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The role of tyrosine kinase 2 (TYK2) in the innate immune defence against cutaneous candidiasis

Master Thesis

University of Veterinary Medicine, Vienna

submitted by

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Table of content

1 INTRODUCTION 3

1.1 Candida *spp*. 3

1.1.1 Significance for humans 3

1.2 *Candida albicans* 4

1.2.1 Forms of *C. albicans* infections in humans 4

1.2.2 Morphology 5

1.2.3 Adhesion to epithelial cells and invasion of epithelial cell surfaces 5

1.3 Immune response to cutaneous candidiasis 6

1.3.1 Innate immune response 6

1.3.2 Th1 and Th17 cells in cutaneous candidiasis 10

1.4 The JAK/STAT signalling pathway 12

1.4.1 Tyrosine kinase 2 (TYK2) 13

1.5 Recombination-activating gene (RAG) and RAG deficiency 15

1.6 Previous work 16

1.7 Aim 16

2 MATERIAL AND METHODS 17

2.1 Material 17

2.2 Methods 19

2.2.1 Mice 19

2.2.2 Experimental setup 19

2.2.3 Fungal strain 20

2.2.4 Cultivation of *C. albicans* 20

2.2.5 Preparation of the *C. albicans* suspension for infection 21

2.2.6 Evaluation of the fungal load in the skin 21

2.2.7 RNA Isolation and cDNA synthesis 22

2.2.8 Quantitative PCR (qPCR) 22

2.2.9 Immunohistochemistry 23

2.2.10 Statistical analysis 24

3 RESULTS 25

3.1 In the absence of T cells and B cells, TYK2 does not affect the local fungal load upon intradermal *C. albicans* infection. 25

3.2 *C. albicans* barely spreads to distal organs in mice devoid of T cells and B cells, irrespective of the presence or absence of TYK2 or TYK2^{K923E}. 26
3.3 *C. albicans* does not invade into deeper skin tissue in mice that lack T cells and B cells, irrespective of the presence or absence of TYK2 and TYK2^{K923E}. 28
3.4 Intradermal *C. albicans* infection induces massive neutrophil infiltration, independently of the presence of T cell and B cells and TYK2. 30
3.5 Absence of TYK2 or its kinase activity impairs the upregulation of *Ifng* mRNA in the skin of *Rag2*$^{-/-}$ mice, whereas it does not affect the upregulation of *Il22* mRNA. 31
3.6 Absence of TYK2 does not grossly affect shifts in blood cell composition in response to intradermal *C. albicans* infection in *Rag2*$^{-/-}$ mice. 32
3.7 Absence of TYK2 or presence of TYK2^{K923E} accelerates wound healing in *Rag2*$^{-/-}$ mice upon intradermal *C. albicans* infection 33

4 DISCUSSION 36

5 SUMMARY 38

5.1 English 38
5.2 Deutsch 40

6 INDEX OF ABBREVIATIONS 42

7 BIBLIOGRAPHY 43

1 Introduction

1.1 Candida *spp*.

1.1.1 Significance for humans

Candida species (*spp.*) belong to the order "*Saccharomycetales*" and were firstly described by Langenbeck in 1839 (Langenbeck, 1839; Oh et al., 2021). More than 200 members, including *C. glabrata, C. krusei* and *C. albicans,* are known (Sardi et al., 2010). *Candida spp.* is part of the commensal skin mycobiome. However, under certain circumstances, several species can turn pathogenic (Kühbacher et al., 2017).

Candida spp. infections are called candidiasis and are a particular problem for immunocompromised patients, e.g., elderly patients or patients who have undergone organ transplantation. In these patients, candidiasis is frequently found as a secondary infection. The number of immunocompromised or immunodeficient people is rapidly increasing, making invasive fungal diseases a threat to global health (Kainz et al., 2020; R and Rafiq, 2021). Each year more than 150 million people encounter invasive infections, resulting in 1.7 million deaths. Mucosal candidiasis affects millions of people and nearly a billion people are thought to have fungal infections of the skin, nails, or hair (Bongomin et al., 2017).

Furthermore, fungal species are becoming increasingly resistant to commonly used fungicides. Azoles, polyenes and echinocandins are the means of choice for the first-line treatment. More and more cases of multi-resistant fungal pathogens are observed, making the treatment extremely difficult (Santos et al., 2018).

New strategies for the treatment of candidiasis are in development, for example, immunotherapy using specifically binding radiolabelled antibodies to kill the fungus via radiation. Other approaches aim at immune cells, such as dendritic cells (DCs), neutrophils, or granulocytes, where the main goal is a faster and better immune cell activation achieved by cytokine and gene therapy to promote fungal killing. Unfortunately, these therapies are still in their infancy due to concerns regarding potential toxicity as well as intrinsic and acquired resistance. Furthermore, it is not yet possible to estimate the costs for the human

application. Therefore it may not be a practical solution for everyone as compared to the inexpensive drugs used today (Nami et al., 2019).

1.2 *Candida albicans*

Among the numerous members of the *Candida spp*., *Candida albicans* is the most common facultative pathogen fungus (Calderone and Cihlar, 2002; Dowd, 2014).

1.2.1 Forms of *C. albicans* infections in humans

Infections with *C. albicans* can range from local to systemic forms. Local superficial infections, mostly affecting the skin, nails and mucous membranes, pose a threat to human health (Bongomin et al., 2017). Cutaneous candidiasis is commonly found in various warm and moist skin folds like cutaneous, vaginal and oral tissues, where it appears as white, moist patches (Haase, 2009). Skin thickening, hyperkeratosis and erythema are possible outcomes of the infection (Kashem and Kaplan, 2016). Oral candidiasis usually affects older, frail and undernourished people, making it very painful to eat. Vulvovaginal candidiasis develops during pregnancy or after broad-spectrum antibiotics treatment. It can cause congenital cutaneous candidiasis during pregnancy and childbirth, which can infect the infant and cause neonatal sepsis since the infant's immune system is still underdeveloped (James et al., 2020). Additionally, mucocutaneous candidiasis can also occur in the region around periodontal pockets and dentures (Sardi et al., 2010). Both psoriasis and atopic dermatitis can include *C. albicans* as a concomitant condition in addition to being the primary disease (Taudorf et al., 2019).

