Cutaneous defense against *Candida albicans*: Modulation of chemokine-driven anti-microbial immune responses

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Abbreviations

Abbreviation	Description
A	
AMP	Antimicrobial peptide
APC	Allophycocyanin
C	
cAMP	Cyclic adenosime monophosphate
CCR	C-C motif receptor
CD	Cluster of differentiation
CLA	Cutaneous lymphocyte-associated antigen
CMC	Chronic mucocutaneous candidiasis
CR3	Complement receptor 3
D	
DAC	Deutschen Arzneimittel-Codex
DAPI	4',6-diamidino-2-phenylindole
DC	Dendritic cell
DMSO	Dimethylsulfoxide
E	
ELISA	Enzyme-linked immunosorbent assay
ET	Extracellular trap
F	
FACS	Fluorescence assisted cell sorting
FCS	Foetal calf serum
H	
HBD-2	Human β-defensin 2
hEGF	Human epidermal growth factor
HICA	Heat-inactivated *Candida albicans*
HLA	Human leukocyte antigen
HPV	Human papilloma virus
I	
IFN	Interferon
IL	Interleukin
ILC	Innate lymphoid cells
IMQ	Imiquimod
L	
LC	Langerhans cell
LSM	Lymphocyte separation medium
M	
MR	Mannan receptor
N	
NE	Neutrophil elastase
NET	Neutrophil extracellular trap
NK	Natural killer
O	
OPC	Oropharyngeal candidiasis
P	
PAMP	Pathogen associated molecular pattern
PAS	Periodic acid-Schiff
PBS	Phosphate buffered saline
pDC	Plasmacytoid dendritic cell
PFA	Paraformaldehyde
PKA	Protein kinase A
PLM	Phospholipomannan

Abbreviation	Description
P	
PMA	Phorbol 12-myristate 13-acetate
PMT	Protein O-mannosyl transferase
PRR	Pattern recognition receptor
PSORS	Psoriasis susceptibility
R	
RPMI	Roswell park memorial institute
RT	Room temperature
S	
SFM	Serum-free medium
SG	Sytox green
SNP	Single nucleotide polymorphism
T	
TIR	Toll/interleukin-1 receptor
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TRAM	TRIF related adaptor molecule
TRIF	TIR-domain containing adaptor inducing IFN-β
U	
UKD	Universitätsklinikum Düsseldorf
V	
VVC	Vulvovaginal candidiasis
Z	
ZETT	Zentrale Einrichtung für Tierforschung und Tierschutz / Central facility for animal experiments and protection

Summary

The opportunistic pathogen *Candida albicans* is a widespread commensal of humans, occupying niches in the gastro-intestinal tract, oral and vaginal mucosae, and the dermal surface. Host defense against invasive *C. albicans* involves a complex regulatory network of immune components and is heavily dependent on both neutrophils and T_H17-derived cytokines. Both these cell types also form the basis of the inflammatory loop characteristic of psoriasis where IL-17 and IL-22 induce keratinocytes towards hyperproliferation. In addition to the shared immune elements involved in both host defense against *C. albicans* infection and psoriatic inflammation, both these processes may also occur within the same anatomical niche in the case of psoriasis intertriginosa.

Our aim was to assess the effect of local exposure to *C. albicans* previous to psoriatic inflammation on the severity of said inflammation as well as on host chemokine responses. To investigate this, we employed both *in vitro* and *in vivo* methods. Cultured human primary keratinocytes were stimulated with live *C. albicans* and expressed inflammatory chemokines including neutrophil-recruiting CXCL8. Moreover, neutrophils stimulated with *C. albicans* responded by undergoing NETosis in a dose-dependent manner. Using an established murine model for psoriasis-like skin inflammation we were able to show that chemokine receptors CCR6 and CCR10 are not essential for the development of psoriasis-like skin inflammation *in vivo*. Furthermore, we adapted this psoriasis-like model to include cutaneous exposure to live *C. albicans* under occlusion for 72 hours prior to the induction of psoriatic inflammation. This combined model showed increased inflammation in response to *C. albicans* even after the initial fungal burden was cleared. Subsequent flow cytometric analyses of draining lymph nodes revealed that the level of IL-17-producing cells was increased in this group. Taken together, these results suggest that cutaneous pre-exposure to C. albicans may trigger or exacerbate psoriatic inflammation.

Zusammenfassung

Der opportunistischer Krankheitserreger *Candida albicans* ist ein häufiger Kommensale des menschlichen Körpers, der vor allem den Gastrointestinaltrakt, die oralen und vaginalen Schleimhäute sowie die Oberflache der Dermis besiedelt. Die Immunantwort des Wirtes gegen invasive *C. albicans* erfordert ein komplex regulierentes Netzwerk bestehend aus Komponenten des Immunssystems und ist abhängig von sowohl Neutrophilen Granulozyten als auch Zytokinen freigesetzt durch T_H17 Zellen. Neutrophile und T_H17 Zellen fördern außerdem die entzündlichen Prozesse der Psoriasis und induzieren durch die Freisetzung von IL-17 und IL-22 die charakteristische Hyperproliferation der Keratinozyten. Sowohl die Immunantwort gegen *C. albicans* als auch die Entzündungsreaktionen der Psoriasis spielen auch im Krankheitsbild der Psoriasis intertriginosa eine wesentliche Rolle.

Ziel dieser Studie war es den Effekt von lokaler *C. albicans* Exposition auf die Entzündungsreaktionen der Psoriasis sowie das assoziierte Zytokinprofil zu ermitteln. Dafür haben wir sowohl *in vitro* als auch *in vivo* Methoden eingesetzt. Nach Stimulation von humanen primäre Keratinozyten mit lebendem *C. Albicans in vitro,* konnten wir eine erhöhte Expression von entzündungsspezifischen Zytokinen, wie dem Neutrophilen Chemokin CXCL8 detektieren . Die Stimulation von Neutrophilen mit *C. albicans* zeigte eine dosisabhängige Induzierung von NETose*, in vitro*. Mittels eines etabliertem Tiermodells für Psoriasis-ähnliche kutane Entzündung konnten wir zeigen, dass die beiden Chemokinrezeptoren, CCR6 und CCR10, nicht essenziell für die Entstehung von Psoriasis-ähnlichen Entzündungen *in vivo* sind*.* Als Adaption unseres Tiermodells haben wir die Maushaut mit lebenden *C. albicans* behandelt und die Stelle für 72 Stunden unter Okklusion gehalten. Die nachfolgende Induktion der Psoriasis validierte eine erhöhte Entzündungsreaktion auf *C. Albicans* in diesem kombinierten Tiermodell. Dieser Effekt war auch, nachdem der Hefepilz nicht mehr vorhanden war, deutlich messbar. Mittels

Durchflusszytometrie konnten wir in den drainierenden Lymphknoten dieser Messgruppe eine erhöhte Anzahl IL-17 produzierender Zellen, im Vergleich zur Kontrollgruppe, detektieren.

Zusammenfassend deuten die Ergebnisse darauf hin, dass kutane Exposition mit *C. albicans* die Entzündungsreaktionen der Psoriasis auslösen oder gar verschlimmern kann.

1 Introduction

1.1 *Candida albicans*

1.1.1 Background

As a common opportunistic commensal of the human gastro-intestinal tract, mucosal membranes, and skin, *Candida albicans* has a clear medical impact worldwide. Once it senses a lapse in immune surveillance, *C. albicans* is capable of infiltrating local tissues and causing severe systemic disease [1]. The latter is a pivotal concern for people with a compromised immune system or specific mutations in genes involved in anti-fungal defense. Localised infections such as oropharyngeal candidiasis (OPC) and vulvovaginal candidiasis (VVC) are wide-spread, with as many as 75% of women of childbearing age being affected by VVC at least once in their lives [2]. Although most research is directed at *C. albicans* involvement at mucosal sites, the relevance of *C. albicans* in the context of cutaneous infections is not to be underestimated.

Attempts to shed light on the components and complexity of the cutaneous mycobiome have been limited to a large extent by their dependency on the necessity of culturing the isolated strains before they could be accurately identified by molecular methods [3]. Only more recently have sequencing techniques advanced enough to allow for positive identification of yeasts and fungi at the genera level, if not also at the species level. A recent study into the fungal microbiome of the human skin using these latest methods assayed 14 distinct anatomical sites and found *C. albicans* to be identifiable most readily on the foot skin [4] of the healthy test subjects. From clinical observations, it is known that skin infections caused by *C. albicans* most commonly occur in the big body folds (intertrigines) where the high humidity, low oxygen concentration and constant friction provide an environment where it can readily grow and invade [5].

One of the most important virulence factors of *C. albicans* is its ability to alter its growth pattern from a unicellular to a hyphal form. The exact mechanism by which this switch occurs and the conditions needed have been the subject of intense research over the years, however much remains unknown. In general, three distinct morphologies are recognised, including an intermediate pseudohyphal stage in addition to the yeast and 'true' hyphal forms [6]. In addition to this, cells in the yeast form can also undergo a polymorphic switch with stages termed white, grey, and opaque, referring to the appearance of the resulting colonies, though this process mainly has functions in the mating process of *Candida* species [7].

Over time, a number of conditions have been identified that are likely to induce a shift from one growth form to another *in vitro*, with a general rule emerging that the more "hostile" its surroundings are (e.g. high temperature, high pH) the more *Candida albicans* is pushed towards an invasive hyphal phenotype [6]. As part of the morphological changes between the growth forms, cell surface components are altered considerably [8]. The outer layer of the cell wall contains of proteins bound to O- and N-linked mannans, whereas the inner layer consists mainly of structural polysaccharides chitin and β-glucan [9]. Both the various specific types of outer layer mannans and glycoproteins, and inner layer polysaccharides are altered in relative frequency between the yeast form and the two filamentous growth patterns [8]. These surface changes have a clear impact on the host's ability and inclination to respond to *C. albicans* in its various forms.

1.1.2 Host response

The first step in any host response to invading organisms is the recognition of pathogen associated molecular patterns (PAMPs) by specific receptors expressed by tissues exposed to the external environment. In the case of *C. albicans* this step is complicated by

its previously mentioned ability to switch from a unicellular to a hyphal morphology. One of the first pattern recognition receptors (PRRs) identified to bind to *C. albicans* outer cell wall N-linked mannans is the mannan receptor (MR) expressed by macrophages. Over the years, more and more PRRs have been identified that are able to recognise *C. albicans* PAMPs. The main players emerging from this field of research are the toll-like receptors (TLR) 2 and 4 as well as Dectin-1 and Dectin-2.

Dectin-1, expressed by phagocytes, mast cels, and dendritic cells, recognises surface β-glucans and can mediate both the release of a host of pro-inflammatory cytokines and chemokines as well as phagocytosis via the intracellular adaptor CARD9. Although the higher level of mannans on the outer layer of the cell wall masks the presence of β-glucan in the hyphal form and Dectin-1 was thought to be unable to bind to it as a result [10], there have been publications showing both mast cells and macrophages do in fact respond to *C. albicans* hyphae [11, 12]. This disparity can be partly explained by the differences in PRR-ligand binding *in vitro* versus *in vivo* [11, 13]. In the yeast form, β-glucan is most accessible at the budding scar, whereas in the hyphal form, Dectin-1 is less effective as β-glucan on the inner layer is masked by the higher concentration of mannans covering it [10]. As a result of Dectin-1 signalling in dendritic cells, differentiation of T cells is skewed towards a T_H17 phenotype [13]. Dectin-1 has also been shown to have an additive effect on inflammatory cytokine release in combination with TLR2 signalling. TLR2 responds to phospholipomannan (PLM), but is not sufficient on its own to mount a strong host immune response [14].

TLR4, in contrast to TLR2 is able to initiate a strong anti-fungal response after ligation to O-linked mannans. Signalling via the adaptor molecule MyD88 leads to release of TNFα as well as release of IFN-γ and IL-12, promoting differentiation of naive T cells to a T_H1

phenotype [15]. TLR4 also interacts with TRIF related adaptor molecule (TRAM) at the inner cell membrane, activation of which leads to the release of type 1 interferons [15]. The ability of both TLR4 and Dectin-1 to bind to their ligands is severely reduced after *C. albicans* switches to a hyphal phenotype. Dectin-2 is one of few receptors specifically able to react to hyphal mannans using the Fcγ receptor as an adaptor molecule [16]. The result of Dectin-2 binding to hyphal mannans is the activation of CARD9 and subsequent release of IL-6, IL-23 and pro-IL-1β, which act to differentiate and activate T_H17 cells as well as, indirectly, neutrophils [17].

The combined results of PRR signalling shape the host anti-*Candida* response partly by way of the direct actions of cytokines and antimicrobial peptides in killing the invader and partly indirectly by the recruitment, maturation and differentiation of leukocytes from the periphery via chemokine gradients. Although there are a number of redundant signalling pathways, a few have been identified as being essential in effective clearance of *C. albicans* during active infection. Control of *Candida* infections is mediated by several cell types, most notably T_H17 cells and neutrophils. Defects in the signalling pathway leading to IL-17 and downstream are a known cause of chronic mucocutaneous candidiasis (CMC) [18].

Neutrophils canonically aid in host defense via the release of antimicrobial peptides (AMPs), cytokines, and chemokines from intracellular granules. More recently, neutrophils have been described to generate extracellular traps consisting of nuclear material (DNA and histones) decorated with granular components [19]. These NETs (Neutrophil extracellular traps) were found to be effective in killing *C. albicans* [20] and also to play a role in various autoimmune diseases by providing complexes of AMP from neutrophilic granules bound to self-DNA [21, 22].

Candida has a number of evasion strategies in addition to the dimorphic switch mentioned above, one of which involves the surface mucin Msb2. This signalling mucin consists of an intracellular domain which is associated with adaptor protein cdc42, a single transmembrane section, and a large, heavily glycosylated extracellular domain. The extracellular part of the protein is continuously cleaved by secreted aspartic proteases, leading to the activation of a signalling cascade that starts with cdc42 activation and results in upregulation of PMT1, 2 and 4 expression. Pmt act as mannosyltransferases that lead to the increase of surface protein mannosylation, promote a hyphal phenotype and reduce β-glucan exposure [23]. Low glycosylation or damaged β-glucans can also increase the rate of Msb2 cleavage. The cleaved portion of the protein has been found to prevent damage to the yeast cells by antimicrobial peptides released by the host [24, 25]

1.2 Psoriasis

1.2.1 Healthy skin

To better understand the pathomechanism of inflammatory skin conditions such as psoriasis, a clear image of the structure and function of the components of healthy skin is essential. The following information on the cellular and structural components of healthy skin can be found in any general human physiology or dermatology textbook. As the largest organ of the human body the skin provides a protective barrier against outside insults. Human skin consists of two parts - the dermis and above that, the epidermis, each of which can be further subdivided into distinct layers.

The dermis forms the structural core of the skin and contains skin appendages such as hair follicles, sweat glands, blood and lymph vessels. The innermost layer of the dermis is named the reticular layer due to its structure being a dense network of connective collagen

and elastin fibres. The main cell type in the dermis is the fibroblast, responsible for the production of these structural fibres, collagen and elastin. Just above the reticular layer is the papillary layer of the dermis so named for the finger-like protrusions towards the epidermis that improve the adherence of the epidermis to the dermis and provide a large surface area to aid in the provision of the upper layers with nutrients. The same connective tissue mesh is present as in the reticular layer, but organised in a much looser fashion. The papillary layer is also home to resident macrophages and a small amount of adipocytes in addition to the ubiquitous fibroblasts. The basal lamina divides the dermis from the epidermis and forms the attachment point for the *stratum basale,* the first layer of the epidermis.

