriasis like reaction in vivo. Multiple models for simulating psoriasis have been proposed and utilised in mice. While it is widely recognised that no single mouse model equates the human disease, the most widely employed in vivo model for psoriasis, is the topical imiquimod challenge model. This model is based on applying the Toll-like-receptor 7/8 agonist imiquimod as cream to the shaved skin (138, 139). Application of imiquimod cream elicits a psoriasis-like skin disease, which has been extensively characterised and, importantly, involves IL-17 signalling. With regard to the IL-17 vaccine, the main point to consider was whether the vaccine would afford a similar degree of protection as monoclonal antibodies targeting IL-17A. Since these monoclonal antibodies

have already shown efficacy in treating human psoriasis demonstration that the vaccine has comparable effects in an IL17-mediated model of psoriasis would provide sufficient evidence to support further development. Therefore, we conducted a head-to-head comparison with an anti-IL-17A monoclonal antibody, which is known to be highly effective for the treatment of psoriasis. As a comparator to the vaccine the most extensively used available monoclonal antibodies known to neutralise IL-17A in vivo in mice were used (MAB421, R&D, ND50 ≈ 10µg/ml).

As shown in figure 3.3, the increase observed in ear thickness caused by application of imiquimod was reduced in mice vaccinated against IL-17A to the same extent as in those treated with IL-17A targeting monoclonal antibody.

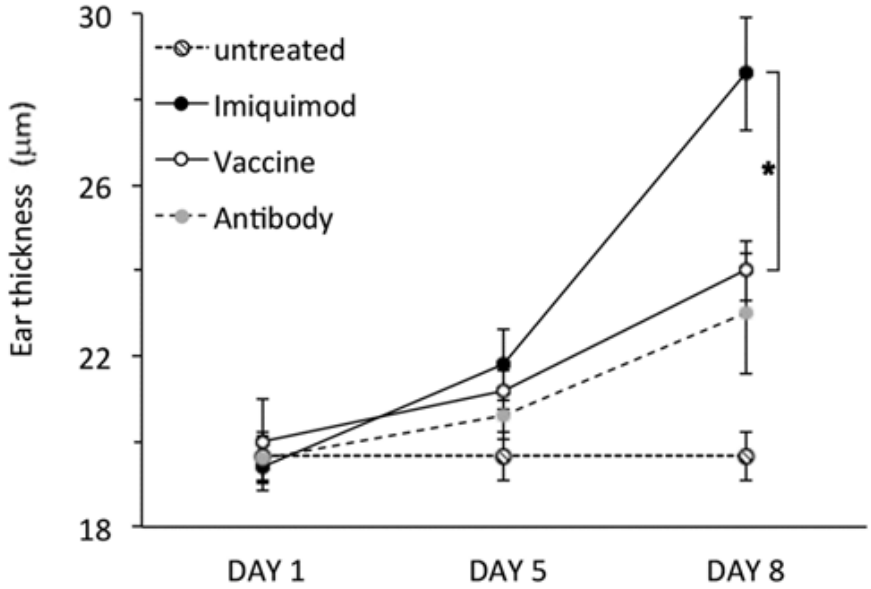

Figure 3.3. Reduction of increase in ear thickness caused by topical treatment with imiquimod in mice receiving anti-IL-17A or in mice pre-vaccinated against IL-17A (n = 5 per group). * $p < 0.05$ of imiquimod-only versus vaccinated mice in a two-sided unpaired T-test. Data shown represent averages and standard deviation.

The epidermal thickness of the dorsal skin treated with imiquimod also showed a statistically significant reduction (p = 0.0007) achieving the same result as the monoclonal antibody, as shown in figure 3.4.

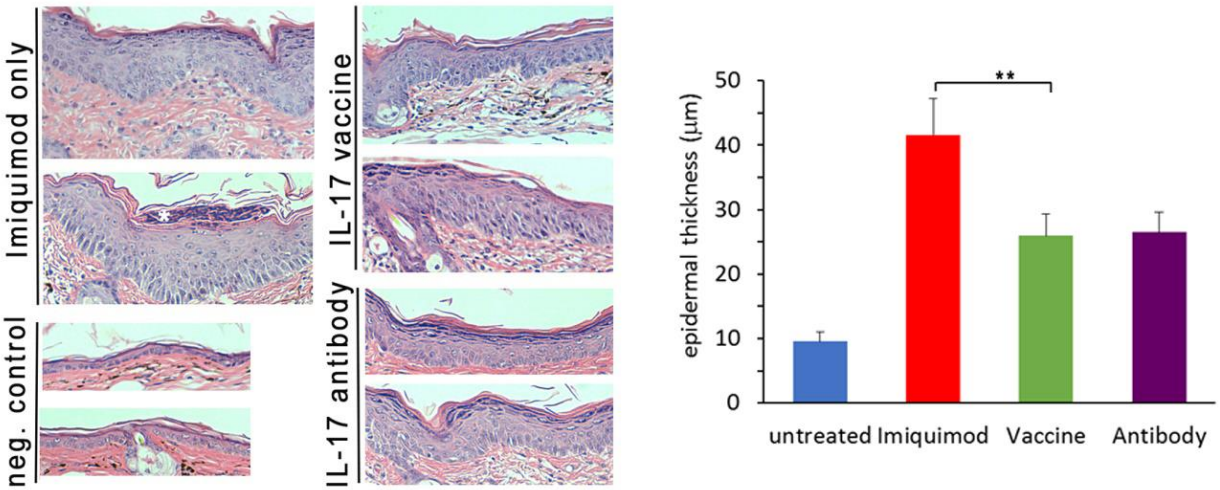

Figure 3.4. H&E stained dorsal skin taken from imiquimod-treated mice, and mice receiving concurrent anti-IL-17A treatment or vaccinated against IL-17A, as indicated. Left: representative samples, shown at 100x magnification. * denotes a sub corneal deposit of neutrophils (a so-called Munro-type micro-abscess), typical of psoriasis, which was occasionally seen in imiquimod-treated mice, but not in vaccine- or antibody treated mice. Right: quantification of n = 5 mice per treatment group. ** p< 0.01 in a two-sided T-test.

Analogous results were also demonstrated in epidermal thickness of the ears from the same mice with a highly significant reduction (p = 0.0004), as shown in figure 3.5. Again, these results were in line with the IL-17 targeting monoclonal antibody.

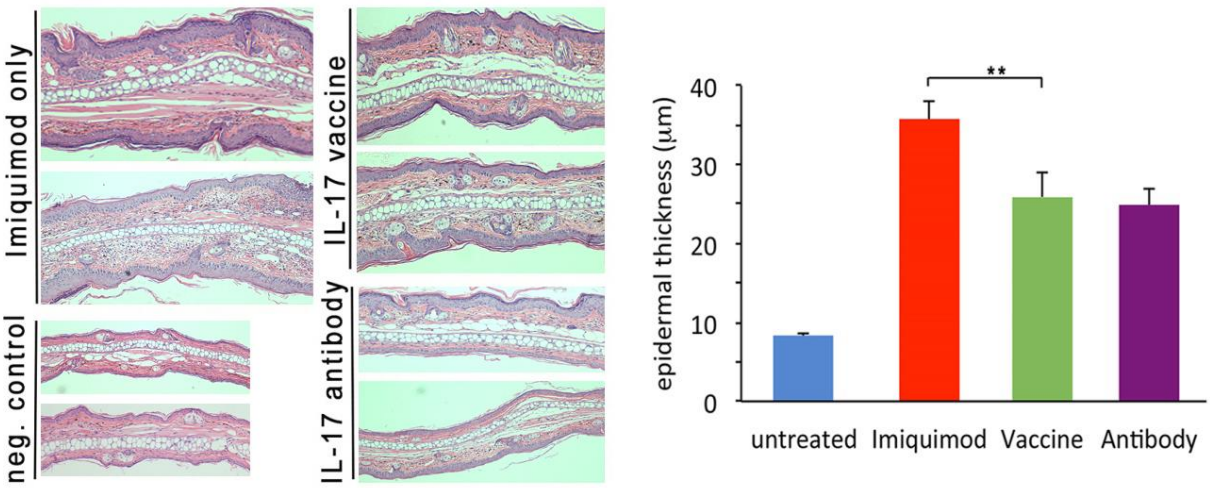

Figure 3.5. H&E stained ear taken from imiquimod-treated mice, and mice receiving concurrent anti-IL-17A treatment or vaccinated against IL-17A, as indicated. Left: representative samples, shown at 100x magnification. Right: quantification of n = 5 mice per treatment group. ** p< 0.01 in a two-sided T-test.