Severe systemic infections occur as the fungus reaches the bloodstream. Venous catheters are frequently the reason for *C. albicans* being able to enter the organism as they diminish the integrity of the skin barrier allowing the fungus to disseminate into the blood circulation. Once in the bloodstream, the fungus spreads to other organs, most commonly the kidneys (Hebecker et al., 2016; Lionakis, 2014). Consequences can be organ damage and, in the worst case, the death of patients (Lionakis, 2014).

1.2.2 Morphology

C. albicans is a dimorphic fungus (Thompson et al., 2011). The budding yeasts' primary structure is oval and unicellular with a diameter of 5 µm. The growth leads to filament development and the formation of hyphae (Figure 1) (Sudbery et al., 2004). An interesting characteristic of *C. albicans* is its ability to switch between those morphologies. The switch is dependent on several factors, such as temperature, pH and CO_2 concentration. During an infection, the yeast form is mainly associated with adherence to epithelial cells, mucosal colonization and systemic dissemination, whereas the hyphal form is involved in tissue invasion, tissue damage and immune evasion (Lorenz et al., 2004).

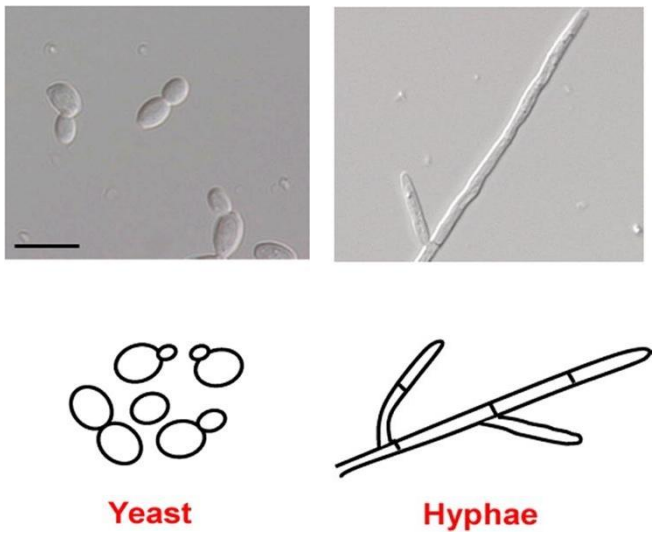

Figure 1: Different morphologies of C. albicans, adapted from Thompson et al., 2011.

1.2.3 Adhesion to epithelial cells and invasion of epithelial cell surfaces

Fungal adhesion to host cells is a critical step to colonize epithelial surfaces, form biofilms and eventually cause infections. To attach to the host, *C. albicans* has developed distinct gene families. The adhesin gene family, which includes *Hwp1*, *Int1* and *Eap1*, is involved in host cell attachment. The agglutinin-like sequence (Als) gene family encodes large cell-wall glycoproteins that bind to serum proteins, extracellular matrix, cadherins and integrins (Williams et al. 2013, Laforce-Nesbitt et al. 2008, Hosseini et al. 2019).

Once attached to epithelial cells, fungal cells can start invading. Since the top layer of the skin is mostly cornified and stratified tissue, a certain effort is required for the fungus to invade the tissue. Therefore, the fungus produces proteases and toxins, such as secreted aspartyl proteinases (Sap) 1-3 and candidalysin, to perforate the host membrane. This causes cell death and allows the fungus to infiltrate the surrounding tissue (Kühbacher et al., 2017).

It is crucial for the host to distinguish between commensal colonization and pathogenic invasion. As a commensal fungus, *C. albicans* is present in smaller numbers and does not induce cell damage, it does not trigger an immune response. As soon as *C. albicans* hyphae start invading and damaging epithelial cells, the production of inflammatory cytokines and antimicrobial agents is activated (Pellon et al., 2020).

1.3 Immune response to cutaneous candidiasis

1.3.1 Innate immune response

The host defence against *C. albicans* starts with the recognition of the pathogen by the innate immune system. Macrophages, DCs and monocytes recognize conserved molecular structures of pathogens by pattern recognition receptors (PRRs). Upon binding to these receptors, the cells respond by secretion of proinflammatory cytokines. As a result, killing mechanisms, cytokine production by other activated cells and, ultimately, activation of the adaptive immune system for long-term immunity is triggered (Drummond et al., 2015).

1.3.1.1 C-type lectins, Toll- and NOD-like receptors

Three main PRR families are relevant in the context of cutaneous candidiasis: C-type lectins, toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) (Kashem and Kaplan, 2016).

C-type lectins are a large family of receptors primarily found on DCs, macrophages and neutrophils, which mainly bind fungal glycans (Bojang et al., 2021). Dectin-1 is a C-type

lectin receptor that detects β-glucans located on the *C. albicans* yeast cell wall. Binding to β-glucans triggers the nuclear factor kappa B (NF-kB) pathway, subsequently resulting in the synthesis of the proinflammatory cytokines interleukin- (IL)-1β, IL-6 and IL-12 (Kashem and Kaplan, 2016).

Two TLRs are crucially involved in *C. albicans* recognition: TLR-2, which spots cell-wall polysaccharides and TLR-4, which detects O-linked mannans also present in the fungal cell wall (Kashem and Kaplan, 2016). These TLRs are found on a variety of cells, including keratinocytes, melanocytes and macrophages (Doering TL, Cummings RD, 2017). Ligand binding to TLRs activates the mitogen-activated protein kinase (MAPK) and the NF-kB intracellular signalling pathways. This results in the production of proinflammatory cytokines, such as IL-1β, tumour necrosis factor (TNF)-α and IL-12 (Kashem and Kaplan, 2016).

NLRs are a family of intracellular PRRs that, upon ligand binding, assemble into multiprotein complexes called inflammasomes. The main inflammasome associated with fungal infections is the NLR family pyrin domain containing (NLRP) 3 inflammasome. During *C. albicans* infections, *Candida*-secreted aspartic proteases (Saps) activate the NLRP3 inflammasome. This results in the cleavage of caspase-1 and proteolytic processing of IL-1β and IL-18 into their biologically active forms (Tavares et al., 2015).