The keratinocytes that make up the epidermis start off here as basal cells and they constantly divide and differentiate. The basal lamina provides an effective barrier restricting the movement of differentiating keratinocytes to a single direction - outward. The epidermis therefore is grown from the inside out; as keratinocytes differentiate further, they migrate outwards forming the remaining layers of the epidermis. Adjacent to the *stratum basale* is the *stratum spinosum* so named for the protrusions visible in histological samples where the keratinocytes (8-10 cells thick) attach to each other. This layer is also home to skin-resident dendritic cells (Langerhans cells) - often the first responders to external threats. The next layer, the *stratum granulosum,* is only 3-5 cells thick and characterised by the presence of keratin and keratohyalin filled granules in the keratinocytes. This represents the final stage of keratinocyte differentiation after which they undergo apoptosis and are pushed further outwards. In some areas with very thick skin such as the soles of the feet, a distinct *stratum lucidum* can be seen consisting of dead keratinocytes and high levels of keratin, giving a slight ‘transparent’ effect to the skin when seen from the outside.

The final layer is the *stratum corneum* which consists entirely of anuclear, dead keratinocytes (corneocytes) in an overlapping ‘roof-tile’ pattern [26].

In addition to the mechanical barrier function of the skin, constant immune surveillance is essential in the quick host response to external threats. For this purpose, a variety of leukocytes are present in the skin under homeostatic conditions [27].

Most T cells present in the skin are found in the dermal compartment, with only a small minority in the epidermis. αβ T cells are the most abundant population of T cells and can be subdivided into $CD4^+$, $CD8^+$ and natural killer (NK) T cells. They migrate to the skin after antigen presentation in the lymph nodes and subsequent differentiation. The chemokine receptors specific for skin-homing of αβ T cells (CCR4, CCR6, CCR8, and CCR10) are upregulated to facilitate migration. γδ T cells, in contrast, do not require priming and are seeded to the skin during embryonic development. They provide both innate and adaptive-type defense against cellular damage and infection [28].

On the innate side, antigen-presenting cells including Langerhans cells, dermal dendritic cells and macrophages all maintain a stable presence in the skin. Finally, mast cells and innate lymphoid cells (ILCs) also provide immune surveillance functions. ILCs are characterised by their lack of T-, B-, myeloid and dendritic cell markers and were originally described in mucosal tissues before being identified as being resident in the dermis as well. Type 2 ILCs have been found to interact with mast cells, release IL-5 and IL-13 [27] and to be elevated in number in atopic lesions [29]. In contrast to this, type 3 ILC populations are expanded significantly in the skin of psoriasis patients [30]. Neutrophil granulocytes are not resident in the skin, but rather circulate in the blood and are recruited to the skin via chemokine gradients by the first responders to any injury or infection. All

these various cell types work in concert to provide an adequate response to external threats and maintain immune homeostasis during normal conditions. Under abnormal circumstances such as autoimmune conditions these same cells become dysregulated and cause damage to the host.

1.2.2 Background

Psoriasis affects around 2% of the population [31] and its character as a chronic recurrent-remitting autoimmune condition means it is often used as a model for chronic inflammation. Apart from its widespread incidence, the relevance of psoriasis is also magnified by the high prevalence of psoriasis-associated morbidities such as diabetes mellitus and cardiovascular diseases [32]. Furthermore, 20-30% of psoriasis patients develop secondary complications such as psoriatic arthritis. Clinically, the most common type of psoriasis, psoriasis vulgaris, presents with sharply bordered erythematous plaques accompanied with silver-to-white scales. Histologically, epidermis affected by psoriasis features pronounced acanthosis, loss of the granular epidermal layer, a thickened stratum corneum, and retention of nuclei in the upper epidermal layers while the basal layer keratinocytes present with accelerated division rates [33]. In the dermis, an infiltrate of mononuclear cells and T cells are additional diagnostic hallmarks of psoriasis.

1.2.3 Pathomechanism

Knowledge about the genetic factors predisposing individuals to the development of psoriasis was limited for a long time in part based on the uncertainty whether it was primarily a keratinocyte-mediated condition or whether immune cells could also play a role. Linkage analysis in several family groups first identified psoriasis susceptibility (PSORS) loci [34] even though the precise genes and their possible functions remained unknown for many years afterwards. With advancing techniques, the genetic component of psoriasis

has been elucidated to a greater degree, such that over 50 regions containing single nucleotide polymorphisms (SNPs) have now been identified as contributing to disease development [35]. Almost all of the positively identified PSORS have a primary function in some aspect of the immune system. The loci in which these point mutations occur contain genes that range in function from T-helper cell polarisation (*RUNX1*) to inhibition of NF-κB signalling (*TNIP1)* and genes involved in the IL-12/IL-23 axis and HLA complex [36]. Despite the many genetic susceptibility factors identified so far, psoriasis is not a purely genetic condition as even monozygotic twins only have a 70% chance of both being affected, and for dizygotic twins this chance drops to as low as 20%. The currently accepted theory is that the genetic predisposition in combination with outside stimuli or trigger factors cause flare-ups of active disease. For instance, psoriasis is one of a number of skin conditions where lesions can appear at the site of traumatic injury. This so-called Koebner phenomenon has been long recognised as a trigger for certain people with psoriasis [33], and the exact molecular mechanism has been investigated in more detail recently [37]. Raychaudhuri et al. found that a subset of patients with psoriasis responded to mild mechanical trauma of the skin with a flare-up of psoriatic lesions at the site. This response correlated with elevated levels of nerve growth factor compared to healthy controls as well as non-responding psoriatic patients [37]. Other environmental factors identified as potential triggers include stress, tobacco smoke [38] and microbial infections, including *C. albicans* [39]. All the above mentioned triggers can converge in the pathogenic pathway in the generation and release of antimicrobial peptides (AMPs) by keratinocytes.

The inflammatory cascade perpetuated in psoriasis begins with keratinocytes triggered by external trauma, stress, or certain microbial infections. Antimicrobial peptides released by keratinocytes in response to these triggers combine with local DNA or RNA from damaged cells to activate resident myeloid dendritic cells, which subsequently release IL-23. This

particular cytokine is known to be essential in maintaining the phenotype of T_H17 cells after the initial stimulus pushing towards differentiation [40]. Resident or recruited neutrophils are also a source for DNA-AMP complexes if they are induced to undergo NETosis by inflammatory cytokines [22], including IL-8 released by activated keratinocytes [41]. Triggered keratinocytes also release the chemokine CCL20, which attracts both T_H17 cells as well as more dendritic cells [42]. Plasmacytoid dendritic cells activated by DNA- or RNA-AMP complexes via TLR9 and TLR7, respectively, produce type 1 interferons. These, in turn, activate myeloid dendritic cells in much the same way as DNA-AMP complexes can do directly via TLR8 [21].

Neutrophils are recruited and activated by chemokines (CXCL1, CXCL2, CXCL8) released from activated keratinocytes as well as T cells driven by IL-36 family members. Although neutrophils can not react directly to IL-17 due to a lack of the corresponding receptors, other cytokines released by T_H17 cells (GM-CSF, TNF-α, IFN-γ) can and do directly activate neutrophils [43].

With both keratinocytes and T_H17 cells recruiting neutrophils to the site, the inflammatory loop is closed by neutrophils releasing IL-17 [44]. This prompts keratinocytes to release more CXCL8 and CXCL1, resulting in increased neutrophil recruitment. Activated neutrophils also release the chemokine CCL20, which attracts more T_H17 cells to further strengthen the positive feedback loop characteristic of psoriasis. Finally, recruited neutrophils undergoing NETosis provide long-lasting DNA-AMP structures as well as strong chemokine gradients for immense cell recruitment [41]. Together, these reactions maintain a positive feedback loop of local inflammation, which supports the physical symptoms characteristic of psoriasis.

1.2.4 Psoriasis intertriginosa

Various subtypes of psoriasis are recognised, either differentiated by physical appearance of the lesions or by the anatomical location primarily involved. Around 5% of people with psoriasis suffer from the intertriginal subtype [45]. Psoriasis intertriginosa, or psoriasis inversa affects the large body folds (intertrigines) such as the gluteal cleft, groin, submammary fold, or the stomach fold in particularly obese patients. These anatomical sites differ from those primarily affected by psoriasis vulgaris in that the skin is thinner due to the mechanical stress of skin-on-skin friction and generally provide a moist and isolated niche for a particular microbiotic profile. This has to be taken into account clinically, as co-infections with either bacterial or fungal species may inhibit the efficacy of topical treatments. In addition to this, systemic therapies targeting those pathways overlapping with host anti-*Candida* defense (such as TNF-α and IL-17) can lead to an increased incidence in the development of infection and affect treatment options [46].

1.3 *Candida albicans* and psoriasis

Considering the overlap in both the anatomical locations for psoriasis intertriginosa and *C. albicans* dermal colonisation and in immunological responses to the commensal and psoriasis in general, the amount of research done to clarify the role of *C. albicans* in this particular condition has been ample over the years. Despite this, there is a distinct lack of consensus regarding the question whether *C. albicans* is more commonly present in or on patients with psoriasis compared to healthy controls. A case study by Wilmer et. al. in 2013 [47] describes a patient with psoriasis intertriginosa previously misdiagnosed as having an intertriginal *C. albicans* infection, highlighting the commonalities between the two conditions. Two separate investigations into psoriasis vulgaris showed a higher incidence of *C. albicans* in saliva and faeces [48] and on the tongue [49] in patients with psoriasis compared to healthy controls. However, a study more specifically aimed at psoriasis

intertriginosa found no evidence of *C. albicans* at the affected sites [50]. As early as 1973 it was suggested that psoriasis could not only be triggered by *C. albicans* but also be exacerbated long after the initial colonisation has resolved due to the Koebner effect [51].

1.4 Aims

The aim of this project was to ascertain whether exposure to *C. albicans* preceding a psoriasis flare up could exacerbate the symptoms of the latter as well as to investigate the role of chemokines and their recruited cell types in this process.

2 Materials and Methods

2.1 Mice

For all experiments female wildtype Balb/c, Balb/c-*Ccr10*$^{-/-}$, wildtype Bl/6J, Bl/6J-*Ccr6*$^{-/-}$ mice between 8 and 16 weeks of age were used. Wildtype mice were purchased from Janvier Labs (France, Europe) or bred in-house at the Central Facility for Animal Experiments and Protection (ZETT) of the Heinrich-Heine-University Düsseldorf (Düsseldorf, Germany) where all mice were kept and all experiments were performed. *Ccr10*$^{-/-}$ mice were a kind gift from Prof. Dr. M. Steinhof, Department of Dermatology, Hamad Medical Corporation (HMC), Doha, Qatar. *Ccr6*$^{-/-}$ mice were developed and bred in-house. Mice were provided with water and food *ad libitum* and kept on a 12-hour light/ dark cycle under specific pathogen free conditions. All animal experiments were performed in accordance to local and international guidelines, under animal licence number 470-13.

2.2 Methods

2.2.1 Human primary keratinocyte cell culture

Primary human keratinocytes were isolated from skin obtained post-surgery and cultured in keratinocyte-SFM containing L-glutamine, recombinant human epidermal growth factor (hEGF), bovine pituitary extract (Thermo Fisher Scientific, Cat. 17005075) and 1% penicillin/streptomycin. For stimulation experiments cells were used in the third or fourth passage at 80% confluence unless otherwise stated.

2.2.2 Stimulation with *C. albicans* / HICA

Primary human keratinocytes were seeded in 12-well plates (Falcon, Fischer Scientific Cat. 08-772-3A) and incubated with varying concentrations of live *C. albicans* or heat inactivated *C. albicans* (HICA) for up to 24 hours at 37℃, 5% CO_2, 100% humidity.

2.2.3 Time-lapse microscopy

Primary human keratinocytes grown in a monolayer and stimulated with various concentrations of *C. albicans* were photographed every 5 minutes over the course of up to 24 hours at 37℃, 5% CO_2 using Zeiss Axio Vert 200M with AxioCam camera and Axiovision 4.8 software (Carl Zeiss, Germany)

2.2.4 qPCR

Cell lysates of primary human keratinocytes co-incubated with live *C. albicans* or HICA were collected in RLT buffer (Qiagen, Cat. 79216) with 1% β-mercaptoethanol. Total RNA was extracted with RNeasy kit (Qiagen, Cat. 74104) according to manufacturer's instructions. RNA concentration and quality were determined using the NanoDrop 3300 (ThermoFisher Scientific) and lysates were adjusted to 4 ng/10 µL. Reverse transcription to cDNA was performed by adding 6 µL DNA digestion mix to each sample and incubating at 37℃ for 30 minutes.

	30x (µL)	1x (µL)	concentration	source
First strand buffer	45	1.5	5x	ThermoFisher Cat. Y00146
RNase inhibitor	30	1	40 U/ µL	Promega Cat. N2611
DNAse I	30	1	2.5 U/ µL	ThermoFisher Cat. 90083
H2O	75	2.5	n.a.	n.a.
Cell lysate	300	10	n.a.	

Table 1. DNA digestion mixture. Reagents used during DNA digestion in isolated RNA from keratinocyte lysates.

	30x (µL)	1x (µL)	concentration	source
Oligo dT	30	1	50 µM	Invitrogen Cat. AM5730G
random hexamer	12	0.4	50 µM	Invitrogen Cat. N8080127
H2O	78	2.6	n.a.	n.a.

Table 2. Random primer mixture. Reagents used for primer annealing.

	30x (µL)	1x (µL)	concentration	source
First strand buffer	135	4.5	5x	ThermoFisher Cat. Y00146
dnTP	45	1.5	2.5 mM	Invitrogen Cat. R72501
DTT	30	1	0.1 M	Invitrogen Cat. D1532
RNAse inhibitor	15	0.5	5x	ThermoFisher Cat. Y00146
H2O	45	1.5	n.a.	n.a.
Superscript	30	1	200 U/µL	Invitrogen Cat. 18064-022

Table 3. Reverse transcription mixture. Reagents used for reverse transcription of RNA to cDNA.

	1x (µL)	concentration	source
TaqMan MasterMix	10	2x	ThermoFisher Cat. 4304437
AoD Mix	1		Applied Biosystems (Table x)

Table 4 Target gene mix Reagents used for target gene qPCR analysis.

	1x (µL)	concentration	source
TaqMan MasterMix	12.5	2x	ThermoFisher Cat. 4304437
18S Probe	1.5	10 µM	ThermoFisher Cat. 4308329
18S 5' Primer	1.5	10 µM	
18S 3' Primer	1.5	10 µM	
H2O	20.5	n.a.	n.a.

Table 5 PCR mix 18S control Reagents used for internal housekeeping control.

After digestion, samples were incubated with 4 µL random primer mix for 10 minutes at 25℃ to allow the primers to anneal to the RNA before adding 10 µL reverse transcription mix to each sample and heating to 50℃ for 50 minutes. Finally, samples were chilled to -20℃ before further analysis.

cDNA was quantified using NanoDrop and diluted to 2.5 ng/µL in water and 10 µL was used per PCR reaction. All primers used were obtained from Applied Biosystems (**Tab. 6**) and 18S was used as a housekeeping control with all used reagents outlined in **Tables 1-5**. All qPCR experiments were performed using QuantStudio 6 system (Applied Biosystems) with the following settings: heated to 50℃ for 2 minutes, followed by 95℃ for

10 minutes before 40 cycles of 95℃ for 15 seconds and 60℃ for 1 minute. The obtained results were analysed using standard spreadsheet software.