3.4 Long-term kinetics of the antibody response

An important safety consideration of auto-vaccines is reversibility of the induced anti-vaccine immune response, in addition to long-term kinetics with repeat challenge. Another important safety aspect to consider is the potential of endogenous boosting of the anti-IL-17A immune response in the context of active psoriasis-like disease. To gain insight into these vaccine-relevant factors, mice received a baseline vaccination regime consisting of three inoculations given two weeks apart. Thereafter, as shown in figure 3.6, one group of mice (dark shaded) received no further treatment, while another (light shaded) was subjected to a simulated psoriasis flare induced by seven days application of imiquimod to shaved dorsal skin (grey box). Application of imiquimod did not trigger endogenous boosting of anti-IL-17A titres.

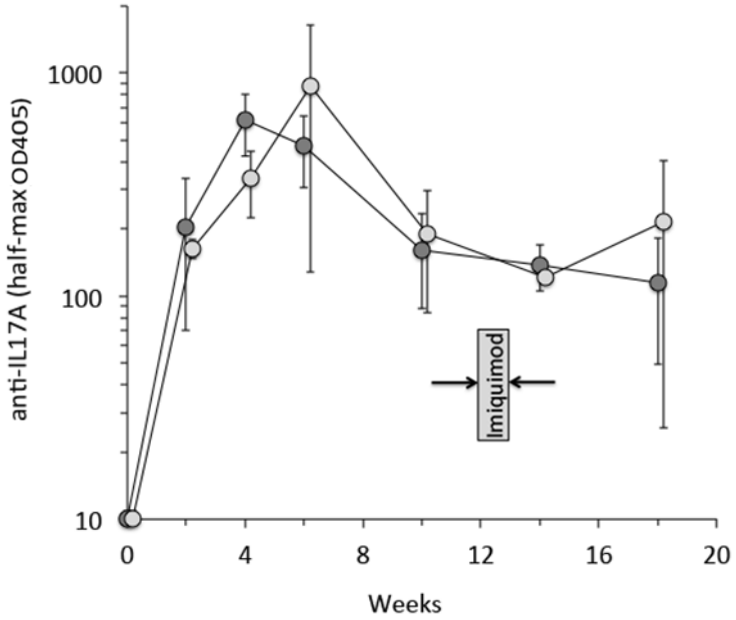

Figure 3.6. Response of anti-IL-17A titres in mice receiving endogenous IL-17 boosting. Light shaded group (n = 4) were subjected to imiquimod challenge at 6 weeks as shown by the grey box. Dark shaded group (n = 4) did not receive any further treatment after initial triple-booster.

Next, the long-term titre development after vaccination and the effect of re-boosting were characterised. As the group receiving the endogenous boost (figure 3.6) did not respond differently to the group without the endogenous boost vaccinated at the same time intervals, these two were subsequently analysed as a single group, as shown in figure 3.7. This group received one single triple-shot booster regimen at 24 weeks (black arrows). An additional group of mice, the second group (white symbols) received single vaccine booster injections every 12 weeks as above up till 36 weeks (white arrows). The resulting IL-17-specific IgG titres show that, as expected, the vaccination effects were reversible. Titre reversal was noted by 14 weeks in the absence of any boosting (black symbols). A single triple-injection boost completely restored antibody titres but did not lead to “super-boosting” (that is, >5 x higher IgG titres

than after initial vaccination). After the triple-injection boost, titres persisted for approximately 6 months at low levels, consistent with the formation of memory. Finally, a single booster-injection, applied every 3 months, was able to sustain antibody titres, and yielded reversal of antibody titres after approximately 5 months.

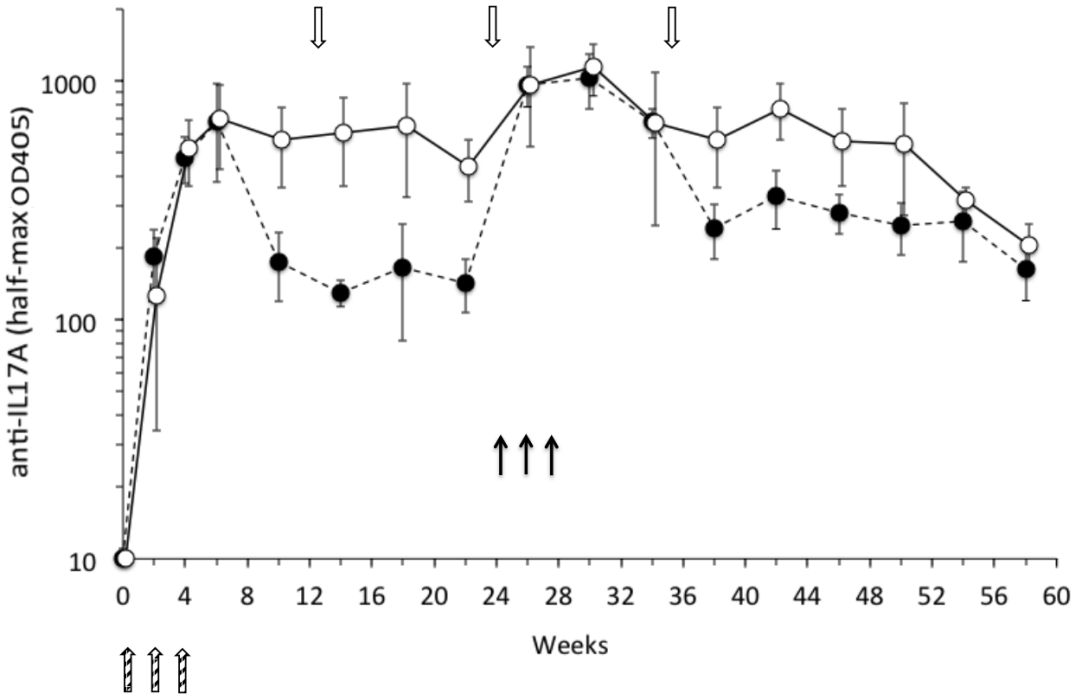

Figure 3.7. Long-term kinetics of anti-IL-17A titres in mice receiving repeat booster regimens and/or after induction of active psoriasis-like disease in vivo. Arrows indicate vaccine injections given to each group by colour code. Baseline immunisation schedule is shown on the bottom with striped arrows. White arrows at the top represent 3-monthly single booster shot (n = 4) whereas black arrows at the bottom represent triple-injection boost (n = 8) respectively.

These data show that antibody generation in response to the IL-17A vaccine is reversible and that antibody titres can be re-boosted either by single-injection or

by injection using three monthly regular boosters, or by full triple-injection vaccine schedule. Importantly, the data show that endogenous psoriasis-like disease with increased local IL-17A release in the skin did not trigger endogenous boosting of the anti-IL-17 response.

3.5 Enhancing vaccine responses by pre-DPT injection

In clinical practice it would be important to induce an anti-IL-17 antibody response above a minimal threshold in as many patients as possible. For most classical vaccines, however, there is a population of low vaccine responders. In recent years evidence has been gathered to suggest that these poor antibody responses are driven by the absence of good T cell epitopes within the vaccine for these particular individuals. In order to overcome this limitation, the IL-17A vaccine used in this thesis has been engineered to genetically incorporate a universal T cell epitope. This universal T cell epitope is derived from the tetanus vaccine, which is used in all individuals exposed to tetanus. Since tetanus vaccination, given as part of the DPT-vaccination, is universally administered in most populations, this epitope provides a vaccine-intrinsic immunostimulatory adjuvant. Thus, one of the key-target populations with decreased responsiveness to vaccines, the elderly, has pre-existing T cell memory to the epitope. Such a feature is expected to reduce the fraction of patients making poor antibody responses, essentially avoiding the non- or low-responders.

In order to test whether previous exposure to tetanus vaccination enhances the response to the IL-17A vaccine, both young and old mice were immunised with DPT (as used in humans) prior to immunisation against IL-17. As shown in figure 3.8, pre-vaccination with DPT induced a statistically significant increase in

mean antibody titres against IL-17 in the older group of mice. These data indicate that the design feature added to the cytomegalovirus-VLP may be able to enhance antibody response in recipients of the vaccine.