1.3.1.2 Epidermal and dermal cells

The outermost layer of the skin serves as a physical barrier that protects against potential pathogens. The most important cells of this layer in the context of cutaneous candidiasis are keratinocytes and melanocytes. Keratinocytes possess TLRs, C-type lectins and NLRs, enabling them to quickly react to infections. Besides the PRRs, keratinocytes express receptors for TNF-α, IL-17A and IL-22. The binding of TNF-α and IL-17A on keratinocytes causes them to produce antimicrobial molecules, such as β-defensins and S100 proteins, and chemokines. The chemokines recruit neutrophils and other inflammatory cells. Aside from inducing antimicrobial peptide production, IL-22 increases keratinocyte proliferation and

shedding, resulting in impaired adhesion of fungi to the epithelial surface (Figure 2) (Kashem and Kaplan, 2016; Kenneth and Weaver, 2017; Kobiela et al., 2022).

Melanocytes are located in the epidermis, producing melanin for skin pigmentation. In the context of *C. albicans* infection, melanocytes recognize the fungus most likely through TLR-4, which increases melanin production. As melanin has antimicrobial effects, it inhibits fungal growth (Tapia et al., 2014).

1.3.1.3 Cutaneous nerves

The interplay between the immune and the nervous systems has already been described in both humans and mice (Ordovas-Montanes et al., 2015). Cutaneous sensory nerves can directly recognize *C. albicans* cell wall components via neuronal PRRs, such as C-type lectins, to sense fungal cell wall properties. Neurons, activated by infections, secrete calcitonin gene-related peptide (CGRP), which stimulates DCs for IL-23 production (Figure 2) (Drummond and Lionakis, 2018; Kashem and Kaplan, 2016; Tang et al., 2012; Underhill and Pearlman, 2015).

1.3.1.4 Antigen-presenting cells

DCs are antigen-presenting cells (APCs) crucially bridging the gap between innate and adaptive immune responses when fighting off pathogens. Immature DCs patrol in the peripheral tissue and are attracted by various chemokines and antimicrobial peptides released by epithelial cells upon infection. Several PRR families, such as like C-type lectins, TLRs and complement receptors, bind fungal properties and trigger DCs responses (Bojang et al., 2021; Richardson and Moyes, 2015). Activated DCs migrate to the lymph nodes three to four days *post infection* and have a crucial role in the activation of the T cell compartment. During cutaneous candidiasis special dermal dendritic cells, called Langerhans cells (LCs) are detecting the fungus in the skin with C-type lectins, galectins and TLRs. Upon stimulation, LCs secrete cytokines, such as IL-1β, transforming growth factor (TGF)-β and

IL-6, which are required for T helper 17 (Th17) cell differentiation (Figure 2) (Kashem and Kaplan, 2016; Kühbacher et al., 2017). When *C. albicans* filaments penetrate deeper into the tissue they are recognized by TLR-2 on $CD103^+$ dermal DCs (dDCs). Activated dDCs secrete IL-12 and migrate into the local lymph node, where they activate naïve $CD4^+$ T cells to differentiate into T helper 1 (Th1) cells (Figure 2) (Kashem et al., 2015).

1.3.1.5 Gamma delta (γδ) T cells

γδ T cells are T cells homing in the skin, intestine and lungs. These cells own a T-cell receptor (TCR) consisting of the γ- and δ-subunit (Cruz et al., 2018). During cutaneous candidiasis, γδ T cells are activated by IL-23, which is secreted by alert monocytes, macrophages and DCs upon stimulation by the fungus. Activated γδ T cells produce IL-17A, which is essential for an adequate immune response (Kashem et al., 2015b; Maher et al., 2015). IL-17 acts on stromal cells to secrete granulocyte colony-stimulating factor (G-CSF), which attracts neutrophils to migrate to the site of infection and stimulates the bone marrow for further neutrophil production. Additionally, IL-17 stimulates epithelial cells to secret β-defensins which form pores in the fungal cell wall (Figure 2) (Li et al., 2018).

1.3.1.6 Neutrophils

To our knowledge, the role of neutrophils in *C. albicans* infections of the skin has not yet been directly assessed. However, neutrophils are crucial for immune defence against oral *C. albicans* infections (Trautwein-Weidner et al., 2015). Early neutrophil recruitment to different tissues such as the spleen, liver and oral mucosa is important to control *C. albicans* growth. Neutrophils engage in fungal phagocytosis upon recognition via dectin-1 and release neutrophil extracellular traps (NETs) to engulf *C. albicans* filaments (Kashem and Kaplan, 2016). Additionally, neutrophils create reactive oxygen species (ROS) to facilitate the fungal elimination (Desai and Lionakis, 2018).

1.3.2 Th1 and Th17 cells in cutaneous candidiasis

The T cell compartment is a central mediator of adaptive antifungal immunity in cutaneous candidiasis (Kashem and Kaplan, 2016).

1.3.2.1 Th1 cells

Th1 cells develop from naïve $CD4^+$ T cell precursors upon activation by IL-12, derived from $CD103^+$ (dDCs). Th1 cells secrete interferon-gamma (IFN-γ), granulocyte-macrophage colony-stimulating factor (GM-CSF) and TNF-α, which promotes maturation of phagocytes and their killing activity (Speakman et al., 2020). Mice lacking Th1 cells have less protection against secondary systemic but not cutaneous *C. albicans* infections (Kashem and Kaplan 2016).

1.3.2.2 Th17 cells

Th17 cells are known for their importance in the immune defence against infections with extracellular bacteria and fungi (Kenneth and Weaver, 2017). IL-6 acts on naïve $CD4^+$ T cells to promote Th17 cell proliferation (Tang et al. 2012, Underhill and Pearlman 2015). Th17 cells primarily secrete IL-17 and IL-22, which increases trafficking and fungicidal activity of neutrophils and β-defensin production by epithelial cells and keratinocytes (Speakman et al., 2020).