Gene name	TaqMan assay number
CXCL8	Hs00174103_m1
CXCL1	Hs00605382_gH
CCL20	Hs00171125_m1
CCL17	Hs00171074_m1
CXCL9	Hs00171065_m1
CXCL10	Hs00171042_m1
CXCL11	Hs00171138_m1

Table 6 Target gene primers Gene assay numbers for the primers used for the target genes. All obtained from Applied Biosystems.

2.2.5 ELISA

Supernatants collected from primary human keratinocytes co-incubated with live *C. albicans* were used to assay the presence of selected chemokines by ELISA according to the manufacturer's instructions (R&D Systems, Cat. DY208) using the Multiskan Ascent (Thermo Electron Corp) plate reader.

2.2.6 Isolation of neutrophils from whole blood

Whole blood was obtained either as leftovers from the blood donation centre at the Blutspendezentrale (Blood donation centre) at the UKD or taken by venipuncture into EDTA-coated vacutainers (BD, Cat. 366450) from volunteers. Neutrophil isolation was performed as follows. Briefly, 12 mL blood was layered on 10 mL lymphocyte separation medium (LSM) (Lonza, Cat. 17-829F) in a 50 mL Falcon (ThermoFischer Scientific, Cat. 14-432-22) and subsequently centrifuged at 600x g for 30 minutes at 4℃ without brakes. The supernatant was discarded and the erythrocyte pellet with granulocyte layer was washed with PBS and subjected to a hypotonic shock for 50 seconds by adding 4.26 mL

H_2O per mL of pellet. After addition of 5M NaCl to reach an isotonic solution and 3 more PBS washes, the cells were counted using a live/dead dye Trypan Blue (ThermoFischer Scientific, Cat. 15250061) in a heamocytometer. The volume of 5M NaCl was determined using the following calculation:

$$5\text{M NaCl(mL)} = \frac{[\text{H}_2\text{O(mL)} \times \text{pellet(mL)}] \times 15}{500}$$

2.2.7 Induction of NETosis

Isolated human neutrophils were adjusted to 1x10^6 cells/mL in RPMI 1640 (ThermoFischer Scientific, Cat. 11875093) containing 1% Penicillin/Streptomycin, 1% FCS and 100 µL of cell suspension was seeded per chamber of a chamberslide (ThermoFischer Scientific, Cat. 154526) coated with 3 mg/mL poly-L-lysine or per well of a 96-well plate (Corning, Cat. 3356). Cells were allowed to rest at 37℃, 5% CO_2 for 45 minutes before being stimulated with 100 µL phorbol 12-myristate 13-acetate (PMA) or live *C. albicans* in RPMI 1640. Dimethyl sulfoxide (DMSO) or RPMI 1640 were used as negative controls. Neutrophils were stimulated for up to 3 hours at 37℃ before further analysis.

2.2.8 Quantification of NETosis

NETosis was quantified using two methods.

SG - After stimulation, Sytox Green (SG) was added to each well to a final concentration of 1 µM and incubated at room temperature (RT) for 10 minutes. Fluorescence intensity was measured by TECAN Infinite 200 Pro (Tecan, Zürich, Switzerland) with an excitation wavelength of 488 nm and emission wavelength of 523 nm. Gain was optimised before each measurement.

Microscopy - NETs generated on poly-L-lysine covered chamber slides were stained with 1 µM SG for 10 minutes at room temperature before being washed gently with PBS and

mounted with Fluoromount G (Thermo Fisher Scientific, Cat. 00-4958-02). Slides were left to harden overnight at 4℃ and representative microscopy images were taken the next day for quantification. The area covered in each field by positive SG fluorescence after exclusion of non-NET fluorescence by size and shape was calculated as a percentage of the total area.

2.2.9 Staining NETs

NETs were stained either directly after generation by addition of SG to a final concentration of 1 µM before a single wash and immediate mounting and covering, or after a 30 minute fixation in 4% PFA with DAPI (ThermoFischer Scientific, Cat. D1306) diluted 1:1000 in PBS.

For IL-26 staining, chamber slides containing NETs were fixed with 4% PFA for 30 minutes, washed with PBS and covered with blocking buffer containing 2% goat serum, 0.1% fish gelatin and 0.05% Tween 20 detergent in PBS for 30 minutes at room temperature before being incubated with 10 µg/mL IL-26 antibody or 10 µg/mL mouse IgG isotype in blocking buffer overnight at 4℃. After the primary antibody incubation, slides were washed with PBS and covered with Alexa fluor 555 conjugated donkey anti-mouse (Invitrogen, Cat. A31570) at 2 µg/mL in blocking buffer for 45 minutes at room temperature. After a final wash with PBS, slides were counterstained with DAPI, mounted, and covered as described above before microscopic analysis.

2.2.10 IMQ mouse model

Established by van der Fits et al. [52], this model was used to mimic psoriasis *in vivo*. Briefly, female mice between 8 and 16 weeks of age were housed singly and shaved dorsally. 62.5 mg Aldara cream (3M, Minnesota, USA) containing 5% IMQ was applied

daily to the shaved back and right ear for 7 days. Mice were sacrificed 24 hours after the last application of IMQ by CO_2 gas inhalation. Samples of back and stomach skin were collected, in addition to both ears, the spleen and draining lymph nodes of the ear. Basiscreme DAC was applied as a negative control. This standard recipe amphiphilic cream contains 40% purified water, 25.5% white vaseline, 10% propylene glycol, 7.5% mid-chain triglycerides, 7% macrogol-1000-glycerol monostearate, 6% cetyl alcohol and 4% glycerolmonostearate weight for weight.

2.2.11 Combined *C. albicans* colonisation and IMQ model

Cutaneous colonisation of the back skin with *C. albicans* was achieved by first shaving the dorsal region and tape-stripping the shaved area x8 with Tegaderm 1624W (3M, Minnesota, USA) to partially remove the stratum corneum. A fragment of absorbent DracoPor dressing (Draco, Germany) approximately 1 cm^2 impregnated with 100 µL of a $2x10^7$ /mL suspension of *C. albicans* wild-type strain SC5314 blastoconidia (kindly supplied by Prof. J. Ernst, Heinrich-Heine University Düsseldorf) in PBS was placed on the

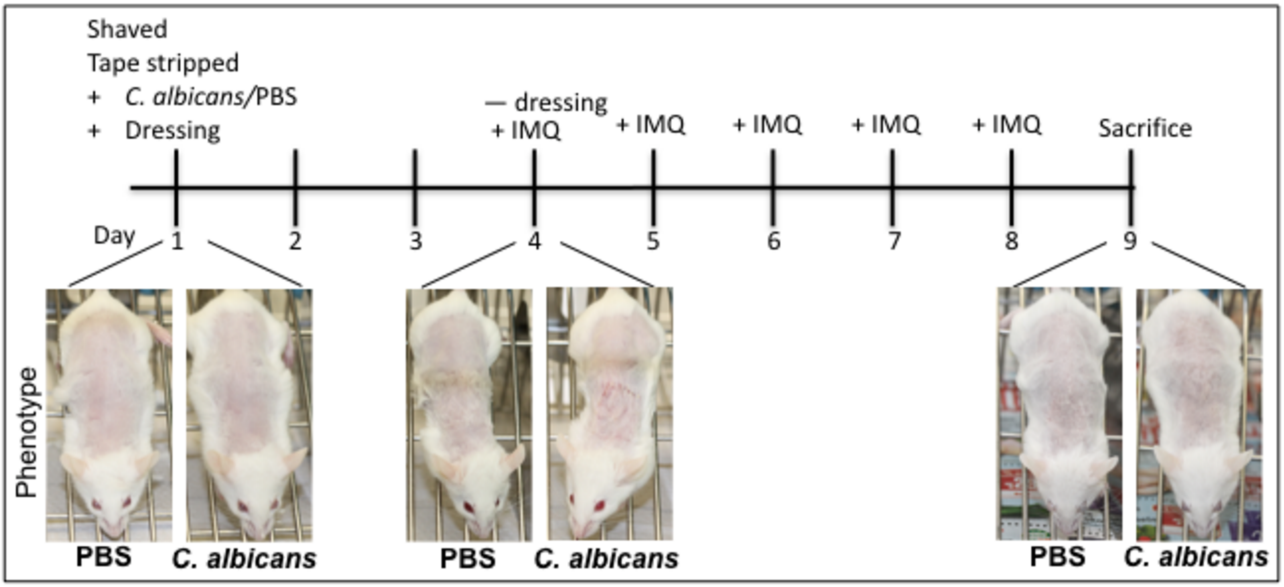

Figure 1. Timeline of murine model combining *C. albicans* colonisation with IMQ-induced psoriasis-like skin inflammation (combination model). Day 1: female mice between 8-16 weeks are shaved dorsally and tape-stripped before application of $1x10^6$ blastoconidia of live *C. albicans* or PBS on an immobilised absorbent dressing. Day 4: dressing is removed. Day 4-8: 31.25 mg Zyclara containing 3.75% IMQ is applied to the shaved back and right ear. Day 9: mice are sacrificed via CO_2 inhalation.

back of each treated mouse and immobilised by a section of Tegaderm. The dressing was covered with a circumferential strip of Coban (3M, Minnesota, USA) elasticated bandage and left in situ for 72 hours. PBS impregnated dressings were applied as a control to half the mice in each experiment. Immediately after removal of the dressing, 31.25 mg Zyclara (3M, Minnesota, USA) containing 3.75% IMQ was applied to the back and right ear daily for 5 days. Mice were sacrificed by CO_2 inhalation 24 hours after the last application of Zyclara. Measurements of the ear and back double skin fold thickness were taken on day 1 before application of the dressing, on day 4 after removal of the dressing and on day 8 immediately after sacrifice. Photographs (EOS 1100D; Canon, Japan) were taken at each of these time points of the whole mouse and the back skin and ears in detail. Control experiments sacrificing mice immediately after dressing removal (day 4) and on day 8 without the application of IMQ were also performed. An overview of the timeline of the method can be found in **Fig. 1**.

2.2.12 Histology of tissue samples

Formalin-fixed tissue samples were processed using the VIPr Vacuum Infiltration Processor Floor (Tissue Tek, Sakura) and subsequently embedded in paraffin and cut into 5 µm sections. Sections were stained with haematoxylin and eosin using the standard protocol as used for routine samples in the histology laboratory of the department of dermatology of the university hospital Düsseldorf. Briefly, sections were submerged for 40 seconds each in the following sequence of reagents:

xylene x2 - 99% isopropyl alcohol - 96% isopropyl alcohol - 70% isopropyl alcohol - H_2O - haematoxylin x5 - H_2O - HCl isopropyl alcohol - H_2O - Scott’s tap water substitute - H_2O - aqua dest - eosin - 96% isopropyl alcohol x2 - 99% isopropyl alcohol x2 - xylene x2

Following this, coverslips were attached using the Tissue Tek coverslipper (SCA-4765 Film Coverslipper, Sakura)

Samples embedded in Tissue-Tek OCT compound (Sakura Finetek Europe) immediately after collection were snap-frozen and stored at -80℃ until being cut into 3 µm sections using a cryotome (ThermoFischer Scientific). Frozen sections were thawed and allowed to dry at room temperature and stained with Haematoxylin and Eosin according to the standard protocol described above with the alteration of skipping the initial decreasing alcohol series, starting the staining directly in the Eosin.

2.2.13 Cell count determination in the lymph nodes

Axillary lymph nodes collected from mice exposed to *C. albicans* in the combined model as described above were mechanically homogenised in 300 µL RPMI 1640 (ThermoFischer Scientific, Cat. 11875093) without supplements. After removal of debris samples were analysed by VetABC (Scil animal care company, Viernheim, Germany) full blood count analyser.

2.2.14 Intracellular cytokine staining of lymphocytes

Axillary lymph nodes collected from mice exposed to *C. albicans* in the combined model as described above were mechanically disrupted by passing through a 60 µm pore mesh (Fisher Scientific, Cat. 08-771-2). The cell suspension was pelleted by centrifugation and resuspended in 500 µL RPMI 1640 containing 10% FCS and 1% penicillin/streptomycin before being seeded in round bottom 96-well plates (Sigma-Aldrich Cat. CLS3367). Cells were incubated at 37℃, 5% CO_2 for 1 hour with 50 ng/mL PMA and 750 ng/mL ionomycin before addition of Brefeldin A (eBioscience, San Diego, USA) to a final concentration of 3

Antigen	Antibody	Fluorophore	Final concentration
IFN-γ	Rat IgG1a (eBioscience	APC	667 ng/mL
IL-17	Goat IgG (RnD Bioscience)	PerCP	1:5 dilution of stock

Table 7. Intracellular marker antibody mixture components.

µg/mL and further incubation at 37℃, 5% CO_2 for 5 hours. After centrifugation of the plate for 4 minutes at 200x g to pellet the cells, the supernatant was discarded and surface marker antibody mix diluted in FACS buffer was added according to the details in **Table 8** and incubated at 4℃ for 20 minutes including 1 µL counting beads (BD Bioscience Cat. 349480) per well. Stained cells were then pelleted by centrifugation and washed with FACS buffer once before being fixed with a 2% PFA solution in PBS for 10 minutes at room temperature. Samples were then washed with permeabilisation buffer twice before incubation for 30 minutes at 4℃ with the intracellular marker antibody mixture diluted in permeabilisation buffer as outlined in **Table 7** above. Cells were finally resuspended in 150 µL permeabilisation buffer after two washes with the same buffer and kept on ice until analysis by flow cytometry with LSR Fortessa (BD Bioscience, Germany) and FACS Diva software (BD Bioscience, Germany). For each sample 5000 events were counted and recorded. Subsequent data analysis was performed using FlowJo software (Flowjo, Oregon, USA).

Antigen	Antibody	Fluorophore	Final concentration
CCR6	Armenian Hamster IgG (BioLegend}	PE	2 µg/mL
CD4	Rat IgG2a (eBioscience	PE-Cy7	667 ng/mL
CD8	Rat IgG2a (BD Bioscience	Bv711	667 ng/mL
Ly-6G	Rat IgG2b (BD Bioscience	APC-Cy7	667 ng/mL

Table 8. Surface marker antibody mixture components.