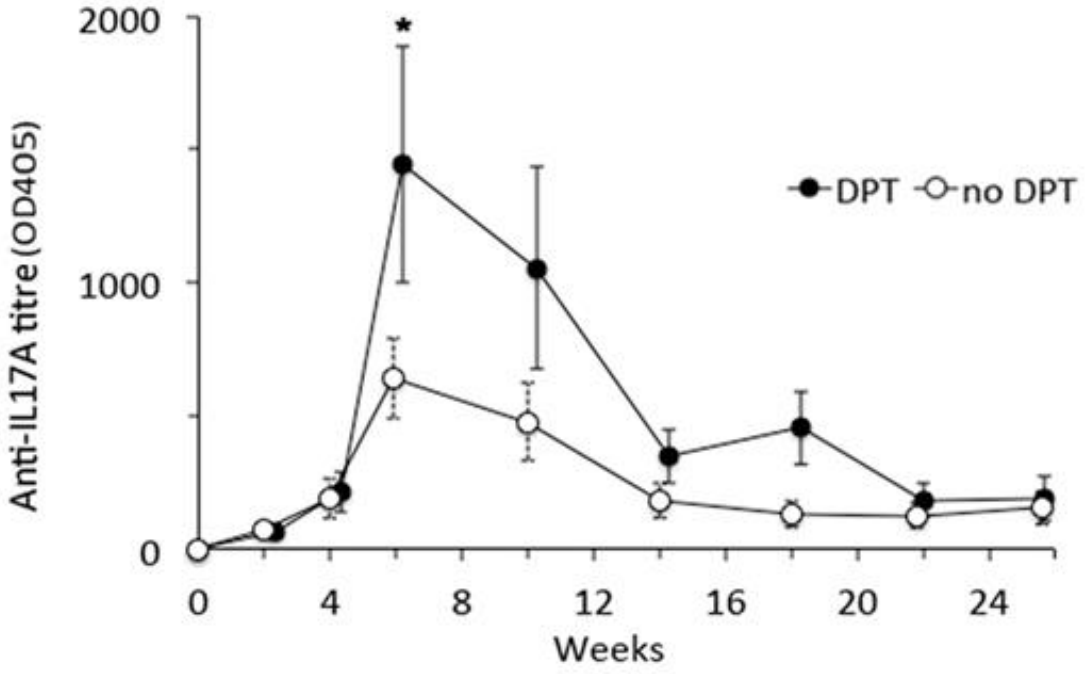

Figure 3.8. The effect of DPT-pre-immunisation on the anti-IL-17A antibody titres in 18 months old mice (n = 5 per group) receiving a sub-optimal dose (10µg) of anti-IL-17A vaccine. Data shown represent average and standard error of the mean. * $p < 0.05$ (T-test)

The group of young mice with DPT-pre-immunisation also showed an earlier increase and higher anti-IL-17A titre level compared to those without DPT immunisation. However this did not reach statistical significance, most likely due to the limited number of animals studied, as shown in figure 3.9.

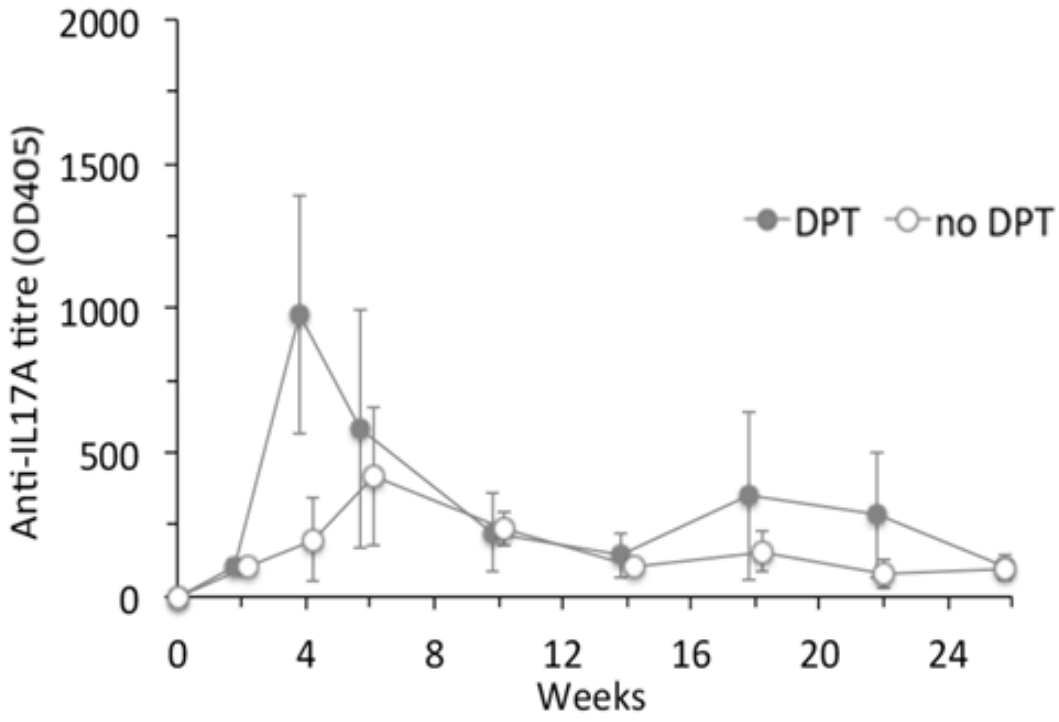

Figure 3.9. The effect of DPT-pre-immunisation on the anti-IL-17A antibody titres in 7 months old mice (n = 5 per group) receiving a sub-optimal dose (10µg) of anti-IL-17A vaccine. Data shown represent average and standard error of the mean.

3.6 Enhancing vaccine responses by blocking IL-22

IL-22 has been shown to have a potent role in the inflammatory process of psoriasis in humans, and as such anti-IL-22 antibodies are used in the treatment of IL-22 mediated inflammatory disorders, including psoriasis. Mice were injected with anti-IL-22 antibody to determine whether this enhances the vaccine or anti-IL-17 antibody. As shown in figure 3.10, a reduction in epidermal dorsal skin thickness was demonstrated in the dorsal skin H&E samples. However neither the vaccine ($p = 0.34$) nor the antibody ($p = 0.33$) caused a statistically significant decrease compared to the group with no treatment and imiquimod only.

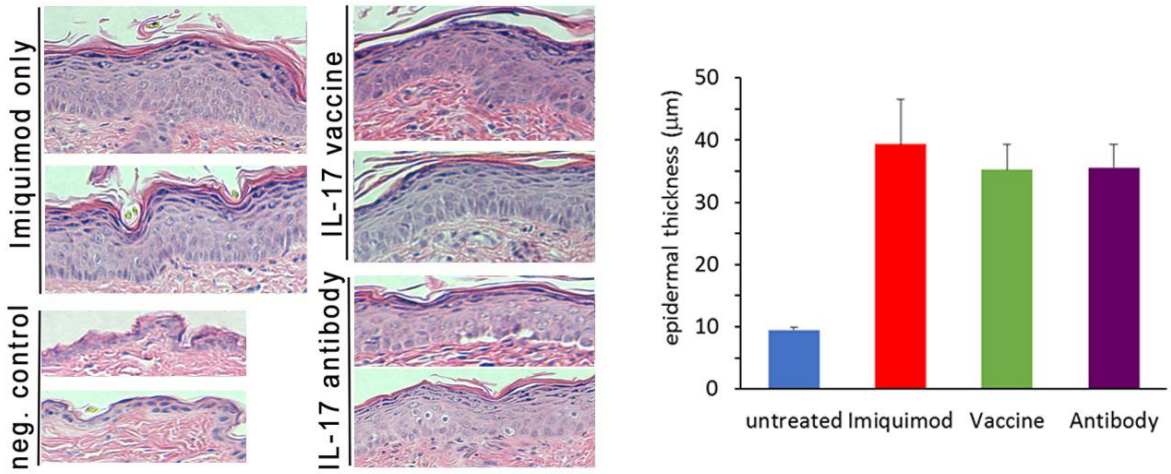

Figure 3.10. H&E stained dorsal skin taken from imiquimod-treated mice, and mice receiving concurrent anti-IL-17A treatment or vaccinated against IL-17A, with concurrent anti-IL-22 knockout as indicated. Left: representative samples, shown at 100x magnification. Right: quantification of n = 5 mice per treatment group.

In contrast to the lack of response in the dorsal skin there was a statistically significant decrease in the epidermal thickness of the ears of these mice ($p = 0.01$), as shown in figure 3.11. Of interest only the vaccine, not the monoclonal antibody ($p = 0.06$), achieved statistical significance in this reduction. However, only half the dose of the antibody was given for this test, which could be the reason for the difference in reduction. Nevertheless such a gap does not exist in the dorsal skin data.