The role of Th17 in cutaneous candidiasis was demonstrated using an *IL-23p19*$^{-/-}$ mice, which lack functional IL-23. *IL-23p19*$^{-/-}$ mice intradermally infected with *C. albicans* showed decreased numbers of mature Th17 cells, which is consistent with the crucial role of IL-23 in the maintenance of Th17 cells. Mice deficient in IL-23 were less able to clear the infection in the skin, indicating an important role of Th17 cells in the defence against cutaneous candidiasis (Kagami et al., 2010; Toussirot, 2012).

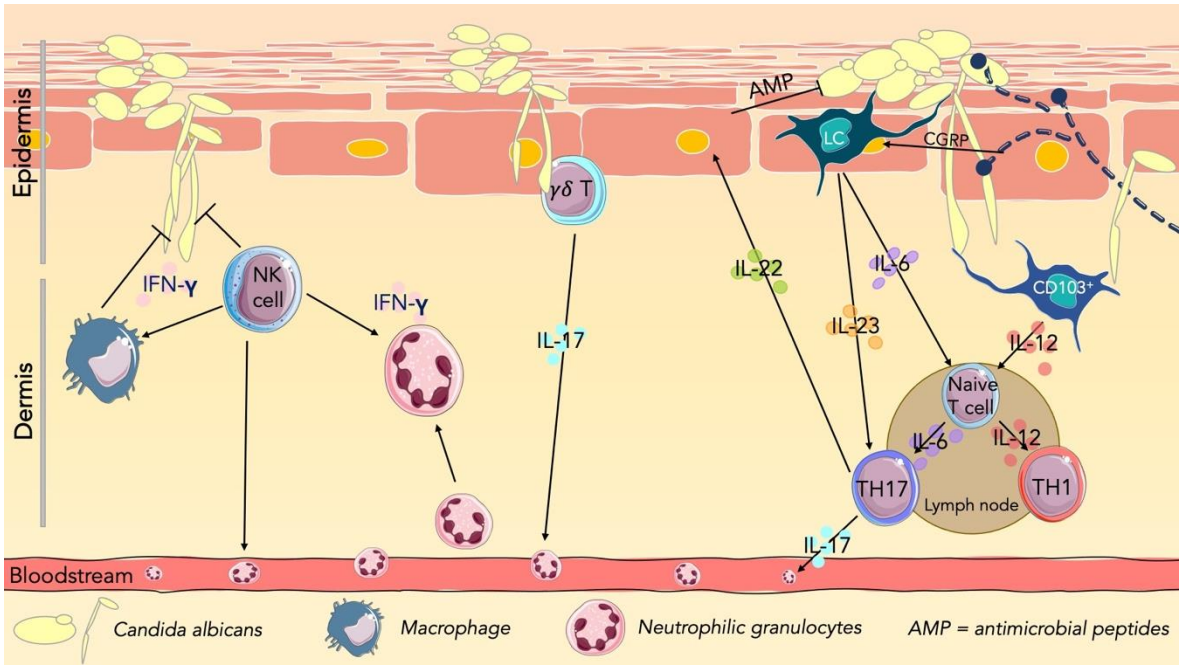

Figure 2: Immunological processes in the skin during local C. albicans infection (Source: Kristina Schmidhofer)

1.4 The JAK/STAT signalling pathway

The Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway is a signal transduction pathway which is evolutionarily conserved from flies to humans. This pathway can be activated by many cytokines, hormones and growth factors which are involved in multiple biological processes, such as cell growth and differentiation, host defence and immunoregulation (Haan et al., 2012).

Four JAKs (JAK1, JAK2, JAK3, TYK2) and seven STATs (STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, STAT6) occur in mammals. JAK1, JAK2 and TYK2 are ubiquitously expressed, while JAK3 is mainly expressed in hematopoietic cells (Rane and Reddy, 1994; Yamaoka et al., 2004). STATs are ubiquitously expressed, except for STAT4, which is mainly expressed in spleen, blood and the brain (Cavaleri and Schöler, 2009; Hertzog et al., 2005).

The JAK/STAT signalling pathway is initiated by binding of a ligand to its respective receptor complex, which is located on the cell membrane. Ligand binding leads to a conformational change of the receptor, which then causes JAKs to auto- and transphosphorylate and phosphorylate receptor chains. Receptor phosphorylation creates docking sites for STATs, which get activated as homo- or heterodimers and translocate to the nucleus to associate with DNA and regulate the transcription of target genes (Figure 3) (Haan et al., 2012).

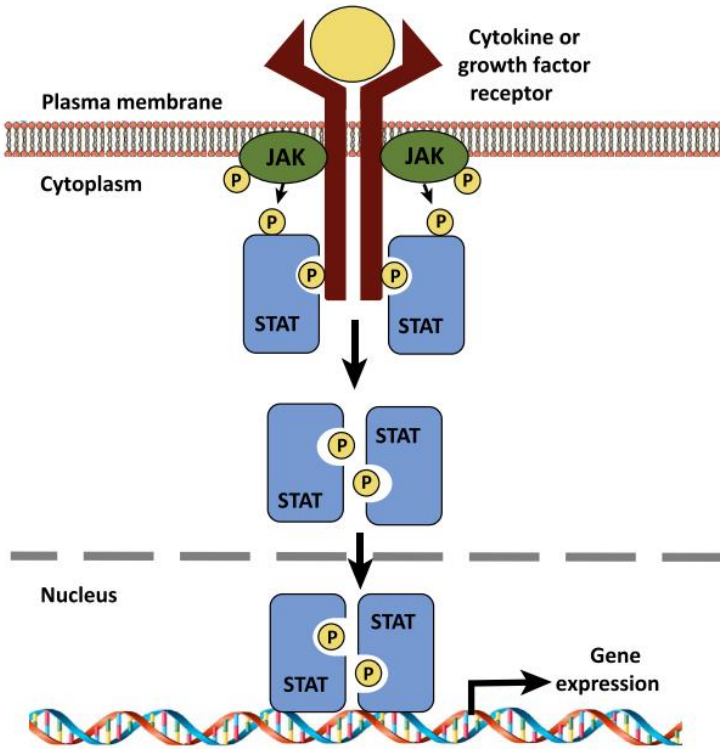

Figure 3: Overview of the JAK/STAT pathway from Dodington et al., 2018.