3 Results

3.1 Effect of *C. albicans* on chemokine expression by human keratinocytes

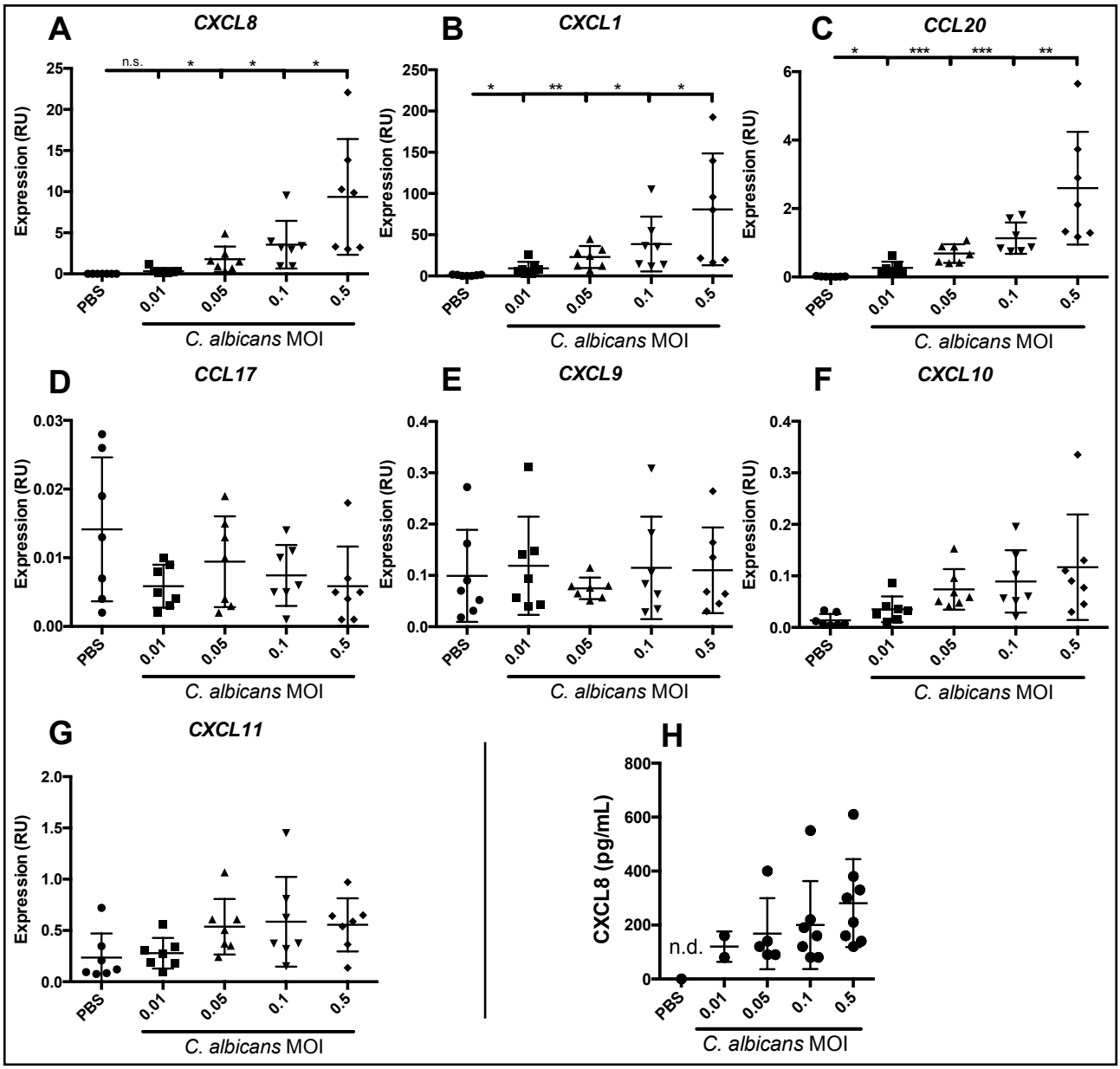

Figure 2. *Candida albicans* induces expression of chemokines by human primary keratinocytes. NHEKs grown in a monolayer to 80% confluence were co-incubated with various MOIs of live *C. albicans* (strain) or PBS for 6 hours. Total cell lysates were collected and qPCR analysis performed on isolated RNA. Expression levels are reported in relative units compared to internal housekeeping control (18S). Dose-dependent upregulation of (**A**) *CXCL8,* (**B**) *CXCL1* and (**C**) *CCL20,* but not of (**D**) *CCL17,* (**E**) *CXCL9,* (**F**) *CXCL10* and (**G**) *CXCL11.* ELISA using supernatants from the same experiments confirms the presence of CXCL8 (**H**) Representative data from 7 individual donors analysed in 3 independent experiments. Significance determined using two-tailed Student's t-test with Welch's correction. N.s. not significant, * $p<0.05$, ** $p<0.001$, *** $p<0.005$, n.d. not detectable.

Keratinocytes are the first cell type to come into contact with *C. albicans* on the skin and form the primary barrier as the most common cell type of the epidermis. Expression of inflammatory cytokines and chemokines by keratinocytes is known to be inducible by both whole *C. albicans* and sterile filtrates [53].

To investigate the host-*Candida* interaction, human primary keratinocytes were grown *in vitro* in the presence or absence of live *C. albicans* for 6 hours. Because chemokines are the first line of response, expression of a panel of chemokines was analysed via real-time qPCR. The expression of neutrophil attractants CXCL8 and CXCL1 as well as $CCR6^+$ T_H17 cell-attracting chemokine CCL20 was induced in a dose-dependent manner after exposure to live *C. albicans* for 6 hours (**Fig. 2A-C**). Expression levels of T_H2 associated chemokine CCL17 remained unaffected by the co-incubation with *C. albicans* (**Fig. 2D**). In the category of T_H1 associated chemokines, neither CXCL9, CXCL10 nor CXCL11 showed any marked upregulation of expression (**Fig. 2E-G**).

An ELISA assay determined that protein levels for CXCL8 were increased in a dose-dependent manner in the supernatants collected after 6 hours of co-culture (**Fig. 2H**).
In summary, *C. albicans* induced T_H17 cell- and neutrophil-attracting chemokines in human primary epidermal keratinocytes.

3.2 Neutrophil extracellular traps

3.2.1 NETosis *in vitro*

As can also be inferred from the chemokine expression pattern upon stimulation with *C. albicans,* neutrophils play an important role in host defense against this opportunistic pathogen. One of the ways neutrophils combat *C. albicans* is by the generation of large structures called extracellular traps (ETs) via the cell death process of ETosis. NETs can

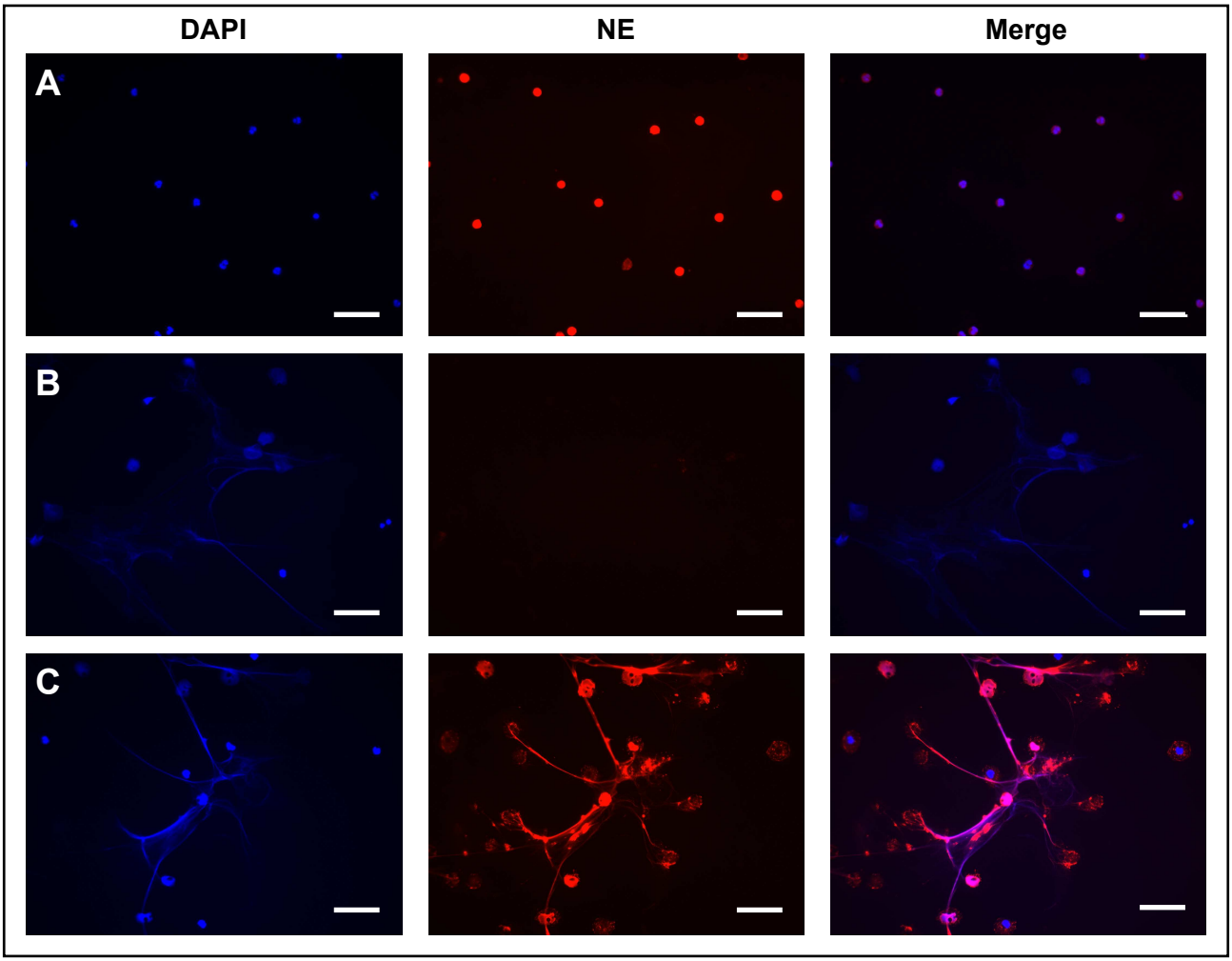

Figure 3. Neutrophil elastase is present on NETs. Fluorescent microscope images of human neutrophils incubated with DMSO (**A**) and PMA (**B, C**) for 3 hours and stained for DNA (blue) and NE (red) using DAPI and anti-NE (monoclonal rabbit anti-human), respectively. **B** this panel shows staining with DAPI and isotype control, **A** and **C** show staining with DAPI and anti-NE. Intact neutrophils stain positive for NE as do NETs generated by PMA stimulation. Images shown are representative of 3 independent experiments using 6 different donors. Scale bar = 40 μm.

be induced *in vitro* simply by the stimulation of isolated neutrophils with PMA, a potent activator of PKA [19]. NETs generated *in vitro* can be visualised with any nuclear stain and stain positive for neutrophil elastase (NE) (**Fig. 3**). Both neutrophil elastase and myeloperoxidase are commonly used as markers to identify NETs [20, 54]. Adherent neutrophils stimulated with PMA respond by generating NETs within 4 hours whereas those exposed to the PMA diluent DMSO do not show any nuclear changes or release of NE (**Fig. 3A**). The presence of NE overlaps specifically with the decondensed chromatin strands of the NETs as well as the cytosol of degranulating cells, whereas in unstimulated cells, NE is closely associated to the nucleus.

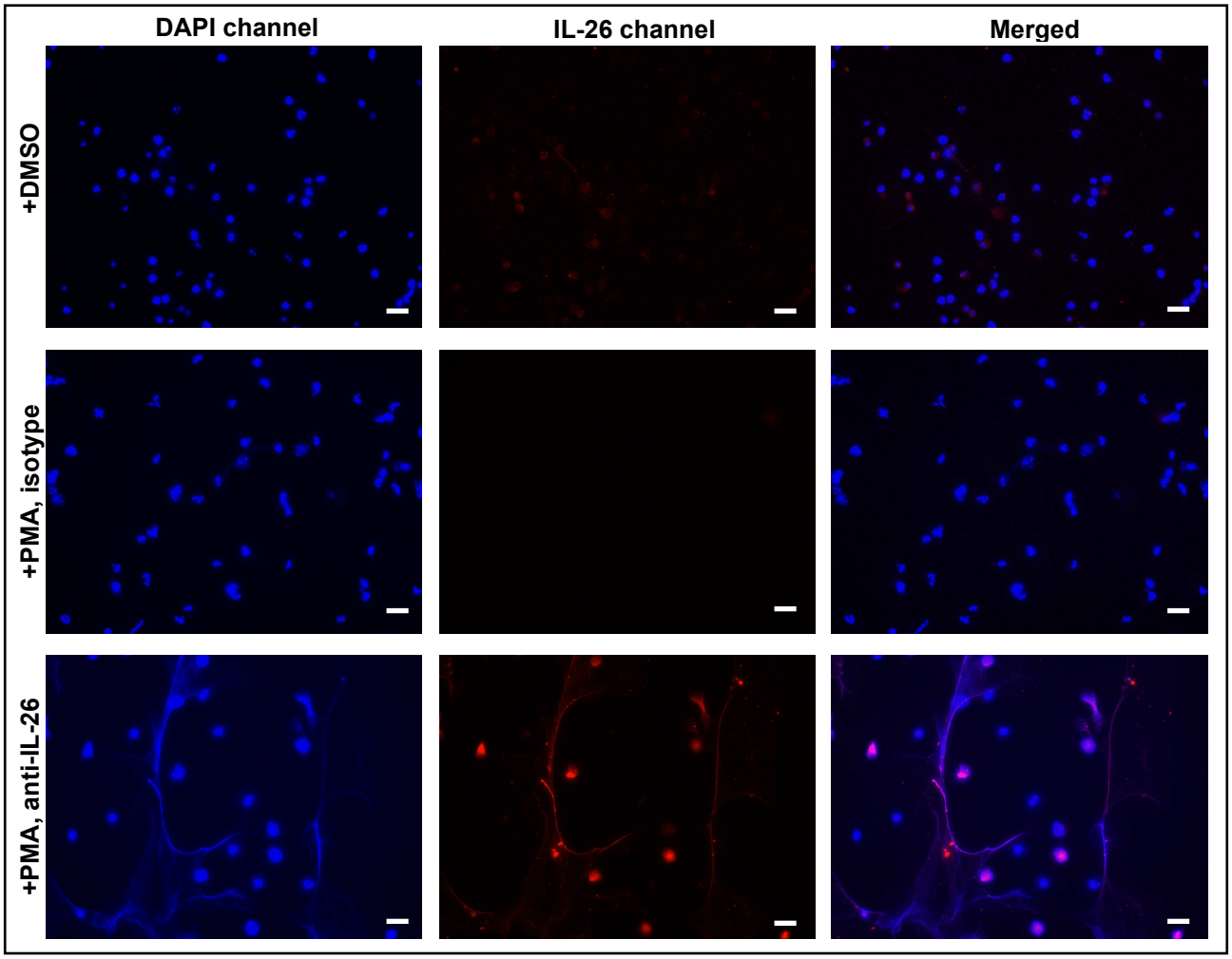

Figure 4 IL-26 is present on NETs Fluorescence microscope images of human neutrophils isolated from whole blood incubated with DMSO (**A**) or 100 ng/mL PMA (**B**, **C**) for 3 hours. Samples were stained for IL-26 using monoclonal antibody (**A**, **C**) or isotype control (**B**) and nuclear material (DAPI) after stimulation and fixation. Images are representative of 3 experiments using different donors. Images were taken using 40x objective. Scale bar = 20 µm.

Small cationic peptides adhere to the anionic chromatin strands, including the cytokine IL-26 (**Fig. 4**). In contrast to NE, which is localised to the nucleus in unstimulated cells, IL-26 is visible in the cytosol of unstimulated cells (**Fig. 4A**). Upon stimulation with PMA and subsequent NETosis, IL-26 is also localised to the chromatin strands.

NETosis can be induced *in vitro* and it was found that NETs form a matrix containing various granular and cytosolic proteins including the cytokine IL-26.