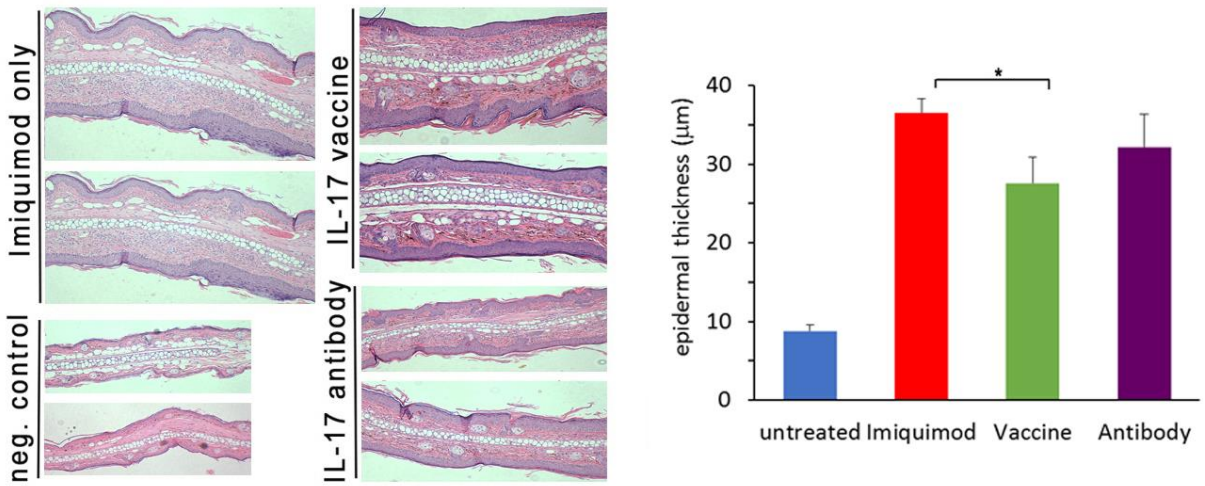

Figure 3.11. H&E stained ear taken from imiquimod-treated mice, and mice receiving concurrent anti-IL-17A treatment or vaccinated against IL-17A, with concurrent anti-IL-22 knockout as indicated. Left: representative samples, shown at 100x magnification. Right: quantification of n = 5 mice per treatment group. * $p < 0.05$ in a two-sided T-test.

Clinical photographs of the mice were taken at the time of scheduling to show phenotypes of the mice. These photographs showed a greater degree of erythema and irritation of the dorsal skin for mice with applied imiquimod compared with either the vaccine or antibody treatment, as shown in figure 3.12.

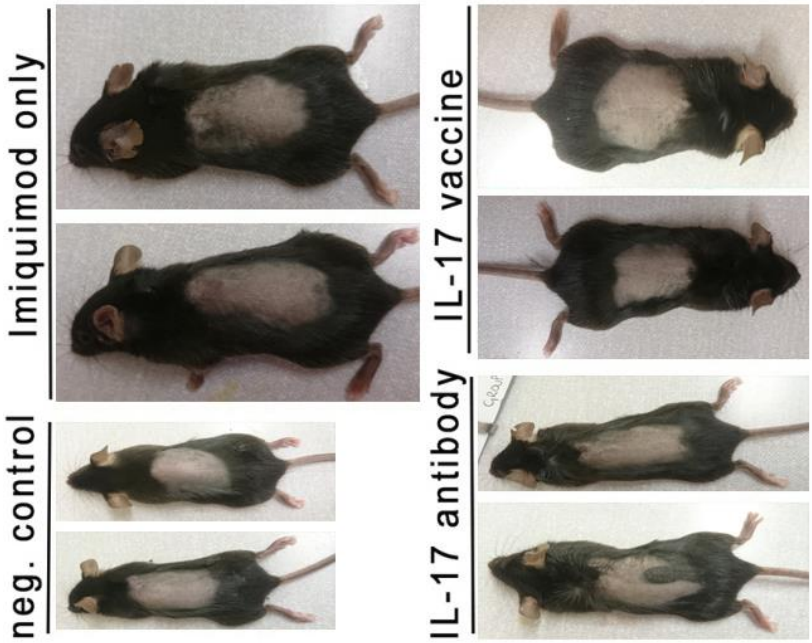

Figure 3.12. Clinical photographs of the mice from anti-IL-22 experiment taken at time of scheduling. Mice in the imiquimod only group can be seen to have more erythematous dorsal skin and ears than that of the other groups in the study.

3.7 Enhancing vaccine responses by formulation with aluminum gel

To determine whether formulation of the anti-IL-17 vaccine with aluminum gel as adjuvant enhances antibody response, this was compared with both the regular IL-17 vaccine and IL-17 targeting monoclonal antibody. As shown in figure 3.13, ELISA analysis 2 weeks after each time interval shows a significantly higher titre level in the group with the formulated vaccine compared to the regular vaccine.

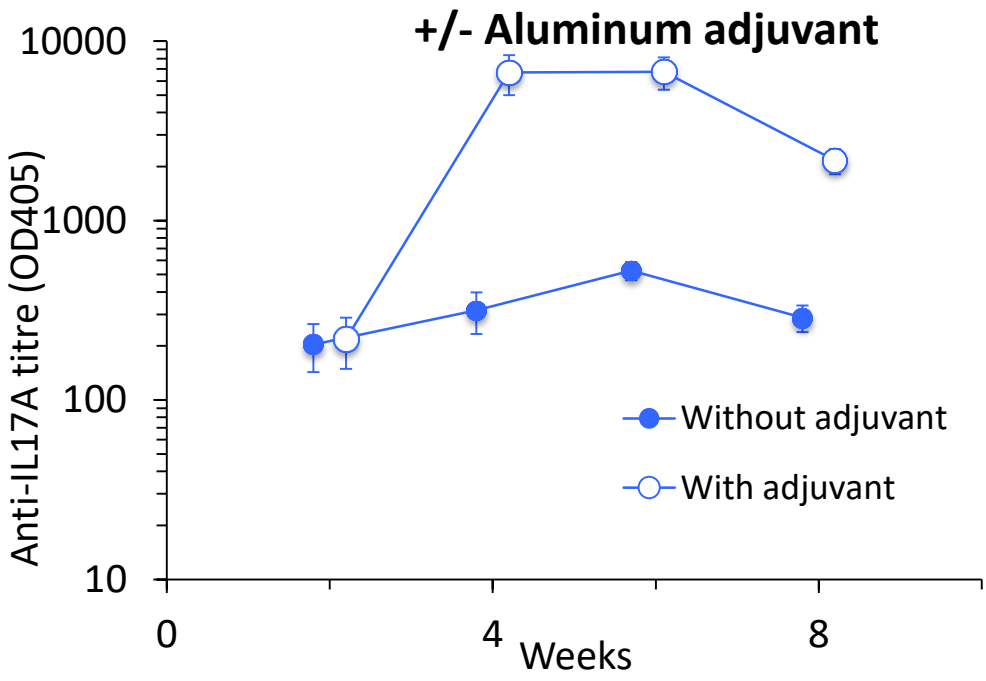

Figure 3.13. Change in anti-IL-17A titre levels when IL-17 vaccine formulated with aluminum gel. Anti-IL-17A titre levels in mice vaccinated without additional adjuvant (blue circles) compared to those with adjuvant (white circles).

Despite the significantly higher anti-IL-17A titre levels in all mice with the adjuvant, the same significance was not achieved comparing the greater reduction of epidermal thickness of the dorsal skin samples with that of the unaltered vaccine ($p = 0.23$). Both the formulated vaccine ($p = 0.10$) and the monoclonal antibody ($p = 0.16$) failed to reach statistical significance with comparison to the imiquimod only group, as shown in figure 3.14.

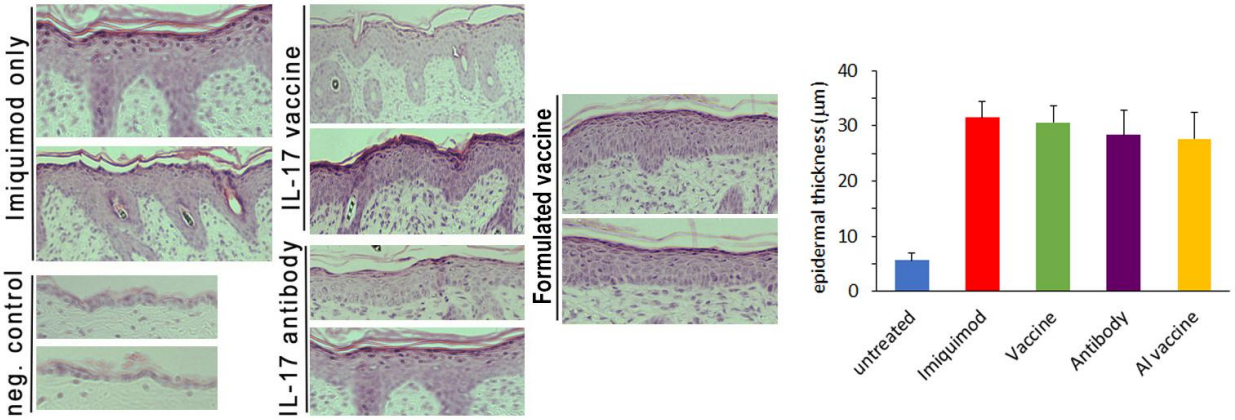

Figure 3.14. H&E stained dorsal skin taken from imiquimod-treated mice, and mice receiving concurrent anti-IL-17A treatment, formulated anti-IL-17A vaccine or vaccinated against IL-17A, as indicated. Left: representative samples, shown at 100x magnification. Right: quantification of n = 5 mice per treatment group. Al vaccine represents the formulated vaccine with aluminum gel.