1.4.1 Tyrosine kinase 2 (TYK2)

Since the first discovery of TYK2 back in 1990, the kinase has been the subject of intensive research (Krolewski et al., 1990; Strobl et al., 2011). TYK2 is activated either with JAK1 or JAK2 but never with JAK3 (Strobl et al., 2011). The *TYK2* gene is located on chromosome 10 in humans and chromosome 9 in mice. It has a molecular weight of approximately 130 kDa and consists of four structural subunits: the N-terminal four-point-one, ezrin, radixin, moesin (FERM) domain, the Src homology (SH2) domain, the pseudokinase domain and the C-terminal kinase domain (Figure 4). Based on sequence homologies, seven JAK homology (JH) domains (JH1-JH7) have been defined. The FERM domain (JH7- JH4) is necessary for binding to the receptor chains and stabilizes this bond. The SH2 domain (JH4-JH3) is also involved in receptor binding. The pseudokinase domain (JH2) possesses a canonical kinase domain and a binding site for adenosine triphosphate (ATP) but lacks catalytic activity. It is thought to be a regulatory unit for the kinase domain. The kinase is

activated by phosphorylation at two tyrosine residues (Y1054/Y1055 in humans and Y1047/Y1048 in mice) (Leitner et al., 2017; Wallweber et al., 2014).

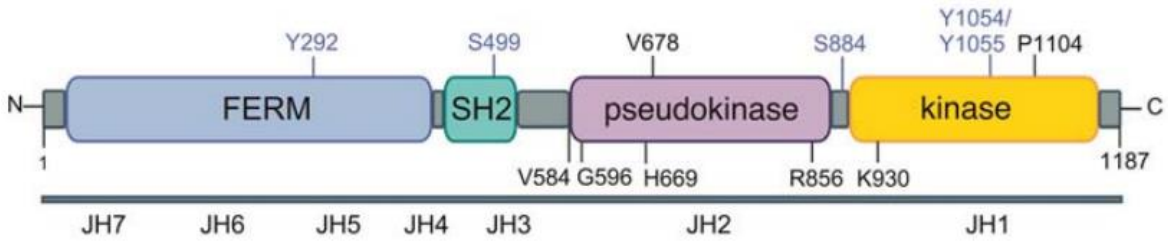

Figure 4: Structural and functional properties of a TYK2 protein, adapted from Strobl et al., 2011.

TYK2 binds to five different receptor chains: IFN α receptor 1 (IFNAR1), IL-10 receptor 2 (IL-10R2), IL-12 receptor β1 (IL-12Rβ1), IL-13 receptor α1 (IL-R13α1) and gp130. Cytokines that utilize these receptor chains are type I IFNs and members of the IL-10/IL-20, IL-12, IL-4/IL-13 and IL-6/gp130 families (Figure 5) (Leitner et al., 2017).

Studies in mice and humans have uncovered the crucial role of TYK2 in immunity (Strobl et al., 2011). The first *Tyk2*$^{-/-}$ mouse was created in 2000 (Karaghiosoff et al., 2000; Shimoda et al., 2000). *Tyk2*-deficient mice show increased sensitivity to viral, bacterial and parasitic infections but, on the other hand, have an increased resistance to autoimmune/inflammatory diseases, including psoriasis, arthritis and colitis (Gorman et al., 2019). In mice it has been demonstrated, that TYK2 is involved in the differentiation of Th1 or natural killer (NK) cell maturation (Muromoto et al., 2021; Simonović et al., 2019). Furthermore TYK2 is essential in tumor surveillance, as seen in *Tyk2*-deficient mice, where the mice displayed decreased cytotoxic capacity of NK/NKT cells (Karjalainen et al., 2020; Stoiber et al., 2004). Many TYK2 inhibitors are in development and in clinical trials for the treatment of autoimmune and inflammatory diseases (Nogueira et al., 2020). One of many promising inhibitors is the TYK2 inhibitor deucravacitinib from Bristol-Myers Squibb. This inhibitor already reached clinical phase III showing promising results in psoriasis (Bristol-Myers Squibb, 2022; Ghoreschi et al., 2021). The first human patient with an inborn *TYK2* deficiency was identified in 2006 (Minegishi et al., 2006). Up to now, around thirteen *TYK2* deficient patients have been reported. Most of the patients show impaired responses to IFN-α, IL-10, IL-6, IL-12 and IL-23. All these patients showed increased susceptibility to infections in

their medical history and four of them showed atopic dermatitis and hyper IgE syndrome (Fuchs et al., 2016; Kreins et al., 2015; Lv et al., 2022; Sarrafzadeh et al., 2020).