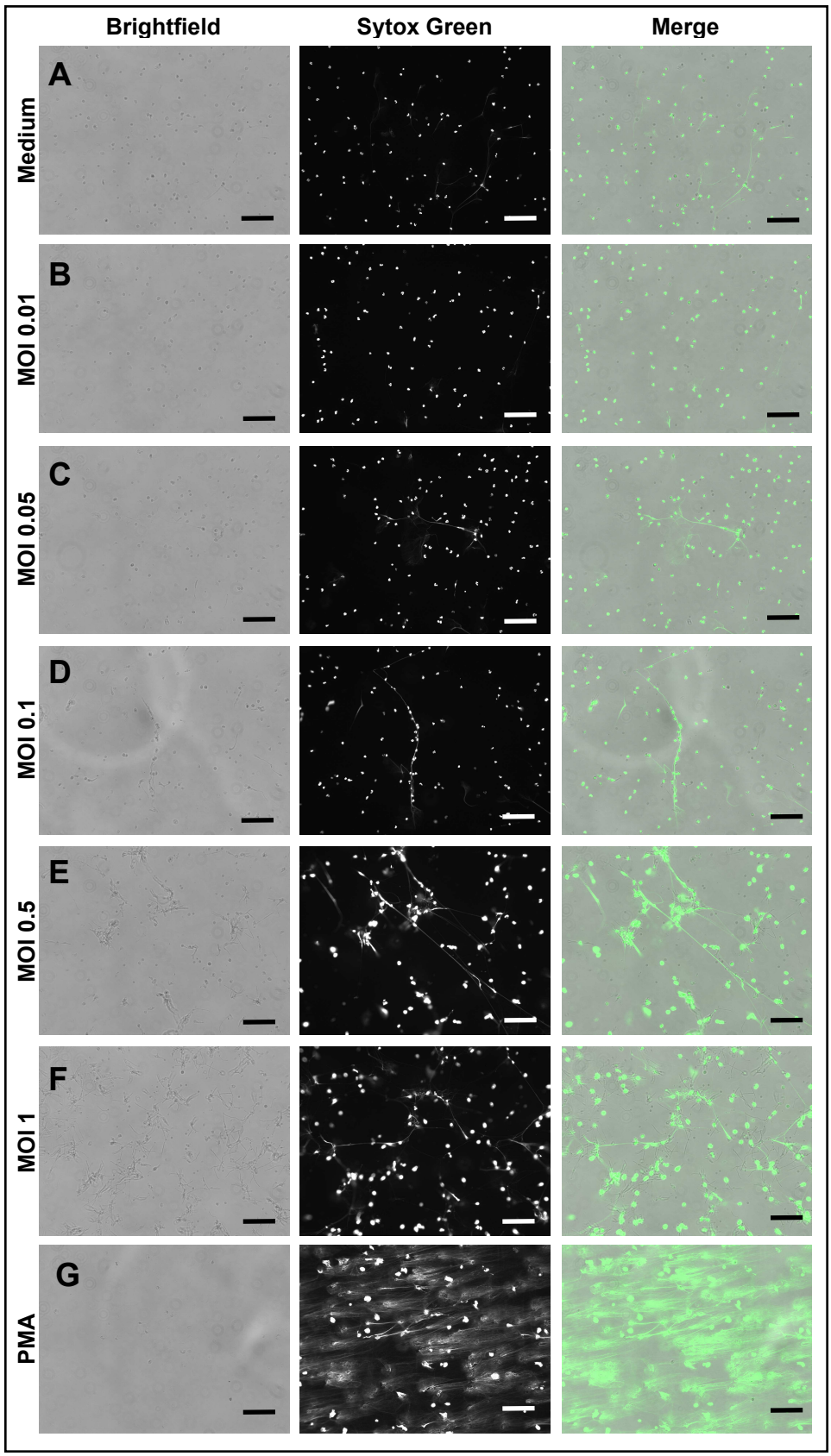

Figure 5. *Candida albicans* induces NETosis in a dose-dependent manner. Brightfield, fluorescence and overlay images of human neutrophils coincubated with Medium (**A**), live *C. albicans* in various concentrations (**B**-**F**), or PMA (**G**) for 3 hours. Images are representative of n=4 independent experiments with 4 different donors. Images were taken using 20x objective. Scale bar = 40µm.

3.2.2 NETosis in response to *C. albicans*

In order to investigate the effect of *C. albicans* in the context of psoriatic inflammation, the response of neutrophils to this yeast had to be clarified. Neutrophils co-incubated with live *C. albicans* for 3 hours undergo NETosis in a dose-dependent manner (**Fig. 5**). Even though the *C. albicans* added to the neutrophils was in yeast form at the start of the experiment, over the 3 hours of co-incubation with the neutrophils in RPMI, pseudohyphae and hyphae were observed.

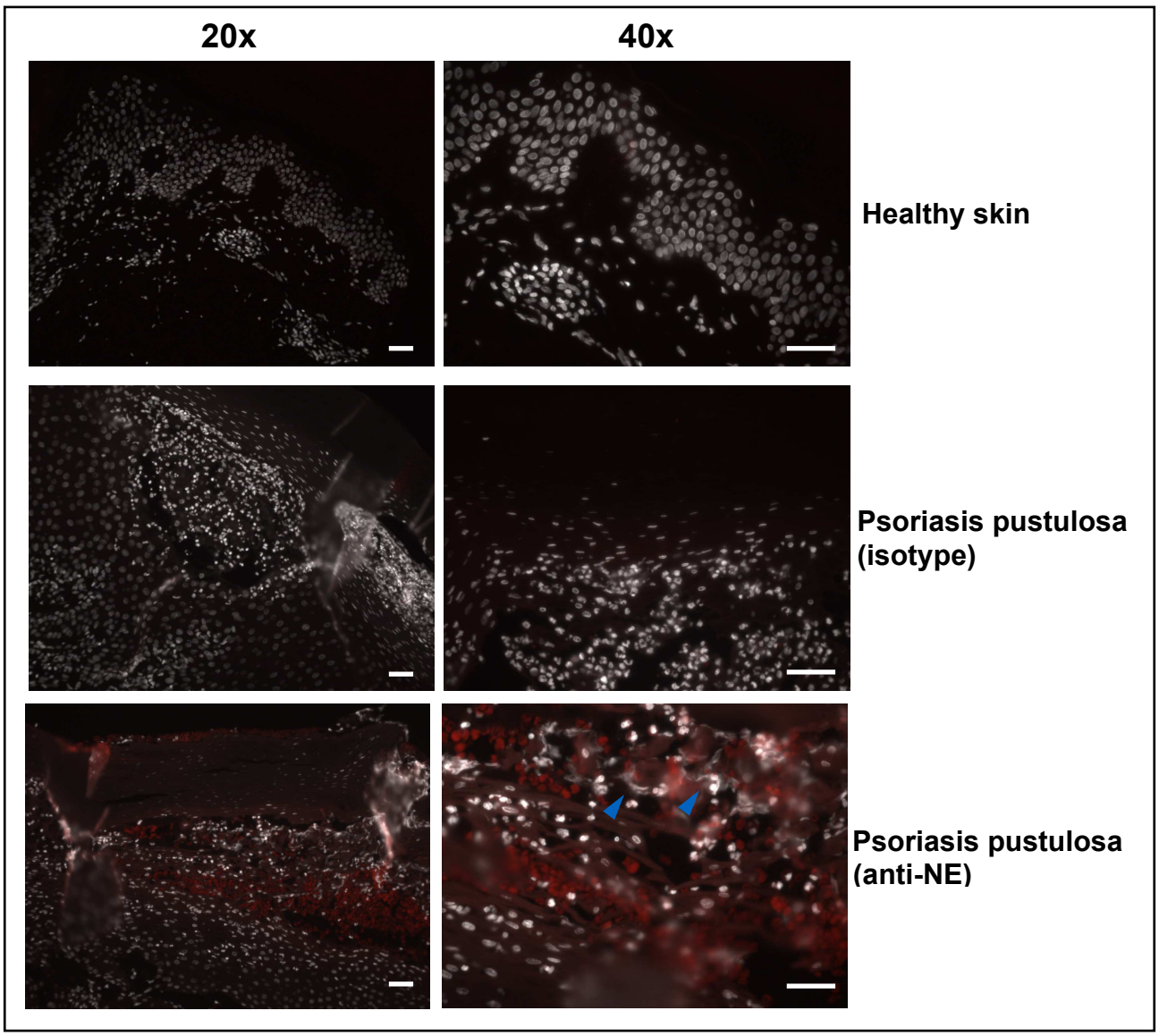

Figure 6. NETs are present in psoriatic skin. Fluorescent microscopy images of skin biopsies of healthy and psoriatic skin stained for DNA with DAPI (white) and with anti-NE (red). Blue arrowheads indicate NETs. Images are representative of n=5 psoriasis pustulosa patients and n=3 healthy volunteers. Images taken with 20x or 40x objective. Scale bar = 40 µm.

3.2.3 NETosis *in vivo*

Massive infiltrates of neutrophilic granulocytes are a hallmark of psoriatic inflammation and especially the pustulosa subtype is characterised by micro-abscesses filled with neutrophils. NETs have already been indicated in other studies to be a source of IL-17 in psoriatic plaques [55]. By employing both a nuclear stain and specific antibody against neutrophil elastase NETs were identified in tissue samples from psoriasis pustulosa patients (**Fig. 6**).

In order to assess whether neutrophils isolated from the peripheral blood of psoriatic patients with an active flare-up of disease are more prone to undergo NETosis in response to both chemical and biological stimuli, two methods of quantification were employed (**Fig. 7**). The first relied on the simple measurement of fluorescence intensity as a result of the expulsion of nuclear material using a cell-impermeable DNA dye after stimulation of isolated neutrophils *in vitro*. The second method involved generating NETs on microscope slides and calculating the surface area of each taken image covered with NETosis as visualised with an extracellular DNA dye. This method allows for the differentiation between necrosis, NETosis and other cell death mechanisms. Neither method indicated any clear difference between neutrophils isolated from the blood of psoriatic patients and healthy controls in the amount of NETs generated. The higher lever of NETosis as measured by fluorescence intensity in healthy controls compared to psoriatic patients in response to PMA (**Fig. 7A**) disappears once measured by percentage coverage (**Fig. 7B**), indicating it may be due to an increase in other cell death mechanisms leading to cell death/permeabilisation.

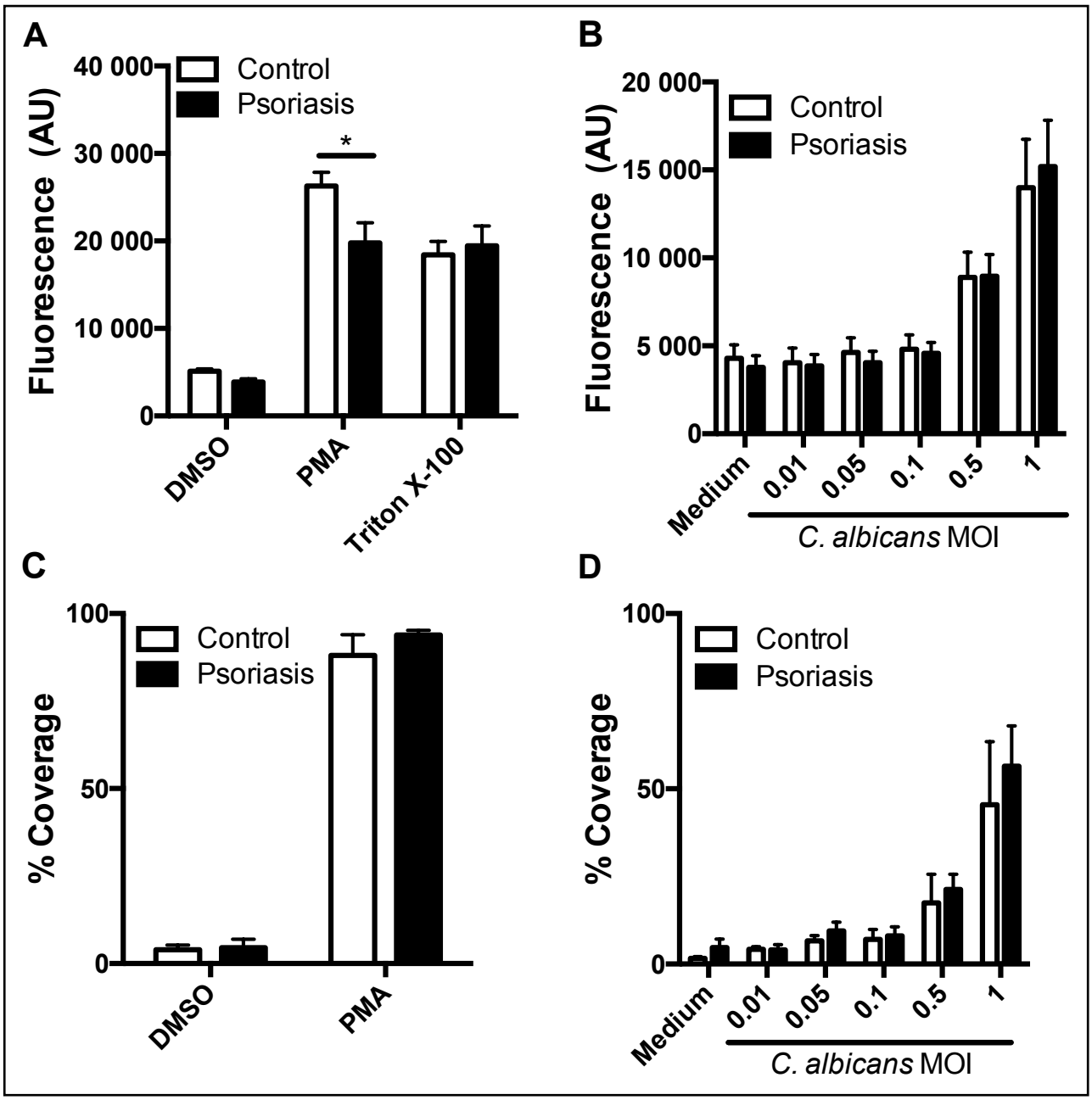

Figure 7. Neutrophils isolated from the blood of psoriasis patients do not generate more NETs than those from healthy controls. NETosis was quantified by measuring fluorescence intensity after SG staining of neutrophils co-incubated for 3 hours with chemical stimuli (**A**) or live *C. albicans* (**B**). Triton X-100 was used as a positive control where all DNA is made available to the SG. DMSO and medium were used as vehicle controls for PMA and *C. albicans,* respectively. Apart from the higher level of NETosis measured after PMA stimulation, no clear differences are visible between healthy controls and psoriasis patients. NETosis was also quantified by measuring the area covered by NET structures on chamber slides as a percentage of the total area after chemical stimulation (**C**), or co-incubation with live *C. albicans* (**D**). This method allows for differentiation between NETosis and other cell death mechanisms and does not show any differences between neutrophils isolated from the blood of healthy controls versus those from psoriasis patients. Fluorescence data is pooled from n=7 independent experiments each analysing 1 psoriasis patient vs 1 healthy volunteer. Surface coverage data is pooled from n=5 experiments each analysing 1 psoriasis donor vs 1 healthy donor. Statistical significance tested with Student's t-test with Welch's correction. * $p<0.05$

Taken together, *in vitro* results and clinical observations of psoriasis patients provide a hint that neutrophils and specifically NETosis form an overlap between host anti-fungal defense and psoriatic inflammation.