Similar to the dorsal skin of these mice the results of the ear sample epidermal thickness H&E analysis showed a greater reduction with the formulated vaccine compared with the equivalent dorsal skin samples from the unaltered vaccine, however this failed to reach statistical significance ($p = 0.35$). Nevertheless, both the vaccine ($p = 0.04$) and even more so with the formulated vaccine ($p = 0.003$) achieved a significant reduction in comparison to the imiquimod only group, as shown in figure 3.15.

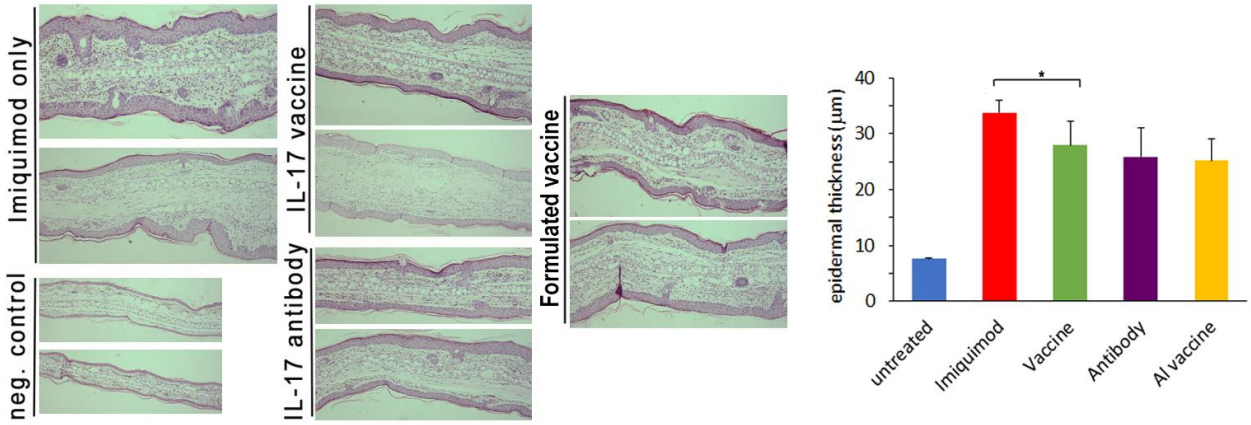

Figure 3.15. H&E stained ear taken from imiquimod-treated mice, and mice receiving concurrent anti-IL-17A treatment, formulated anti-IL-17A vaccine or vaccinated against IL-17A, as indicated. Left: representative samples, shown at 100x magnification. Right: quantification of n = 5 mice per treatment group. Al vaccine represents the formulated vaccine with aluminum gel. * $p < 0.05$ in a two-sided T-test.

Clinical photographs of the mice were taken at the time of scheduling to show phenotypes of the mice. These photographs showed a greater degree of erythema and irritation of the dorsal skin for mice with applied imiquimod compared with the vaccine, formulated vaccine or antibody treatment, as shown in figure 3.16. Despite shaving at the time of scheduling, many mice had overlying hair which made the phenotype more difficult to exhibit a change than could be evidenced by the histological analysis.

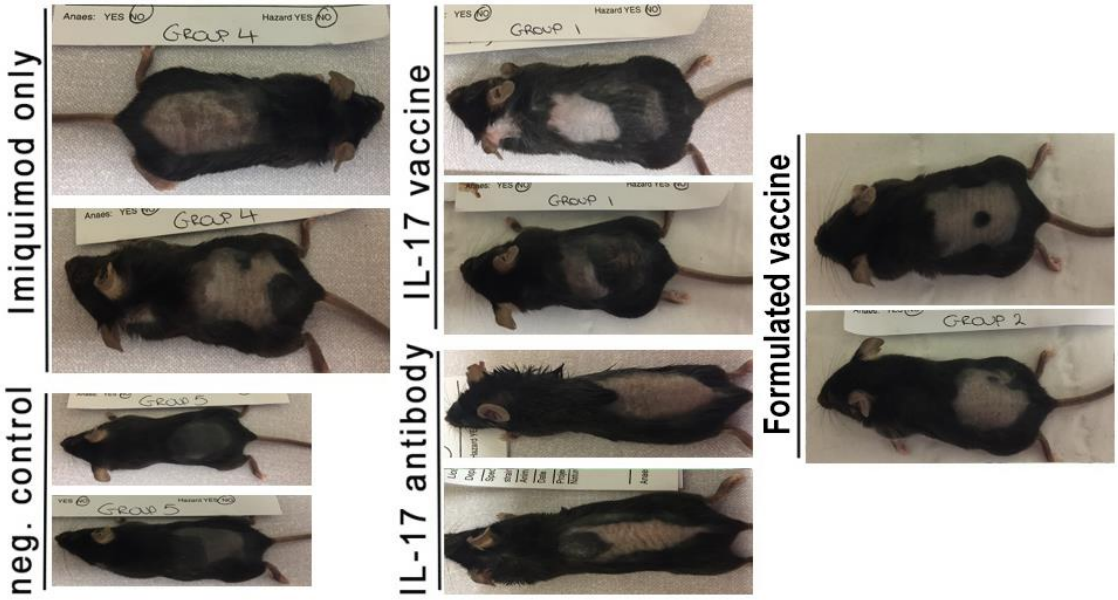

Figure 3.16. Clinical photographs of the mice from formulation of the IL-17 vaccine with aluminum gel experiment taken at time of scheduling. Mice in the imiquimod only group can be seen to have more erythematous dorsal skin and ears than that of the other groups in the study.

3.8 Reproducibility and long-term stability of anti-IL-17A vaccine

Overall, three independent experiments were carried out to determine the efficacy of the vaccine. Two of these were carried out with different batches of IL-17 vaccine. A third experiment was carried out with the same batch as used earlier but after prolonged storage at -20^0C. Therefore, the sum of these data afforded tentative information on the long-term stability of vaccine stored under these conditions, as well as the reproducibility of clinical efficacy of the vaccine between various batches. Pertinent protocol features of each experiment are summarised in the table 3.1.

Table 3.1. Summary of key protocol design features and outcomes of IL17VAX prototype non-clinical efficacy in vivo studies in mice. For experiments 1 and 2 the primary outcome was measured on day 8, for experiment 3 (elimination of IL-22 signalling) it was measured on day 11.

Experiment	**1**	**2**	**3**
Specific Aims	First in vivo efficacy test of IL-17 prototype	Replicate in vivo efficacy with independent vaccine batch	Assess vaccine stability (storage - 20^0C > 6 months) Assess efficacy after elimination of IL-22 effect in imiquimod model
Primary Endpoint	Reduction of ear swelling after challenge with topical imiquimod		
Outcome	**Percent reduction of ear thickness compared to Imiquimod only:**		
	Vaccine: 76% **Antibody: 73%**	**Vaccine: 50%** **Antibody: 60%**	**Vaccine: 23%** **Antibody: nil***
Statistical significance	P < 0.001 (vaccine vs control), two-sided t-test	P < 0.001 (vaccine vs control), two-sided t-test	P = 0.005 (vaccine vs control), two-sided t-test
Safety signals	None, normal weight gain, no discomfort, no clinical distress throughout		

* Anti-IL-17A was administered only on day 1 in experiment 3.

The data summarised in table 3.1 confirm that the IL17VAX vaccine is as effective as a validated monoclonal anti-IL-17 antibody, as validated using two independent vaccine batches, that the vaccine displays significant efficacy after storage at -20^{0}C for six months, and that efficacy can also be detected in a modified imiquimod assay after elimination of IL-22 signalling. Ear thickness measurements in each experiment are summarised graphically below in figure 3.17. It is necessary to mention in figure 3.17 that although the results of the day 11 experiment achieves significance in the third experiment, had the experiment been discontinued at day 8, as was the case for the other two experiments, the vaccine would have failed to achieve a statistically significant reduction in ear thickness. Using the day 11 ear thickness measurements may therefore show bias towards promoting the efficacy of the vaccine. A later end date was used because the methods had a prospective predefined endpoint as clinically evident psoriasis like phenotype. At day 8 in the third experiment group erythema and scaling were not visible and thus, the mice were scheduled when visible at day 11.