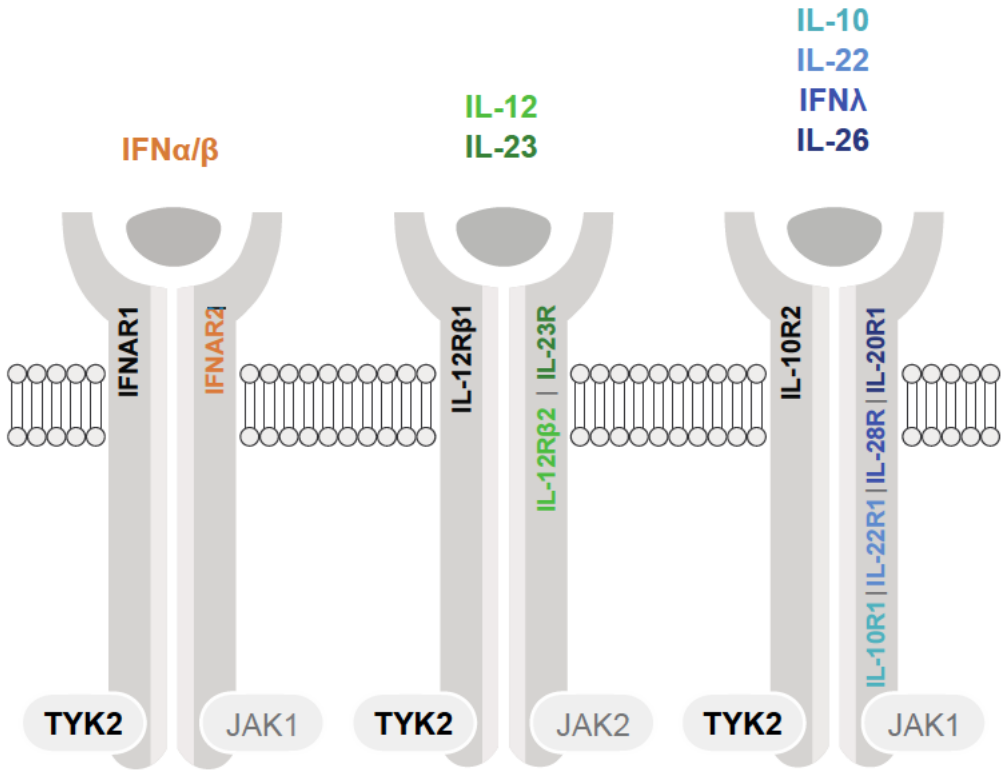

Figure 5: Cytokine families signalling via the JAK/STAT pathway, adapted from Wöss et al., 2019.

1.5 Recombination-activating gene (RAG) and RAG deficiency

Recombination activating gene 1 (*RAG1*) and recombination activating gene 2 (*RAG2*) are genes found on chromosome 11 in humans and chromosome 2 in mice (Oettinger et al., 1992). Their significance became evident in 1992, when the *Rag2* gene was knocked out in mice resulting in degenerated T and B cells (Shinkai et al., 1992). RAG1 and RAG2 are recombinases that form a complex which creates double-stranded DNA breaks resulting in the recombination of T-cell and B-cell receptors (Reza et al., 2018). *Rag1* and *Rag2* knockout mice are frequently used to investigate the role of the immune system in cancer, autoimmunity and a variety of other diseases (Rios et al., 2020).

1.6 Previous work

Ongoing studies in our research group are directed towards characterizing the role of TYK2 signalling in cutaneous *C. albicans* infections in mice. For this purpose, wildtype (*WT*) and TYK2 knockout (*Tyk2*$^{-/-}$) mice were intradermally infected with *C. albicans*. Additionally, mice with a kinase-inactive form of TYK2 (*Tyk2*^{K923E}) were used to unravel potential TYK2-scaffolding functions.

It was shown that *Tyk2*$^{-/-}$ and *Tyk2*^{K923E} mice have reduced fungal load in the skin on day two and day four after infection when compared to *WT* mice. Furthermore, in contrast to *WT* mice, *Tyk2*$^{-/-}$ and *Tyk2*^{K923E} animals did not show systemic dissemination of *C. albicans* to the kidneys and spleen. These results indicate that TYK2 has a detrimental role in the early immune defence against cutaneous candidiasis and prompt the hypothesis that this is mediated by TYK2 signalling in innate immune cells.

1.7 Aim

Aim of this project is to study the role of TYK2 in the immune defence against cutaneous candidiasis in the absence of a functional adaptive immune system. To address this objective, *Rag2*$^{-/-}$ mice were crossed with *Tyk2*$^{-/-}$ and *Tyk2*^{K923E} mice to generate *Rag2*$^{-/-}$*Tyk2*$^{-/-}$ and *Rag2*$^{-/-}$*Tyk2*^{K923E} mice and infected intradermally with *C. albicans.* The fungal burden in the skin, spleen and kidneys should be assessed, blood parameters used to analyse the systemic response and wound healing upon infection monitored. Furthermore, local gene expression, immune cell evasion into tissue surrounding the fungus and fungal penetration into deeper tissue should be investigated.

2 Material and methods

2.1 Material

Table 1: Equipment

Equipment		
Name	**Company**	**Identifier**
Histology Cassettes	Kabe Labortechnik	Cat. #053771
Innova 4230 Refrigerated Incubator Shaker	New Brunswick Scientific	Cat. #NB-4230
MiniBatch D-9	Miccra	Cat. #N/A
Pellet Pestle	Thermo Fisher Scientific	Cat. # 12-141-364
Pellet Pestle Cordless Motor Adapter	Thermo Fisher Scientific	Cat. # 12-141-362
Mini G 1600 Homogenizer	SPEx Sample Prep	Cat. # N/A
NanoDrop 2000 Spectrophotometer	Thermo Fisher Scientific	Cat. # ND-2000

Table 2: Buffers

Buffers		
Name	**Company**	**Identifier**
Dulbecco´s Phosphate Buffered Saline (PBS)	Sigma	Cat. #D8537
RNA Later	Invitrogen	Cat. #AM7201

Table 3: Chemical reagents and commercial assays

Chemical reagents and commercial assays		
Name	**Company**	**Identifier**
Agar-Agar	Carl Roth	Cat. #5210.2
Yeast Extract	Formedium	Cat. #YEA03
Peptone	Formedium	Cat. #PEP03
D (+) Glucose	PanReac AppliChem	Cat. #141341.0914
Ethanol 70%	Scharlau	Cat. #ET00051000
RNeasy Fibrous Tissue Mini Kit (50)	Qiagen	Cat. #74704
iScript cDNA Synthesis Kit	Biorad	Cat. #1708891
$MgCl_2$ 25 mM	Solis BioDyne	Cat. #05-11-00025
HOT FIREPol DNA Polymerase	Solis BioDyne	Cat. #01-02-01000

dNTP Mix, 10mM	Thermo Fisher	Cat. #R0182
10X Reaction Buffer B	Solis BioDyne	Cat. #01-02-01000
Anti-Neutrophil Antibody (NIMP-R14)	Abcam	Cat. #Ab2557
Goat Anti Rat Antibody	Bio-Rad	Cat. # STAR131A
Avidin/Biotin Blocking Kit	Vector Laboratories	Cat. #SP-2001
Super Block	Scytec	Cat. #AAA125
Gomori Methenamine-Silver Nitrate (GMS) Stain Kit	Scytec	Cat. #KAA-1
DAB-Substrate	Vector Laboratories	Cat. #SK-4100
Haematoxilyn	Sigma-Aldrich	Cat. #GHS316