3.3 *In vivo* models

3.3.1 Imiquimod model for murine psoriasis-like inflammation

To investigate psoriatic inflammation *in vivo* the imiquimod model was used as previously described [52]. Two chemokines with functions related to host defense against *C. albicans* and psoriatic inflammation are CCL20 and CCL27, ligands for CCR6 and CCR10, respectively. Using *Ccr6*$^{-/-}$ mice in the imiquimod model showed no difference in inflammatory disease markers (**Fig. 8A-C**) compared to wildtype controls as a robust

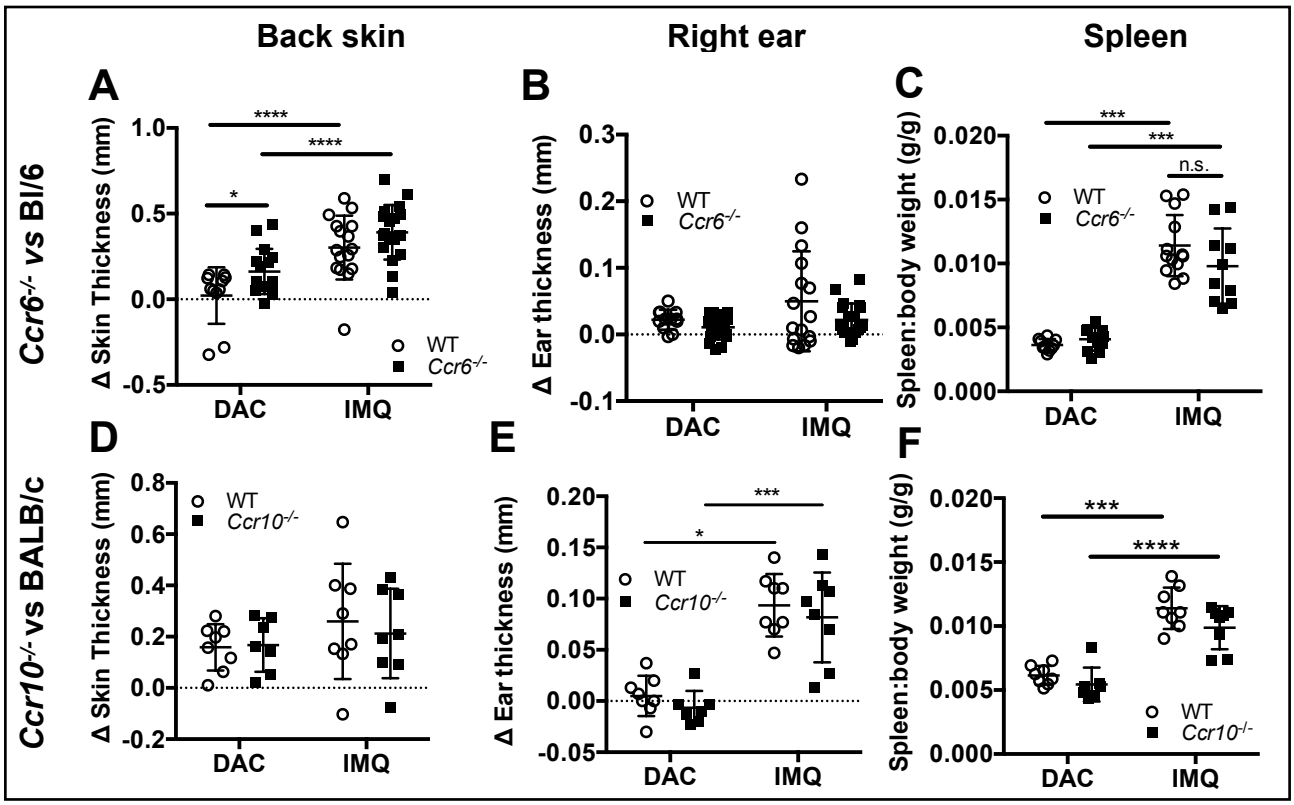

Figure 8. IMQ-mediated psoriasiform inflammation is not affected by a lack of either *Ccr6* or *Ccr10.* Measurements taken of skin (**A, D**) and ear (**B,E**) thickness before and after daily treatment for 5 days with 62.5mg Aldara containing 5% IMQ. DAC was applied as a negative control and in all mouse strains there is an increase in thickness in response to IMQ, but no effect of the chemokine receptor knockouts. Spleen to body weight ratio (**C**, **F**) also indicates systemic inflammation as a result of IMQ treatment, but no differences between knockout strains and WT controls. *Ccr6*$^{-/-}$ data collected from n=4 independent experiments, *Ccr10*$^{-/-}$ data collected from n=3 independent experiments. Statistical significance determined by Student's t-test, witch Welch's correction. N.s. not significant, * p<0.05, ** p<0.001, *** p<0.005.

response was induced in both. Mice lacking the Ccr10 receptor also did not show any difference in inflammation as measured by skin and ear thickness as well as splenomegaly compared to wildtype controls (**Fig. 8D-F**). As a trend, Bl/6 mice respond to imiquimod with increased skin thickening compared to Balb/c wildtype mice, but phenotypically both strains of mice exhibit a clear inflammatory response mimicking psoriasis after 5 days of treatment with imiquimod.

Taken together these results imply that neither Ccr6 nor Ccr10 alone are essential for the development of imiquimod-induced skin inflammation in mice and redundant mechanisms may be at play.

3.3.2 Combined model for *C. albicans* colonisation and psoriasis

In order to more closely simulate the situation of a pre-existing colonisation of *C. albicans* triggering a stronger psoriatic flare-up in predisposed individuals, an adapted imiquimod model was developed. As *C. albicans* is not a natural commensal of mice, it was applied to back skin with a disrupted stratum corneum and kept under occlusion for 72 hours to allow the yeast to establish colonisation but no strong invasive infection. The occasional small lesions that developed after the three day occlusion resolved within two days and within 4 days *C. albicans* could not be detected either on the surface of the skin or within the dermis (data not shown).

Relative splenic mass as an indicator for the systemic effects of imiquimod indicated a higher level of inflammation in pre-treated mice compared to those exposed to PBS under occlusion (**Fig. 9C**).

Although a trend is visible, the live measurements of skin and ear thickness taken by calliper to assess the level of local inflammation did not show any clear indication that pre-

colonisation with *C. albicans* had an effect on the imiquimod-mediated inflammation (**Fig. 9A, B**).

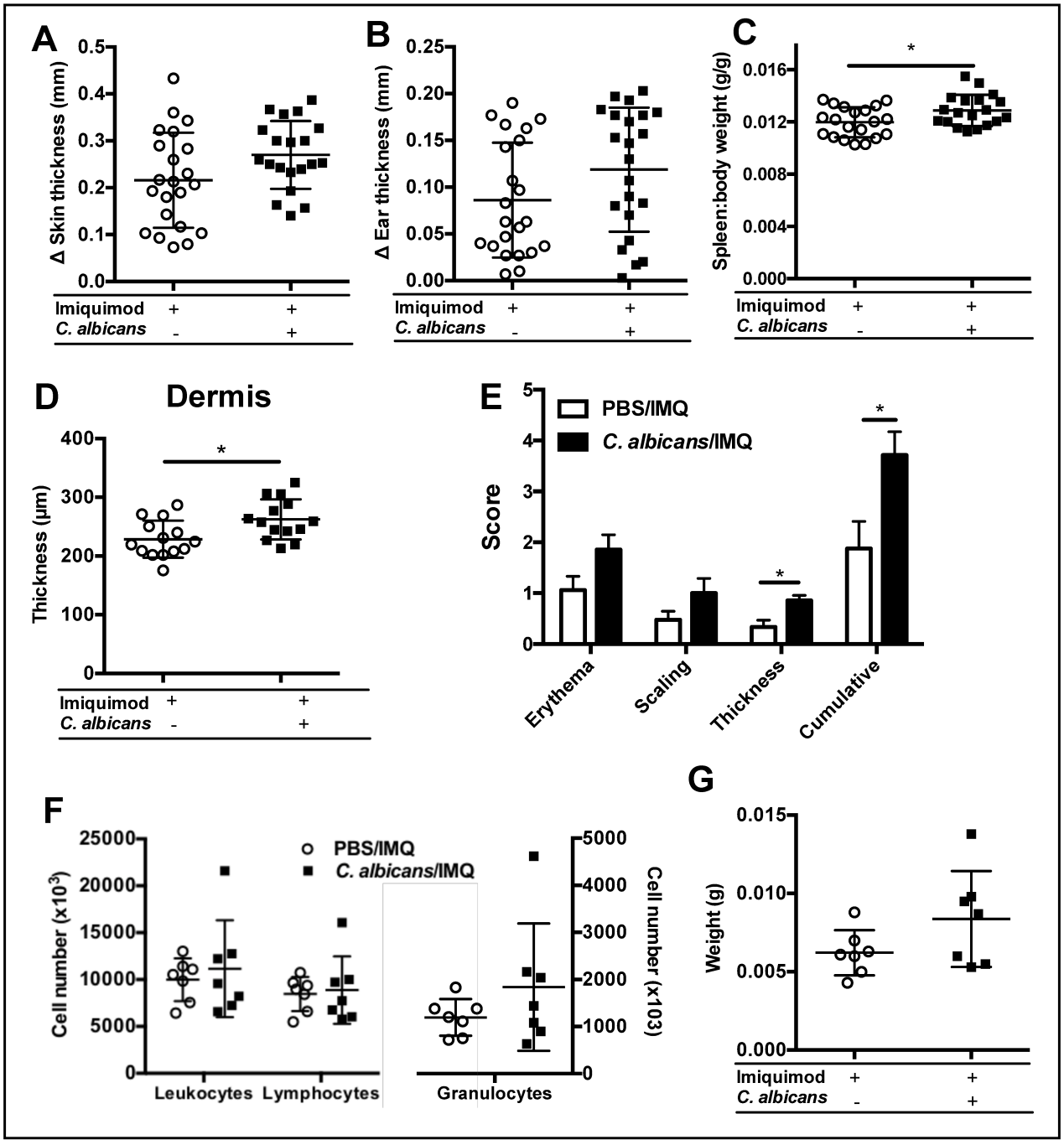

Figure 9. WT mice pre-exposed to *C. albicans* show increased psoriasiform inflammation after IMQ treatment. Measurements taken of skin (**A**) and ear (**B**) show a trend towards increased thickness in Balb/c mice exposed to *C. albicans* for 72h before application of 31.25mg of a 3.75% imiquimod cream daily for 5 days. Splenomegaly as expressed by the ratio of spleen weight to body weight is more pronounced in *C. albicans* pre-treated mice (**C**). Measurements of the dermis taken from formalin-fixed, paraffin embedded sections show an increase in thickness in mice pre-exposed to *C. albicans* (**D**) and the same is seen in clinical scoring on a 4 point scale (**E**). Homogenised axillary lymph nodes were weighed (**G**) and analysed using VetABC full blood count analyser, with cell types expressed as number of cells per lymph node (**F**). **A**-**D** data collected from n=7 independent experiments. **E** data collected from n=3 blinded scorers. **F**,**G** data collected from n=3 independent experiments. Statistical significance determined by Student's t-test, witch Welch's correction * $p<0.05$.

Due to the inherently high level of variance in the skin thickness measurements, micrographs of formalin-fixed, paraffin embedded back skin samples were used to obtain more accurate data on the thickness of both the epidermis and the dermis at the end of the experiment. As shown in **Figure 9D**, the dermis of pre-colonised mice responds to imiquimod by an increased thickness compared to the uncolonised controls. Photographs of the mice on the last day of the experiment were scored by 3 independent blinded assessors on a scale of 1-5 for the level of skin redness, thickness and scaling (**Fig. 9E**), the results of which show a significantly higher index of inflammation for the pre-colonised mice.

The relative cellular composition data of the axillary lymph nodes was inconclusive in determining whether granulocytes or lymphocytes became more enriched in pre-colonised mice (**Fig. 9F**). The trend towards increased numbers of granulocytes in the lymph nodes collected from *C. albicans* treated mice may be skewed by the high level of variance between samples.

The sampled lymph nodes were weighed before cell content analysis and again the trend is towards increased immune activity in the pre-exposed mice compared to the PBS controls (**Fig. 9G**), although statistically these two groups are not significantly different from each other.

Considering the combination model, it was found that both local inflammation as measured by dermal thickness as well as systemic reactions (spleen, lymph nodes) are increased in response to cutaneous pre-colonisation with *C. albicans.*

3.3.3 Cell populations in axillary lymph nodes of mice pre-exposed to *C. albicans* before psoriatic inflammation model

In order to classify the cell types recruited to the site of inflammation, the collected lymph nodes were used in an intracellular cytokine staining protocol. The surface markers CD4, CD8 and CCR6 were used to discriminate between cell populations present in the lymph nodes at the end of the combined *C. albicans* + imiquimod treatment. Together with the cytokine markers IL-17 and IFN-γ, the amount of cytokine-producing cells could be determined for each experimental group. For the purposes of distinguishing the effects of the applied *C. albicans* over time, one experimental group underwent a truncated experiment, being sacrificed immediately after removal of the occlusive dressing (“0d”), another remained untreated after *C. albicans* or PBS inoculation (“5d rest”), while the third went through the standard experimental procedure as described above (“5d IMQ”).

The gating strategies applied to all samples are exemplified in **Fig. 10A-D**, including the gate demarcating the counting beads used to obtain absolute cell numbers (**Fig. 10A**, upper left) per two lymph nodes. Gates were placed so that they contained less than 0.1% specific marker positive cells in negative control samples to exclude false positive signals.