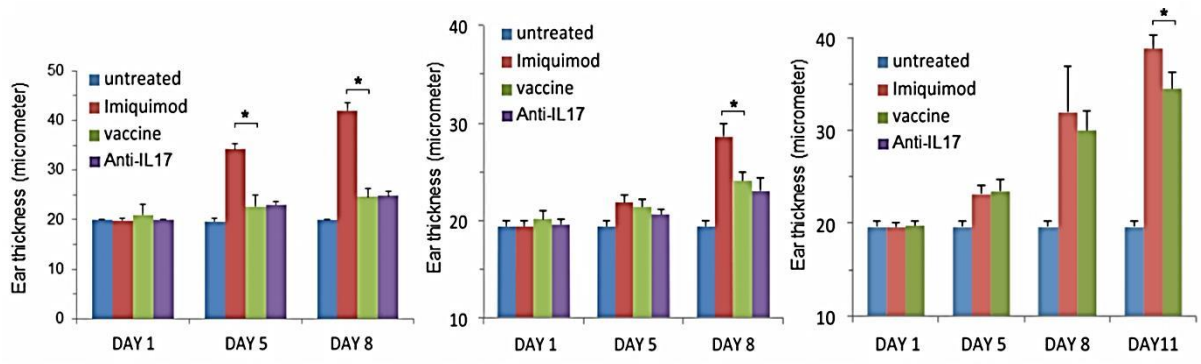

Figure 3.17. Ear thickness after challenge with imiquimod cream. For summary details of the protocols applied, see table 1. Shown are the data for experiment 1 (left), experiment 2 (middle), and experiment 3 (right), respectively. Key protocol aspects are as follows. Experiment 1: test of vaccine batch 1. Experiment 2: test of independent vaccine batch; Experiment 3: test of batch 2 after six months of storage at -20^{0}C and after disruption of IL-22 signalling. * $p < 0.05$ in a two-sided T-test.

Discussion

The below discussion evaluates the significance of the results of this thesis, as well as limitations, considers pertinent published literature, and what future studies would be warranted based on the current results. In particular, attention is paid to what future steps may be made in line with further pre-clinical and clinical trial development.

4.1 Safety of vaccination against interleukin 17

4.1.1 Specificity and safety

Evidence is accumulating to support the transition away from the traditional view that vaccines act only on their intended target molecule (161). Rather, heterologous effects may occur as memory T cells created due to the vaccines are able to adapt and target other molecules (162).This can have positive outcomes, as is the case of the non-specific outcomes for measles vaccination which reduces mortality from diseases other than measles by 45% when given at 4.5 months of age. A similar reduction in mortality has been demonstrated for other live vaccines such as Bacillus Calmette–Guérin (BCG) (163). DPT vaccine - a non-live vaccine, had been shown to have non-specific actions which increased mortality from infections other than diphtheria, pertussis and tetanus. Nevertheless, further studies and meta-analysis studying this have shown the effect to be deleterious (164). Increased mortality from such vaccines off target effects is of particular concern in countries with less healthcare provisions and higher infection associated mortality rates.

Therefore it was important to determine how specific a response was produced from the vaccine. The antibody response generated from the IL-17 vaccine was highly specific for IL-17A, as no significant antibodies reactive to the most closely related isoform, IL-17F, were detectable, as shown in figure 3.2. These results are in agreement with those made with another VLP-type IL-17A vaccine used to treat inflammatory arthropathy (165). This is important for safety consideration, as this result shows the vaccine is targeted in its action and to our knowledge does not affect unintended molecules, which could lead to potential toxicity in mice. Nonetheless, the non-specific effects of such a vaccine ultimately will have to be verified in human studies. The concerns of infection from childhood vaccination studies may be less important in psoriasis cohorts, as the main treatment group is adults, who are past the childhood vaccination schedules.

4.1.2 Treatment associated adverse events

Therapeutic vaccines based on the VLP technology employed in the IL-17A vaccine, but targeted at other self-proteins, have previously been tested in humans (147, 148). These clinical studies have not shown any concerning safety signals, in particular, no non-specific off-target immune activation. Furthermore, existent data have not uncovered evidence of severe vaccine-related adverse effects in rodents or non-human primates immunised with anti-IL-17A vaccine.

No unexpected adverse events were reported in the mice across all experiments. The only adverse event, which was to be expected, was erythema of the dorsal skin and ears where imiquimod was applied. This was, in fact, a

treatment-emergent adverse effect due to TLR7 activation and independent of the vaccine. No adverse events were observed with the IL-17 vaccine, or formulated IL-17 vaccine with aluminum gel.

4.1.3 Mortality and premature scheduling

Two mice were scheduled prematurely and a post-mortem was performed on both by a veterinary surgeon. One mouse had a swollen abdomen which revealed a large liver tumour and the other had a peptic ulcer. Of these mice only one mouse was vaccinated with the IL-17 vaccine, a first dose given one day before scheduling. Given the short time between vaccine administration and scheduling it seems unlikely the vaccine is the cause, as the formation of a hepatic tumour in such a timeframe would be unheard of. Therefore, the cause of these outcomes remains uncertain but is extremely unlikely to be due to vaccination.

4.1.4 Prolonged exposure and safety record

Immunisation of human patients with auto-vaccines using the same vaccine technology and immunisation of rodents and non-human primates with the IL-17A vaccine have consistently shown that the production of anti-IL-17 is reversible and continued antibody production is dependent on booster vaccination (166). The precise kinetics of reversal has not been studied in humans to date but no VLP-based vaccine has been shown to elicit antibody titres persisting beyond six months in the absence of follow-up booster (166).

The long-term kinetics experiment demonstrates the safety of the vaccine over a 58 week period. Currently available data of the prototype IL-17 vaccine

studied in mice also shows reversibility, as shown in figure 3.7. These data indicate that vaccination against IL-17A is unlikely to trigger irreversible long-term neutralising antibodies in vaccine recipients. Data shows that even with repeated exposure to the vaccine there are no increased risk of treatment associated adverse events or mortality in the mice. Moreover, one group of mice given an endogenous boost with imiquimod did not respond differently to those without an endogenous boost, suggesting flares in the disease would not significantly raise the titre levels, as shown in figure 3.8.

4.1.5 Safety of anti-IL-17 biologic agents

Data available from published clinical trials of IL-17 targeting monoclonal antibodies reveals more than 20,000 patient years of long-term inactivation of IL-17. These have shown for the three monoclonal antibodies, seckunimab, ixekizumab and brodalumab, that no concerning safety worries are apparent (167). Across the different studies dose-dependent candida infections, which were severe enough to be treatment limiting, represent the main adverse effect experienced. Moreover, there have been no reported safety concern signals in patients with other conditions being treated with anti-IL-17 therapies.

4.2 Treatment rationale and efficacy of the vaccine

4.2.1 Efficacy of anti-IL-17 biologic agents

The treatment of moderate to severe psoriasis has been revolutionised by monoclonal antibodies and recombinant proteins. The chief limitations of currently licensed anti-TNF and anti-IL-12/23p40 biologics are systemic immunosuppression leading to increased risk for severe infection, in particular

mycobacterial infection such as tuberculosis, as well as the lack or loss of efficacy in a subset of patients. IL-17 targeting monoclonal antibodies bear the prospect of overcoming these limitations. However, the complex and demanding manufacturing process of biologics translates into exceedingly high treatment costs, which is increasingly limiting access of effective treatment for patients. Injection of a monoclonal antibody represents a form of passive vaccination. Replacing this by active vaccination, in order to elicit the generation of antibodies by the vaccinated person, bears the potential of dramatically reducing treatment cost and therefore opening access to effective disease control to many more patients.

4.2.2 Dose response

Vaccines can produce measurable effects even at very low doses. In this thesis doses as low as 0.5μg of IL-17 vaccine were shown to produce measureable anti-IL-17 titre levels. In addition, the vaccine shows a dose response effect with increasing efficacy with dose increase from 0.5μg, as shown in figure 3.1 (in the figure labelled with equivalent microliter dosing amounts). The decrease in efficacy for the 5.0μg group may be attributable to a sampling error as only a small number of mice (n = 4) were used per group and one mouse in the 2.5μg group had a significantly higher titre than the other three mice in that group.

4.2.3 Storage and handling

Preventing loss of vaccine potency is becoming an increasingly important consideration as more expensive vaccines are introduced. Several factors pertinent to the storage and handling of vaccines are important to maintain

potency and maximise shelf life, including correct storage temperatures and minimising freeze-thaw reactions (168). Freeze-thaw reactions have not been thoroughly studied however Petrakis showed that as few as one freeze-thaw reaction significantly reduced serum IgG and IgM levels to below 25% of pre-freezing levels (169). Instability of antibody levels following freeze-thaw reactions are thought to occur due to protein denaturation. In aqueous environments, proteins normally fold spontaneously in such a configuration that their hydrophobic side chains are encased by polar chains with a surrounding bulk hydration layer. Freezing disrupts this bulk hydration layer rendering it inactive. Subsequent unfolding allows hydrophobic reactions to occur, which denatures the protein (170).