Table 4: Software

Software		
Name	**Company**	**Identifier**
Prism 9	Graphpad Software	N/A

Table 5: qPCR primers, assay and dye

qPCR primers, assay and dye		
Name	**Company**	**Identifier**
Ube2d2-F 5′ AGG TCC TGT TGG AGA TGA TAT GTT -3′	Sigma Aldrich	N/A
Ube2d2-R 5′ TTG GGA AAT GAA TTG TCA AGA AA -3′		N/A
Ube2d2-probe 5′- CCA AAT GAC AGC CCC TAT CAG GGT GG -3′		N/A
INFg-F 5′- TGA GTA TTG CCA AGT TTG AGG TCA -3′		N/A
INFg-R 5′- CGG CAA CAG CTG GTG GAC -3′		N/A
IFNg-probe 5′- CCA GCG CCA AGC ATT CAA TGA GCT -3′		N/A
Mm *Il22* 1 SG QuantiTect Primer Assay		Cat. #QT00128324
EvaGreen Dye	Biotium	Cat. #31000

2.2 Methods

2.2.1 Mice

Rag2$^{-/-}$ (*B6.129S6-Rag2*^{tm1Fw}) mice (Shinkai et al., in 1992) were kindly provided by Veronika Sexl, University of Veterinary Medicine Vienna. To generate mice that lack both RAG2 and TYK2 or express enzymatically inactive TYK2, *Rag2*$^{-/-}$ mice were crossed with *Tyk2*$^{-/-}$ (Karaghiosoff et al. 2000) and *Tyk2*^{K923E} mice (Prchal-Murphy et al. 2012), respectively. Age-matched (8–12 week) male and female mice were used for all experiments. *Rag2*$^{-/-}$, *Rag2*$^{-/-}$*Tyk2*$^{-/-}$ and *Rag2*$^{-/-}$*Tyk2*^{K923E} mice were bred at the University of Veterinary Medicine Vienna under specific pathogen-free conditions according to Federation of European Laboratory Animal Science Associations (FELASA) guidelines. All animal experiments were approved by the institutional ethics and animal welfare committee and the national authority according to §§ 26ff. of Animal Experiments Act, Tierversuchsgesetz 2012: TVG 2012 (BMBWF-68.205/0173-V/3b/2019). All *in vivo* experiments were performed by trained personnel.

2.2.2 Experimental setup

Rag2$^{-/-}$, *Rag2*$^{-/-}$*Tyk2*$^{-/-}$ and *Rag2*$^{-/-}$*Tyk2*^{K923E} mice were infected by intradermal injections with $1x10^8$ colony forming units (CFU) of *C. albicans* in 50 µL (divided into two injections of 25 µL at independent sites). Skin, spleen, kidneys and blood were collected on day 4 or day 7 after the infection. Fungal load in the skin, spleen and kidneys was evaluated by CFU assay. The collected blood was used for differential blood analysis to monitor systemic immune responses. To investigate the local immune response in the skin, the expression of genes encoding inflammatory cytokines was evaluated by qPCR and the infiltration by immune cells was assessed by immunohistochemistry. Additionally, mice were kept for long-term monitoring to evaluate the disease kinetics and the wound healing process. The setup is illustrated in Figure 6.

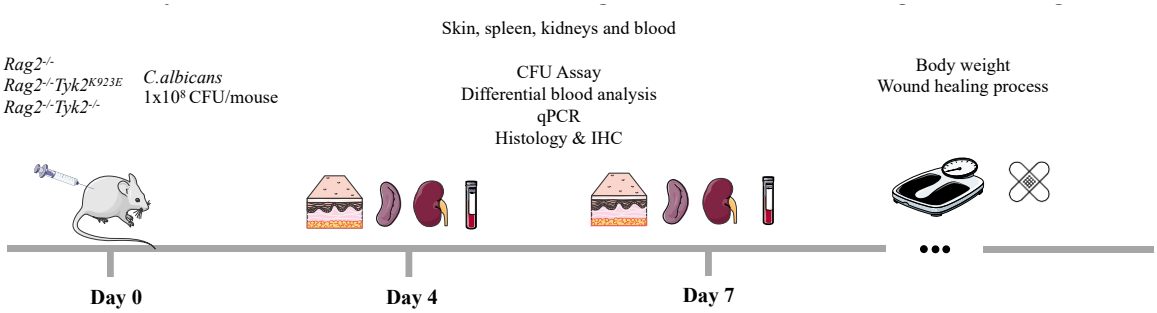

Figure 6: Schematic illustration of the experimental setup (Source: Kristina Schmidhofer).

2.2.3 Fungal strain

The wild-type *C. albicans* strain SC5314 (Gillum et al., 1984) used in this thesis was kindly provided by Karl Kuchler. The strain was stored at -80 °C until further use.

2.2.4 Cultivation of *C. albicans*

Liquid medium

10 g/l	Yeast extract (Y)
20 g/l	Peptone (P)
2% (w/v)	D-Glucose (D)

Yeast extract and peptone were dissolved in ddH_2O and subsequently autoclaved. Glucose was dissolved in ddH_2O (20% stock solution) and added to the autoclaved yeast extract peptone dextrose (YPD)-medium for the final concentration of 2%.

YPD plates

10 g/l	Yeast extract
20 g/l	Agar
20 g/l	Peptone
2% (w/v)	D-Glucose

Yeast extract, agar and peptone were dissolved in ddH_2O and subsequently autoclaved. Glucose was dissolved in ddH_2O (20% stock solution) and added to the autoclaved

YPD-medium to a final concentration of 2%. The warm solution was added to sterile petri dishes. Petri dishes were stored at 4 °C until further use.

2.2.5 Preparation of the *C. albicans* suspension for infection

C. albicans inoculum stored at -80 °C was spread on a YPD plate and allowed to grow at room temperature for 3 days. Afterwards, several colonies were removed from the plate, immersed in 5 mL YPD liquid medium and incubated in the incubator shaker at 30 °C and 200 RPM for 6 hours to create the stock suspension. The optical density (OD) of the stock was evaluated with a cell density meter in a 1:20 dilution.