The number of IFN-γ producing cells is slightly elevated in *C. albicans* exposed mice compared to PBS controls immediately after the 3 days of inoculation. This elevation is reduced after 5 days of rest (**Fig. 10F**) when the *C. albicans* colonisation has resolved. This shows a transient recruitment of pro-inflammatory cytokine-releasing cells being recruited to the draining lymph node closest to the site of insult. When mice were treated with *C. albicans* for 3 days and imiquimod for 5 days, the level of IFN-γ producing cells was once again significantly elevated compared to the PBS controls; to a significantly greater degree than seen immediately after exposure to *C. albicans.* More relevant to

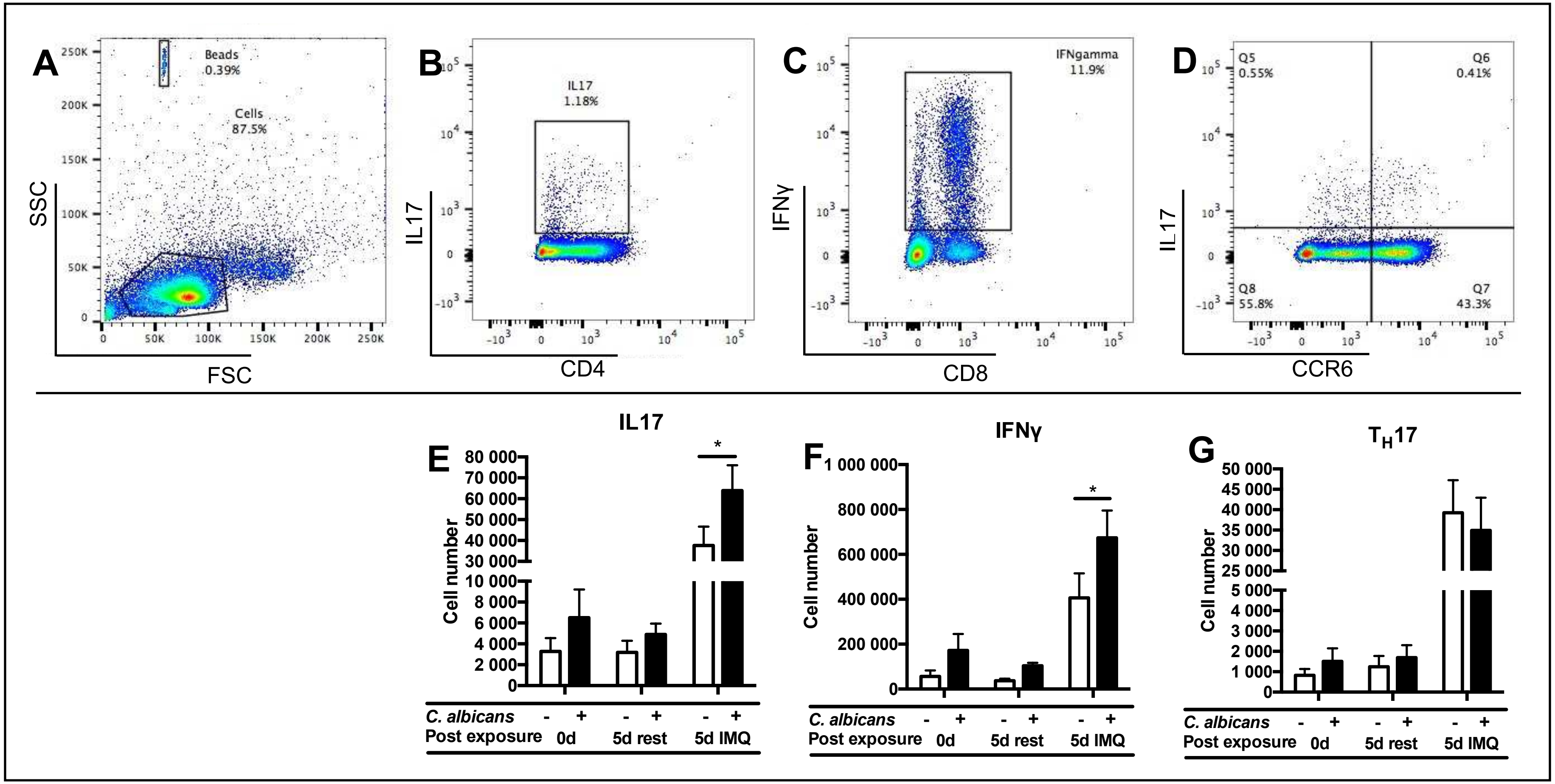

Figure 10. Intracellular cytokine staining of homogenised lymph nodes from mice pre-exposed to *C. albicans* before IMQ treatment. Gating strategies employed to identify cells (**A**), from which IL17$^+$ (**B**), IFNγ$^+$ (**C**) and T_H17 (**D**) populations were characterised. Absolute numbers of cells in those populations after each experiment show both IL17 and IFN-γ producing cell populations are expanded in *C. albicans* pre exposed mice (black bars) compared to PBS controls (white bars) (**E**, **F**) . In contrast, T_H17 cells (characterised as CCR6$^+$IL17$^+$) are not increased when pre exposed to *C. albicans* (**G**). (**A**-**D**) representative data from n=3 independent experiments. (**E**-**G**) pooled data collected from n=3 independent experiments for each post-exposure condition. Statistical significance determined by Student's t-test, witch Welch's correction * $p<0.05$.

psoriasiform inflammation specifically, the number of IL-17 producing cells was also significantly increased in the draining lymph nodes of the experimental group pre-exposed to *C. albicans* (**Fig. 10E**).

An obvious candidate for the source of the heightened IL-17 is the T_H17 ($CCR6^+/IL17^+$) subset. Although the application of IMQ for 5 days did lead to an obviously raised number of T_H17 cells in the axillary lymph nodes, no clear difference could be detected between the PBS- and *Candida*-treated groups (**Fig. 10G**).

In summary, while the data shows there is an increase in IL-17 producing cells as a result of priming by *C. albicans* in the IMQ model for psoriasis, there is no concordant increase in the number of $CCR6^+$ cells, meaning another subtype of IL-17 producing cells is selectively expanded under these circumstances.

4 Discussion

4.1 Host-pathogen interactions

4.1.1 Keratinocyte response to *C. albicans*

The chemokine expression pattern observed after co-culture of human keratinocytes with *C. albicans* (**Fig. 2**) corresponds to previously published findings by Tani et al. [56]. Recognition of *C. albicans* by keratinocytes is often mediated by TLR2 interacting with phospholipomannans (PLM) [57] coating the cell walls of *C. albicans* in its filamentous form. The relevance of various PRRs known to respond to *C. albicans* in human cutaneous colonisation and infection has long been a matter for debate. In addition to TLR2, keratinocytes also express other PRRs capable of recognising *C. albicans* including c-type lectin receptor Dectin-1 [58]. However, Dectin-1 is likely to be of less relative importance in this scenario as the β-glucan it recognises is masked when *C. albicans* grows in filamentous form [10].

The clear, dose-dependent increase in expression of CXCL8, CCL20 and CXCL1 underlines the importance of the attracted cell types in the defense against *C. albicans.* $CXCR1^+$ neutrophils in particular shape the host response to a great extent [59] through degranulation, release of pro-inflammatory cytokines and NETosis [20].

Sterile filtrates of various *C. albicans* subtypes can induce cytokine release by keratinocytes including CXCL8 [53] as soon as 6 hours after stimulation. In addition to this, intact yeast and filamentous form *C. albicans* as well as sterile filtrates can trigger a cytokine response in keratinocytes [53, 60]. The other tested cytokines are also known to be expressed by activated keratinocytes after stimulation [61].

Expression assays have revealed that CCL20 is constitutively expressed at low levels in a wide range of tissues and organs, including barrier organs such as the intestine and lungs as well as the skin [42].

In the context of skin immunity it is known that CCL20 expression is elevated in psoriasis [62], eczema [63] and mycosis fungoides [64] and that it has antimicrobial functions at higher concentrations [65]. Although Hoover et al. could initially find no evidence of any significant microbicidal activity of CCL20 against *C. albicans* [66], subsequent research has shown that CCL20 is in fact capable of killing *C. albicans*, albeit only at high local concentrations [65]. As it appears that CCL20 may have a different species specificity in effective antimicrobial action that does not include *C. albicans,* its chemotactic function in recruiting $CCR6^+$ cells plays a more dominant role in this scenario.

Another chemokine known to be expressed by keratinocytes in response to fungal stimuli is CXCL1, previously known as keratinocyte chemoattractant [56]. Canonically inducible by IL-17 and TNF-α, this chemokine recruits neutrophils via CXCR2 signalling and promotes keratinocyte proliferation and wound healing [67]. The level of CXCL1 produced by epithelial cells in response to live *C. albicans* hyphae is used as a virulence marker for various strains of the fungus [68]. The clear upregulation of expression of CXCL1 by keratinocytes after exposure to live *C. albicans* seen in **Fig. 2B** confirms these previously published results.

The other tested chemokines showed no significant response to stimulation of keratinocytes with *C. albicans,* which corresponded to previous findings [69]. CCL17 for instance is known to play a role in several skin conditions including atopic eczema, contact dermatitis, hypersensitivity reactions and cutaneous T cell lymphoma [70] and recruits

CCR4^{+} T_H2 cells to the skin. Very little is known about any role CCL17 may play in host responses to fungal infection, with most of the connections in the literature being in relation to allergic reactions to fungal species [71].

CXCL9, CXCL10 and CXCL11 are closely related chemokines with anti-microbial functions inducible by IFNγ in keratinocytes, recruiting CXCR3^{+} T_H1 and natural killer cells. In addition to the canonical IFN-γ mediated pathway, all three of these chemokines were found to be over-expressed in keratinocytes stimulated with either T_H22 supernatant or a mixture of IL-22 and TNF-α [72]. Under these conditions keratinocytes could effectively inhibit the growth of *C. albicans* and maintain barrier function of a 3D skin model [72]. Our results suggest that even though they have anti-*Candida* functions, IFN-γ or T_H22 cytokine-mediated activation is a prerequisite for CXCL9, CXCL10, and CXCL11 expression by keratinocytes and the mere presence of *C. albicans* is not enough to induce the upregulation of expression of these chemokines.

Primary human keratinocytes grown in a monolayer *in vitro* provide an initial insight into the capacity of the cell type to respond to a particular stimulus. However, without the more complex context in which keratinocytes find themselves *in vivo,* results cannot be simply extrapolated without confirming them in more accurate models.

4.1.2 NETosis in anti-fungal defense

Neutrophils *in vitro* do not readily attach themselves to surfaces, which is why poly-L-lysine was used to promote adherence for the NETosis assays. Poly-L-lysine itself does not induce NETosis, whereas fibronectin in combination with β-glucan may be a stimulus to an alternative NETosis pathway [73]. Previous research indicates that NETosis can occur in a relatively short timeframe [19, 73], which is why the 4 hour time-point was chosen for the

NETosis assays. Each assay was performed using neutrophils isolated from blood collected at most 3 hours previously, as neutrophils do not survive for long *ex vivo.* NETosis as a host defense mechanism is especially effective against pathogens too large to be phagocytosed [20]. It is likely that the AMPs and granular contents immobilised on the chromatin strands diffuse much more slowly than if they were released, maintaining a high local concentration. As shown previously by Lin et al. and in this thesis, under “sterile” conditions NETs are not only decorated with cytokines and AMPs but also with chemokines, possibly providing a longer-lasting chemotactic gradient for recruited cell types. This supports the finding of the present study that the IL-10 family member and cationic protein IL-26 is present on NETs. IL-26 has both an antimicrobial function as a pore-former in extracellular bacteria as well as an indirect role in immune cell recruitment as an enhancer of CXCL8 expression.

Experiments comparing different pathogenic species of *Candida* show neutrophils are more likely to respond with NETosis to *C. albicans* [74, 75]. We were able to reproduce previous research showing that even *in vitro* human neutrophils undergo NETosis in response to *C. albicans* [20, 75]. There is a clear dose-dependent response to *C. albicans* as can be seen by the different concentrations applied. From MOI 0.1 and upwards neutrophils underwent NETosis to an appreciably higher level than the negative control as seen by the distribution of Sytox Green staining.

Not only *in vitro* do neutrophils react to fungal species with NETosis, this has also been shown to occur *in vivo*. Bruns et al. were able to show NETs overlapping with hyphal structures in lung tissue of *Aspergillus fumigatus* infected mice [76]. Although NETs are an effective host mechanism for killing fungi, it does depend to a great extent on the neutrophil’s ability to recognise specific surface markers and then for the calprotectin and

other antimicrobial peptides on the chromatin strands to reach the pathogen. Recognition of *C. albicans* hyphae by neutrophils is mediated through recognition of β-glucan by complement receptor 3 (CR3) as previously described by Lavigne et al. [77]. Although Dectin-1 is also expressed by human neutrophils, its role in recognition of β-glucan is less clear cut [78]. The surface antigens on the yeast form of the dimorphic fungus recognised by the host immune system are slightly different [10], with Dectin-1 and TLR2 playing a bigger role, though both morphotypes can induce NETosis [20]. *C. albicans* can mask PAMPs by forming biofilms *in vivo,* especially the hard surfaces of dental prostheses, urinary catheters and voice prostheses are often affected [79]. These biofilms are especially resistant to host clearance, because of the thick layer of mannosylated extracellular matrix components covering the pathogens [80]. In the context of fungal skin infections biofilms are not often a concern, although neutrophil infiltrates do occur as a response to invasive fungi [81].

4.1.3 NETosis in psoriasis

One of the defining characteristics of psoriasis is the presence of Munro's microabscesses, which are filled with neutrophils. The high levels of CXCL1, CXCL2 and CXCL8 in lesional psoriatic skin account for the high numbers of neutrophils present at those sites, although there are reports that neutrophils may be absent in some chronic lesions [34]. Neutrophils have also been found to be a source for both IL-17 and DNA-AMP complexes capable of activating pDCs as a consequence of the process of NETosis [82].

Since the first description of NETs by Brinkmann et al., their clinical relevance has been investigated for a large number of inflammatory and auto-immune conditions, including rheumatoid arthritis [83] and systemic lupus erythematosus [84]. With regards to psoriasis, the clinical role of NETosis is slowly gaining more interest. Recently, Hu et al. were able to

show that serum from psoriasis patients was capable of inducing NETosis in neutrophils isolated from healthy controls and that neutrophils from psoriasis patients induce the production by keratinocytes of the antimicrobial peptide human β-defensin 2 (HBD-2) [85]. HBD-2 is known to be strongly overexpressed in the epidermis of psoriasis patients, though the molecular trigger for this upregulation was not clear before [86]. Hu et al. were also able to demonstrate that a higher number of neutrophils undergo NETosis without any additional stimulus after isolation from the peripheral blood of psoriasis patients compared to healthy volunteers. This was shown by manually counting the number of NETs and number of non-NETting cells per microscopic field [85].

Two different methods for the quantification of NETosis between healthy controls and psoriasis patients are shown in this thesis. Neither Sytox Green intensity of NETosis occurring in a 96-well plate, nor surface area covered by NETs on a chamberslide showed any significant increase in NETosis by neutrophils isolated from the whole blood of psoriasis patients compared to healthy controls. The first assay measuring Sytox Green fluorescence can not differentiate between NETosis and other cell death mechanisms such as necroptosis and apoptosis. Despite this, in combination with the microscopy observations that *C. albicans* can induce NETs it can give a good indication of the level of NETosis *in vitro*. The second method is dependent on manual observation of the NETs on a microscope slide. This allows for the exclusion of any possible false positive signals due to the unspecific nature of the Sytox Green staining. However, this method is much more labour-intensive and time-consuming. Methods for the quantification of NETosis published previously include both manual and automated systems. Hu et al. manually counted the number of NETs per total cell number in random fields of microscopy slides [85], whereas an automated method based on the same principles was used by Brinkmann et al. [87].

NETosis quantification based on extracellular DNA dye fluorescence intensity without microscopic visualisation has been described previously in the literature [88, 89].
In order to determine whether NETs could also be seen *in vivo* in psoriatic skin, frozen sections from psoriasis pustulosa patients and healthy controls were stained for DNA and neutrophil elastase. Large amounts of neutrophilic infiltrate could be detected in the patient's samples. Under greater magnification distorted nuclei were seen to overlap with neutrophil elastase staining, indicating the presence of NETs.

NETs are delicate structures by nature, consisting of decondensed chromatin, which makes identifying them *in vivo* challenging. In order to be certain a DNA structure is a NET in the context of fixed tissue, other markers known to be present on NETs such as neutrophil elastase, MPO or citrullinated histones [55, 90] have to be stained for an overlap.

In summary, neutrophils are known to play an important role in psoriatic inflammation and neutrophils isolated from the peripheral blood of patients with psoriasis undergo NETosis to a higher degree without any additional stimulation [85]; although those last results could not be convincingly replicated with our methods.

4.2 Chemokine responses in psoriasis

4.2.1 IMQ model

Originally developed as a nucleoside analog capable of inducing a strong interferon response to treat superficial skin cancers, actinic keratosis and human papilloma virus (HPV) infections, imiquimod was soon reported to induce flare-ups of disease in psoriasis-prone patients using the medication/cream/compound. From this starting point, the murine

model for psoriasis was developed after it was found to reliably induce skin inflammation in mice when applied to the skin daily [52].