Despite being stored at suboptimal temperature of -20^{0}C, as opposed to -80^{0}C, the vaccine showed excellent long-term stability, generating antibody response after greater than six months of storage at this temperature. Freeze-thaw reactions were minimised by aliquoting the vaccine into smaller volumes suitable for injections prior to long-term storage. Across all the experiments, IL-17 vaccine injected into mice never underwent greater than two freeze-thaw reactions. Nevertheless, the experiment evaluating the use of the vaccine with IL-22 used vaccine which had been stored for over 6 months at -20^{0}C. Decreased efficacy was noted in this experiment which could be a result of loss of stability possibly due to retained overall titre but decreased titre of neutralising antibodies by antigen degeneration. This could have been studied to determine if this was the cause using neutralising assay of the sera.

4.2.4 Batch-to-batch consistency

To confirm the reliability of the manufacturing process demonstration of batch-to-batch consistency has become a mandatory step in vaccine development. Many factors can interfere with the antigen content of the vaccine resulting in different immune response of the final product, including different manufacturing processes and interactions with adjuvants added to the final vaccine product. Therefore, demonstration of batch consistency is necessary should the manufacturer wish to gain a licence for their vaccine (171, 172).

Two independent batches of the IL-17 vaccine were supplied by the Jenner Institute. Both batches demonstrated significant ability to reduce epidermal thickness of the ears and dorsal skin, as shown in table 3.1. In addition, mice injected with both batches of the vaccine had high anti-IL-17 titre levels post vaccination. These findings provide strong evidence of the efficacy of the vaccine, in addition to the manufacturing process in producing consistent vaccine batches.

4.3 Modification of vaccine responses by pre-DPT injection

Conventional vaccines often suffer from varied efficacy especially in elderly patients, a phenomenon sometimes called immunosenescence. Immunosenescence has an effect on both the innate and adaptive immune system. Defects and deficiencies of the antigen presenting cells of the innate immune system results in diminished activation of the cells of the adaptive immune response (173). Approaches to overcome these limitations in the elderly have been trialled for the influenza vaccine, namely higher doses and intradermal delivery, and have shown improved vaccination response (174,

175). DTP vaccine is included in the UK childhood immunisation schedule beginning from 2 months of age, and has also been introduced for pregnant mothers (176). Studies have also shown it to be safe to administer to patients over the age of sixty-five (177).

The IL-17 vaccine undergoing pre-clinical evaluation was modified in the manufacturing process such that prior vaccination against tetanus (performed in close to 100% of the population) acts as a vaccine booster. The results of the thesis show that in the older group of mice, aged 7-8 months at the beginning of the experiment, this was the case, as shown in figure 3.8. There was a significant rise in anti-IL-17 titre from as early as 2 weeks after triple induction scheme, which continued to be the case until 22 weeks, without the need for further DPT injections. Notably this was the case despite sub-optimal dosing with a vaccine dose of only 10μl per vaccination, equivalent to only 0.5μg. This small vaccine dose, in conjunction with the limited number of mice involved in the experiment (n = 5) may explain why the same experiment in the younger group of mice did show a rise in titre, but not significantly higher than those without prior DPT vaccination, as shown in figure 3.9. Provided this is one of the intended benefits added into the manufacturing stage, repeating these experiments with a higher dose of IL-17, suggested 2.5μg, and/or larger numbers of mice would be useful to see if this yields consistently higher titre.

4.4 Modification of vaccine responses by blocking IL-22

IL-22 is a cytokine produced from Th1, Th17 and Th22 subsets. IL-22 inhibits terminal differentiation of keratinocytes and can induce epidermal alterations found in psoriasis. Moreover, elevated levels of IL-22 mRNA have been

identified in psoriatic skin lesions compared with healthy skin (178). The use of IL-22 neutralising antibodies has been shown to prevent the development of psoriatic lesions, reduce acanthosis and decrease expression of Th17 cells (179). Van Belle *et al* demonstrated that psoriasiform reactions caused by daily imiquimod application were almost absent in mice deficient in IL-22 or treated with anti-IL-22 antibodies. Studies investigating the mechanism of action of the imiquimod assay show it appears to harbour a synergistic effect of IL-17 and IL-22 (180). Therefore the IL-17 blocking effect could be stronger after eliminating IL-22. These mice also showed decreased expression of chemotactic factors such as CXCL3 and biomarkers such as S100A8 which reflect hyperproliferative responses of keratinocytes (180). Fezakinumab is a monoclonal antibody targeting IL-22 which has completed phase II trials for rheumatoid arthritis and a phase I trial investigating subcutaneous and intravenous routes of administration. Despite this the results of these trials conducted in 2010 were never published, suggesting this may not provide optimal therapy for psoriasis patients (181).

The results of inhibition of IL-22 activity obtained in this thesis were inconclusive. Reduction in dorsal skin samples treated with IL-17 vaccine after pre-elimination of IL-22 signalling by injection of anti-IL-22, failed to reach statistical significance, as shown in figure 3.10 despite a significant decrease in analogous ear thickness samples, as shown in figure 3.11. Of note, the anti-IL-17 antibody combined with the anti-IL-22 antibody also failed to achieve a statistically significant decrease in dorsal skin thickness. Nevertheless, this group of mice only received half the dose of the anti-IL-22 antibody therefore the results must be treated cautiously. Given the statistically significant decrease in epidermal ear thickness however, this is an approach which merits

further investigation in NHP studies. No adverse events were associated with additional blockade of IL-22. Moreover, the lack of toxicity seen in the mice with this additional antibody is encouraging from a safety point of view of targeting greater than one pro-inflammatory cytokine.

4.5 Modification of vaccine responses by adjuvants

Adjuvants are components of a vaccine capable of enhancing or shaping antigen-specific immune responses. Development of adjuvant therapy in vaccines balances improving immune responses without compromising safety by selectively adding well-characterised molecules or formulations (182). Advantages of using adjuvants include dose sparing, less immunisations, broadening of vaccine response and rapid response to pathogens. There are many adjuvants used, which can be divided into organic and non-organic, but the most commonly used are aluminium salts, principally aluminium hydroxide and aluminium phosphate (183). Aluminium potentiates the immune response as a powerful pro-oxidant. The biologically reactive form of aluminium is primarily the Al^{3+} ion which is avidly bound by oxygen and fluoride-based functional groups. Aluminium potentiates the activity of extracellular adenosine triphosphate (ATP), which is implicated in the release of pro-inflammatory cytokines, by two possible mechanisms. One mechanism involves prolonging the lifetime of the nucleotide-receptor complex and the other mechanism via reducing ATP hydrolysis by ectonucleotidases (184).

Enhancement of the vaccine with aluminum gel raised the anti-IL17 titre significantly after each vaccination point in all mice (n = 5) tested, as shown in figure 3.13. Despite this considerable rise in titre, the standard vaccination and

formulated vaccine failed to achieve a statistically significant reduction in epidermal dorsal skin relative to the positive control group, as shown in figure 3.14. However, a significant reduction was demonstrated in the ear epidermal thickness, as shown in figure 3.15. The formulated vaccine did show greater reduction in epidermal thickness, of both ear and dorsal skin samples, than the standard vaccine, and also compared to the anti-IL-17 antibody. It is unclear why none of the three treatment options failed to significantly reduce the dorsal skin epidermal thickness in this experiment. It is possible this could be due to a decrease in neutralising antibodies as a result of antigen degradation after long-term storage. The majority of B cell epitopes are conformational epitopes created by multiple protein loops acting as a 3D surface. These are lost if the protein is denatured, as could have occurred here. However peptide epitopes could have been recognised by T cells, which would give rise to high ELISA titres but no neutralising titre by binding to epitopes buried deep inside the native protein. It is unlikely due to long-term storage of the vaccine at -20^{0}C as this was stored for less time than in the IL-22 blocking experiment which achieved significance for ear epidermal thickness reduction after 6 months of long-term storage at this temperature. Moreover, this would not account for the fact the antibody similarly did not cause a significant reduction, as this was only bought in 1 week before starting the experiment. The aluminum gel was only mixed with the vaccine before injection into the mice, thus it was never stored in less than room temperature as instructed. A possible explanation might be too much imiquimod application onto the dorsal skin and ears, however the same amount (one sachet) was used in previous experiments and did not cause this problem. With this in mind formulation of the vaccine with aluminum gel nevertheless did reduce epidermal thickness. Although the reduction was not

statistically significant, the lack of previously significant data with this experiment suggests it may be worth repeating in another mouse group to identify whether it would be of benefit before NHP studies.

4.6 Further studies

The current study has been conducted in accordance with the 3Rs principle - replacement, reduction and refinement - designed to minimise the number of animals used in testing while still allowing enough to yield statistically significant data. The results of this thesis support continuation of studies into a NHP study.