The mechanism of imiquimod-induced skin inflammation involves several different cellular pathways; the relative importance of which is still a matter for investigation [91, 92]. The canonical interaction partner for IMQ is TLR7. The effective activation of TLR7 leads to the transcription of pro-inflammatory cytokines via transcription factors Myd88 and NF-κB. In addition to this, IMQ's structure as an adenosine analog allows the blocking of adenosine receptors, leading to decreased levels of the anti-inflammatory mediator cAMP [93]. Regardless of the precise pathways activated, the resulting inflammation after daily application of IMQ on murine skin closely mimics psoriasis [52] and variations of this model have been used by numerous researchers in the years since its development [94, 95].

In order to investigate the roles of chemokine receptors CCR6 and CCR10 in the context psoriasiform inflammation, we established a slight variation of the model as first described by van der Fits et al. [52] in both Balb/c and C57Bl/6 wildtype strains as well as Ccr6- and Ccr10- knockout strains. CCR6 ligand CCL20 is constitutively expressed by keratinocytes and markedly upregulated during inflammation in order to recruit $CCR6^+$ T_H17 and $CD4^+$ memory T cells.

Choosing the same metrics for local and systemic inflammation used in the original paper describing the model, we were not able to see any clear differences between the wildtype and knockout mice. A slight trend can be seen in both cases towards a reduction of inflammation in the chemokine receptor knockout mice, but nothing pointing more clearly at an essential or even important role for those receptors in the pathomechanism of IMQ-

induced psoriatic inflammation. These findings are supported by research from Cochez et al. who found that the lack of Ccr6 did not reduce the overall production of IL-22 in the skin, even though most IL-22 producing cells are $CCR6^+$ [96]. An alternative mouse model for psoriasis commonly used is the intradermal injection of IL-23. Previous publications have shown that in this model, CCR6 is essential for the development of psoriatic inflammation as $Ccr6^{-/-}$ mice subjected to IL-23 injection did not develop any plaques or inflammation [97, 98].

Ccr10 deficiency did not lead to any clear reduction in inflammation following the application of IMQ in the psoriasis model. Although keratinocytes express much higher levels of CCR10 ligand CCL27 in psoriatic lesions [62], *$Ccr10^{-/-}$* mice still responded to IMQ by developing cutaneous inflammation and acanthosis to the same degree as wildtype controls. Human studies into CCL27-CCR10 interactions pointed towards a role in the skin-specific recruitment of CLA^+ memory T cells in the skin under inflammatory conditions [99]. The absence of CCR10 does not appear to make a difference in the murine IMQ model, indicating either a redundant system for the recruitment of T cells or differences in the relative importance of CCR10 in the murine immune system compared to the human one in the context of IMQ-mediated inflammation.

Although we were not able to show any significant role for either CCR6 or CCR10 in the murine IMQ model, some changes to the experimental protocol could improve its accuracy. For example, use of digital callipers at precisely defined anatomical locations marked clearly on the ear and back could reduce variation in the measurements. Measurements taken of ears and skin as first indications of inflammation tend to be inherently imprecise to the degree to which the callipers were accurate. Also if the measurements are not taken by the same person at the exact same anatomical location,

more variation arises. This was somewhat mitigated by taking 3 separate measurements and taking the average value at each time-point as part of the protocol. Due to time constraints, we were forced to use wildtype controls from the same general breeding stock. Littermate controls generated from a heterozygote-heterozygote breeding pattern would be a much closer genetic match to the knockout mice and may show a lower intra-individual variance.

4.2.2 Combination model

Establishing the murine psoriasis model was only the first step in the investigation of whether the presence of *C. albicans* is able to exacerbate psoriatic inflammation. The second step was to develop a protocol by which *C. albicans* colonises murine skin without establishing an invasive growth pattern before inducing psoriatic inflammation via IMQ. Since *C. albicans* is not normally a murine commensal, the skin was prepared beforehand by tape-stripping, thereby removing the stratum corneum and thus facilitating the colonisation by *C. albicans*.

Using the same metrics for local and systemic inflammation as described by van der Fits et al. in the IMQ model as well as other research using the imiquimod model [94, 100], here differences could be shown between mice pre-treated with topical *C. albicans* and those treated with PBS. In addition to the calliper measurements of skin thickness taken during the course of the experiment, formalin-fixed paraffin-embedded histological skin sections were used to measure skin thickness. Those measurements showed that the dermal compartment of *C. albicans* + imiquimod treated mice became enlarged compared to the control animals. This also corresponds to the scoring results where both the thickness and cumulative scores were higher for the *C. albicans* + imiquimod group. In addition to this, splenomegaly was also observed to be present to a greater degree in the

pre-colonised mice compared to the healthy controls, giving an indication that not only local inflammation was exacerbated.

These effects are not a result of any enduring presence of *C. albicans* on or in the skin, as both surface cultures and PAS stains of histology sections of the back skin taken at a range of time points demonstrate the disappearance of *C. albicans* a maximum of 3 days after the removal of the dressing (data not shown). Any small lesions present at the time of dressing removal likewise resolve well within the 5 days of IMQ treatment following the exposure to *C. albicans* (data not shown). Furthermore, previous research employing a similar cutaneous colonisation model for *C. albicans* confirms this timeline of *C. albicans* clearance [101].

To further clarify the mechanism by which pre-exposure to *C. albicans* appears to exacerbate psoriatic inflammation, axillary lymph nodes were subjected to two different methods of investigation. Firstly, homogenised lymph nodes were examined by a full blood count analyser to type the distribution of the various types of lymphocytes present in each experimental group. As shown in **Figure 10**, this approach gave no clear answers beyond a trend towards increased numbers of granulocytes to be present in the *C. albicans* + imiquimod group. Considering the important role that neutrophils play in both psoriatic inflammation and the host response against *C. albicans* this increase was not entirely unexpected.

The second investigation performed on the lymph nodes was to test via intracellular cytokine staining how many cells positive for IFN-γ, IL-17, and various surface markers were present. Due to the nature of this assay, only the number of cells capable of producing a certain cytokine can be determined, rather than the specific level of cytokine

production at any given time. The level of IL-17 producing cells was clearly higher in the *C. albicans* group after 5 days of IMQ, but diminished after 5 days of rest. This indicates that the increase is specific to the synergistic effect of *C. albicans* in combination with IMQ inducing psoriatic inflammation. Keeping in mind that IL-17 signalling is a driving factor in the pathogenesis of psoriasis [82] and therapies targeting this pathway have been found effective [102], this result supports the hypothesis that pre-exposure to *C. albicans* may exacerbate psoriatic inflammation. An initial attempt to identify the cellular source of the IL-17 revealed that IL-17 producing $CCR6^+$ cells are not likewise increased, potentially indicating an alternative source. Although the role of IL-17 in both psoriasis and host defence against *C. albicans* is well described [44, 103] its precise cellular origin appears more complex than initially assumed [104]. After the first descriptions of T_H17 cells as a distinct subtype and their subsequent identification in psoriatic lesions [105], it was considered logical that these cells were the main source of the increased levels of IL-17 in psoriasis. However, more recent results indicated a more dominant role for neutrophils, mast cells [55], γδ T cells [106] and innate lymphoid cells type 3 [30]. CCR6-negative T cells sub-populations or terminally differentiated dendritic cells may be an additional source for IL-17 and may be selectively expanded in response to IMQ after pre-exposure to *C. albicans.* Recent studies in the role of CCR6 in the IMQ model for murine psoriasis also show that while $CCR6^+$ cells are essential for the epidermal expression of IL-22, but not for the local production of IL-17 [96].

To provide a clear overview of both the hypothesis and the elements thereof that are supported by the results discussed above, **Figure 11** was created. It shows some of the immune components involved in *C. albicans* infection (left side) and how they could be a trigger towards psoriatic inflammation (right side).

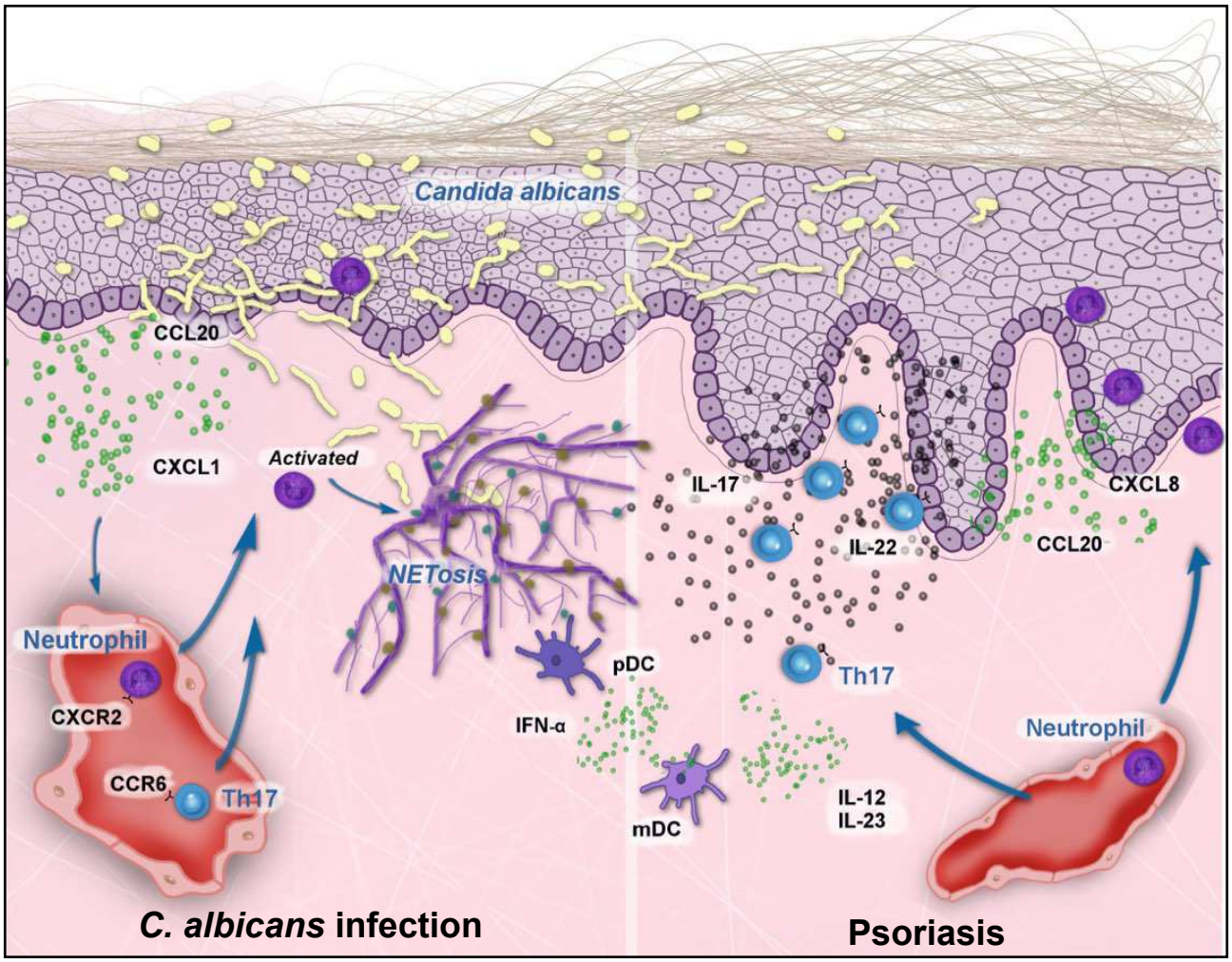

Figure 11. Model outlining the overlapping elements in the immune response to *C. albicans* and during psoriatic inflammation After recognition of *C. albicans* by epidermal keratinocytes, the released chemokines recruit both neutrophils and CCR6⁺ T cells. Upon contact with *C. albicans* neutrophils undergo NETosis, providing a high local concentration of antimicrobial peptides and cytokines bound to DNA. These complexes can activate pDCs and subsequently mDCs into releasing IL-12 and IL-23, providing the basis for psoriatic inflammation. At the same time, high levels of released IL-17 from both neutrophils and T cells act to perpetuate the inflammatory loop that is the basis of the pathomechanism of psoriasis.

Keratinocytes exposed to *C. albicans* release high levels of chemokines including CXCL8, CCL20 and CXCL1 which act to recruit both neutrophils and CCR6⁺ T_H17 cells to the site of insult. Subsequently, *C. albicans* is capable of activating neutrophils into releasing granular contents containing both antimicrobial peptides and a potent mix of cytokines via NETosis. In the context of psoriasis, these same NETs are an important source of the IL-17 that drives the inflammatory feedback loop at the basis of the disease [55]. In addition to this, NETs are also known to be a source of DNA-AMP complexes capable of stimulating pDCs into releasing IFNα [21], which in turn leads to mDC activation and release of IL-23,

a potent T_H17 cell activator [82]. Analysis of the draining lymph nodes in a mouse model for psoriasis preceded by short term cutaneous exposure to *C. albicans* showed an increase in IL-17 producing cells. This model was designed to mimic the conditions in humans whereby the presence (even temporary) of *C. albicans* on the skin may exacerbate psoriasis flare-ups in predisposed individuals.

Although these results provide evidence towards a role for *C. albicans* as a possible trigger or exacerbating factor during psoriatic inflammation, more research is necessary to fully clarify the extent to which it is active.

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8 Curriculum Vitae

Name: Flora Smit

Born: 19/07/1989 in Rotterdam, The Netherlands

Education

5/2013 — 11/2016	Doctoral student in the “Molecules of Infection II” graduate school at the Heinrich-Heine-Universität Düsseldorf, Klinik für Dermatologie, Forschungslabor für Dermato-Immunologie und Onkologie, funded by the Jürgen Manchot Stiftung Supervisor: Prof. Dr. Bernhard Homey Subject: Role of *C. albicans* in promoting psoriatic inflammation
10/2012 — 4/2013	Short term exchange student at the Okinawa Institute for Science and Technology, G0 Research Group. Supervisor: Prof. M. Yanagida Subject: Oxygen metabolism in a mutant library of *S. pombe*
9/2011 — 9/2012	Masters student in Biomedical Sciences at the University of Glasgow, College of Medical, Veterinary & Life Sciences Supervisors: Prof. G. Ramage / Prof. R. J. S. Burchmore Subjects: Effect of IL-17C on *C. albicans* biofilms / optimisation of 2-dimensional gel electrophoresis protocol for the use with human milk Degree obtained: MRes Biomedical Sciences
9/2007 — 5/2011	Bachelor student in Biomedical Sciences at the University of Abertay Dundee, School of Science, Engineering and Technology

	Supervisor: Prof. D. Lester
	Subject: G-protein coupled receptor internalisation and recycling
	Degree obtained: BSc (Hons) First class
9/2001 — 6/2007	Secondary education at the “Rotterdams Montessori Lyceum” with Nature and Health Profile
	Degree obtained: VWO (Gymnasium) Diploma

9 Publications

None.

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11 Declaration

Ich versichere an Eides Statt, dass die Dissertation von mir selbständig und ohne unzulässige fremde Hilfe unter Beachtung der „Grundsätze zur Sicherung guter wissenschaftlicher Praxis an der Heinrich-Heine-Universität Düsseldorf“ erstellt worden ist.