Careful consideration of NHP species protein sequence identity and similarity of IL-17A to the human equivalent would be most helpful for evaluating both efficacy, and more importantly, toxicity of the vaccine. Cynomolgus monkey are an example of a species with similarities to humans in these sequences and have been used successfully for NHP studies of medications in the past (185). NHP studies could be used to simultaneously assess toxicity and the pharmacodnymanic action of the vaccine as neutralising anti-human IL-17 antibodies also neutralise monkey IL-17 (186).

Should the vaccine continue to provide promising safety and efficacy results in NHP studies progression would follow into human clinical trials. Vaccine development follows the same four phases of clinical trials as other therapeutic agents (187). Successful outcomes at clinical trial stages could potentially lead to clinical use of the vaccine for psoriasis patients in the future.

4.7 Limitations

4.7.1 Longevity of mice compared to humans

The lifespan of mice represents a limitation in monitoring the long-term kinetics and repeated booster effects of the IL-17 vaccine. The life expectancy of C57Bl/6j mice is approximately two years which is significantly shorter than human lifespan (188). Therefore only three repeat boosters were given to mice in this thesis, where in human clinical practice the intended use would most likely extend beyond this, given psoriasis is a chronic incurable condition, potentially continuing treatment over many years. It cannot therefore be seen if further boosters would significantly raise titre or continue to have the same effect. Moreover, the long-term titre levels without boostering and the development of late onset adverse events were not able to be studied. Nevertheless, had this study been a human clinical trial, these studies do not often extend beyond a two year period. A recent study developed a multiple regression model to identify the mean overall length from 14,319 completed Phase I, II or III drug intervention trials at 21.4 months. This was due to factors including cost and patient compliance (189). Such a short trial length would make studying these long-term effects of this vaccine difficult to analyse until post surveillance monitoring stages. Taking this into account, the 58 weeks of safety and efficacy data from the long-term kinetics study seems a good proxy marker before progression onto NHP studies.

4.7.2 Inbred strains of mice

Inbred strains examine only the phenotype of a single genotype that may not be representative of the species. This can lead to misleading results and can be

overcome by two potential methods: using multiple different species of inbred stock or by using outbred stocks. Experiments need to factor in the significance of phenotypic differences between inbred strains. Strains may differ in characteristics that are not related to the phenotype under investigation, but may indirectly influence experimental results. An example would be C3H mice become blind in early adulthood due to a mutation in their strain, which would impact upon behavioural analysis studies requiring sight (190). The C57Bl/6j strain of mice are commonly used to investigate psoriasis and other diseases, however results could differ if other strains were used. For example Balb/c inbred strain have been used extensively in imiquimod induced psoriasis models and the effects of the vaccine could have been studied in these mice and potentially different results yielded (191, 192). Inbred studies are generally preferred because they require fewer mice on average to produce statistically significant results. Outbred stocks introduce genetic heterogeneity into the subjects being researched, offering a greater range of subjects more representative of a diverse human population. However, outbred stocks have several disadvantages compared to inbred strains including the genotype of individual mice is unknown, the extent of genetic variation in the stock is usually unknown and the mice are genetically labile (193).

To introduce even greater diversity different animal species could be used. Studies testing medications for psoriasis have been completed in rabbits in the past to evaluate potential safety concerns (194). Rabbits offer advantages for studies in that they are licensed under small animals, providing a more economical alternative to larger animals such as dogs or pigs. In addition they are widely bred and docile animals, making them easy to handle (195). Testing

the response of the vaccine in rabbits could offer further evidence to support the safety of the vaccine before considering NHP and human studies thereafter.

4.7.3 Use of only one disease model to induce psoriasis like reaction

This study used only one disease model to simulate psoriasis, the imiquimod application model. This represents a potential limitation as psoriasis does not naturally occur in mice and thus, using greater than one method could reduce any important phenotype differences that are absent from the imiquimod model. In addition, different brands of imiquimod have been shown to have different effects. A study by Luo *et al* (145), showed Aldara cream, the same brand of imiquimod used in this thesis, showed more characteristic histology of psoriasis as opposed to other brands. However, other brands or dosing regimens of imiquimod application could have been used to demonstrate effectiveness in various psoriasiform lesions.

A variety of transgenic and gene knockout mouse models have been created which simulate psoriasis phenotype features. The majority of these methods increase expression or knockout specific genes directed to the basal layer using promoters for keratin genes *KRT5* or *KRT14*, or involucrin promoter sequences. Moreover manipulation of the STAT3 pathway, key to wound healing and activated in psoriasis, has been shown to induce acanthosis, parakeratosis and loss of the granular layer of mice (196). Targeting growth factors involved in psoriasis pathology, such as VEGF, has also been shown to result in characteristic inflammatory changes of psoriasis lesions (197). Xenotransplantation of psoriatic skin lesions into immunocompromised mice offers the closest similarity to human psoriasis with regard to genetic and

histological changes (198). Nevertheless, this is an expensive method and takes longer to achieve desired results than the imiquimod method.

Using at least one of the above methods for an additional experiment could provide further support for the efficacy of the vaccine to reduce psoriasis like reactions before progressing to NHP studies. Despite this, the role of IL-17 in disease pathogenesis of psoriasis is well recognised and the imiquimod model importantly induces the IL-17/IL-23 axis. In addition a similar IL-17 targeting vaccine has been shown to have effect on other IL-17 mediated disorders, namely RA and MS (165).

4.7.4 No neutralising antibodies tested

A potential limitation may be having not identified whether anti-drug antibodies were present in the serum of the mice treated with the IL-17 antibody. High levels of these have been shown in humans treated with biologics, and some studies have associated high levels of these antibodies with decreased efficacy of the treatment (199). High levels of the serum neutralising antibodies might explain why some mice responded better to the antibody treatment than others. Similar neutralising antibodies against the IL-17 vaccine could also be tested for and might provide a reason for different responses from mice given identical therapies. To overcome such limitations, in clinical practice patients are treated with supplemental non-biologic therapies such as methotrexate. Adding supplemental therapies at this stage would most likely complicate rather than aid the project.

4.8 Summary

In summary, the IL-17A vaccine is a vaccine designed to elicit the production of antibodies blocking the function of interleukin 17A, which is identical to the target blocked by monoclonal antibodies already in clinical use in the treatment for psoriasis. Pre-clinical data in rodents and non-human primates suggest the IL-17A vaccine is capable of eliciting high-titre anti-IL17A as well as blocking the development of IL-17A-mediated inflammatory disease. By increasing the number of mice used per experiment a greater statistical significance reduction in epidermal thickness could have been shown for the vaccine group, particularly for the later experiments of IL-22 blockade, DPT pre-immunisation and formulation of the vaccine. This was not done due to the observed effect size with the second experiment - the primary efficacy experiment - which yielded highly statistically significant data. Thus in order to comply with the principle of the 3Rs, similar numbers of mice were used as were for the primary efficacy experiment. Clinical trials of anti-IL-17A monoclonal antibodies have demonstrated that neutralising IL-17A is safe and sufficient to treat moderate-to-severe psoriasis. The benefit of replacing monoclonal antibodies by active vaccination is that this approach would vastly decrease treatment cost and could ultimately provide access to treatment to a much greater number of patients. An added benefit is that successful proof-of-concept would also be applicable to development for other monoclonal antibodies targeting cytokines.

Conclusion

This thesis details the preclinical evaluation of an IL-17A vaccine for psoriasis. The results show the vaccine to be as effective a treatment option as IL-17 targeting monoclonal antibodies. H&E histology data shows a statistically significant reduction in mouse dorsal skin and ear epidermal thickness compared to groups with imiquimod application only. Long-term kinetic studies showed that antibody generation in response to the IL-17A vaccine is reversible and can be re-boosted by single injection of booster or full vaccination schedules. Moreover, endogenous psoriasis-like disease with increased release of IL-17A in the skin does not trigger an endogenous boosting of the anti-IL-17 response. Another safety issue was addressed with the vaccine proving to be highly specific as no significant antibodies reacted to the most closely related isoform IL-17F. Tentative data shows that the IL-17 vaccine response, particularly in older mice, may be enhanced by pre-vaccination with the common DPT vaccination. Furthermore, the vaccine has been shown to effectively minimise imiquimod applied ear thickness after storage at -20^0C for six months, and in two independent batches. These results offer potential for replacing passive immunisation by an active vaccination against IL-17A. In addition to significantly reducing treatment cost, this approach could potentially be advantageous in other important areas such as, ease of dosing, minimising treatment related staffing requirements, and avoidance of anti-drug antibodies. Moreover, if effective, an IL-17A vaccine has the potential to provide improved treatment access for other common conditions amenable to anti-IL-17A treatment, including rheumatoid arthritis and multiple sclerosis. To progress

towards use in clinical practice, the next step for the development of the IL-17A vaccine would be a non-human primate study, before beginning clinical trials.

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