We used this formula to calculate an appropriate amount of *C. albicans* suspension for infection.

$$x = \frac{V}{2^{18} * OD}$$

Where x is the volume of stock suspension required to achieve an OD of 1 after doubling 18 times in a defined volume (V). In our hands, it took 16 hours at 30 °C and 110 RPM.

After the 16 hours of cultivation, *C. albicans* culture was then centrifuged at 1780 x g for 5 minutes and the pellet was washed twice with sterile PBS. The pellet was resuspended in enough PBS to allow each mouse to receive 50 µL of the infection solution. The fungal suspension was transferred into sterile tubes and mice were injected intradermally at the lower back.

2.2.6 Evaluation of the fungal load in the skin

Mice were sacrificed and their skin, spleen and kidneys were harvested and submerged in PBS (500 µL for skin, 5 mL for spleen and kidneys) and stored on ice. 4 skin biopsies with a diameter of 4 mm were collected, weighed and homogenized using a pellet pestle. Spleen and kidneys were weighed and processed using a tissue homogenizer. Skin homogenates were plated in dilutions of 1:100, 1:1000 and 1:10000. For the spleen and kidney, the

undiluted and 1:10 diluted homogenates were plated. All homogenates were plated in duplicates on YPD plates and incubated at 37 °C for 48 hours. Colonies were counted and the fungal load was calculated.

2.2.7 RNA Isolation and cDNA synthesis

The skin was harvested and immersed in RNA Later for at least 24 hours at 4 °C. For RNA isolation the buffer was removed and replaced by lysis buffer and the skin was homogenized using 1600 MiniG tissue homogenizer with 2 cycles at 1500 RPM for 60 seconds. RNA was isolated with the RNeasy Fibrous Tissue Mini Kit according to the manufacturer's instructions. The amount and quality of the isolated RNA were measured using NanoDrop microvolume spectrophotometer. RNA degradation was evaluated by loading approximately 500 ng of isolated RNA into a 0.8% agarose gel. cDNA was synthesized using the iScript cDNA Synthesis Kit and according to the manufacturer's instructions. Samples without reverse transcriptase (RT) were included to control for DNA contaminations.

2.2.8 Quantitative PCR (qPCR)

Target gene expression was measured using previously synthesized cDNA for qPCR. RT-negative samples were run for every gene in the prerun, to ensure that genomic DNA was not amplified.

Unless a commercial master mix (IL-22) was used, the following master mix was prepared: 4 mM $MgCl_2$, 100 nM Evagreen® or FAM-labelled probe, 1 U/rxn Hotfire polymerase, 1x Hotfire B buffer, 200 µM of deoxynucleoside triphosphate (dNTP) and the respective primers for each gene of interest (Table 5). Each reaction contained 18 µL of qPCR master mix and 2 µL of cDNA or water for the no template control samples (NTC). All samples were analysed in duplicates. The qPCR program consisted of 15 minutes at 95 °C and 40 cycles of 20 seconds at 95 °C followed by 60 °C for 1 minute. Serial 4-fold dilutions of the sample with the greatest expression level, which was identified in a pre-run experiment, were used to create a standard curve. Gene expression was calculated relative to the housekeeping

gene *Ube2d2*. To ensure the quality of the data, quality control was performed with the following criteria: the difference between the first and last C_T-value in the housekeeping gene is limited to 3 C_Ts; the difference between duplicates is limited to 0.5 C_Ts; no samples lower than the lowest or higher than the highest C_T-value of the standard are accepted; the efficiency of the run must be between 80-115% and R^2 must be between 0.98-1.

2.2.9 Immunohistochemistry

Skin biopsies were fixed in 4% formaldehyde for 24 hours at room temperature, washed with 70% ethanol, dried and embedded in paraffin. Samples were cut into 3 µm slices.

To stain *C. albicans*, a GMS Stain Kit was used. Tissue slides were deparaffinized and hydrated afterwards using distilled water. The slides were placed in a loosely capped Coplin jar. Chromic acid was added and the slides were microwaved for 10-12 seconds at 800 W and subsequently incubated for 3 minutes. Slides were rinsed once in tap water and twice in ddH_2O. The rinsed slides were incubated with sodium bisulphate solution for 1 minute at room temperature and rinsed again once with tap water and twice in ddH_2O. The silver working solution was added and the slides were microwaved for 25 seconds at 800 W and rinsed 4 times in ddH_2O. Afterwards, the slides are incubated in gold chloride solution for 20 seconds and rinsed 4 times in ddH_2O. The slides were incubated in sodium thiosulfate for 2 minutes and rinsed twice in ddH_2O afterwards. The slides were incubated in light green solution for 2 minutes and rinsed afterwards in 100% ethanol and capped.

To detect Ly-6G and Ly-6C positive cells (neutrophils and inflammatory monocytes), the rat monoclonal anti-neutrophil NIMP-R14 antibody was used. As secondary antibody, goat anti-rat antibody was used. All steps were carried out according to the manufacturer´s instructions, except that blocking was done by incubating in Avidin/Biotin Block and Superblock for 10 minutes each. In between, the slides were washed once with PBS. For detection, Vectastin ABC Kit and DAB substrate and counterstain with haematoxylin was used.

2.2.10 Statistical analysis

Statistical analysis (ANOVA) was performed using GraphPad Prism 9 Software. One-way ANOVA was performed. Differences were considered significant when the p-value was less than 0.05 ($p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$) and # show significant difference towards the PBS control, wherein # $p \leq 0.05$, ## $p \leq 0.01$, ### $p \leq 0.001$, #### $p \leq 0.0001$.

3 Results

3.1 In the absence of T cells and B cells, TYK2 does not affect the local fungal load upon intradermal *C. albicans* infection.

Previous experiments in our laboratory have shown that the absence of TYK2 results in a decreased load of *C. albicans* in the skin and decreased fungal dissemination to spleen and kidneys at day 2 and day 4 after intradermal infection (Miranda et al., unpublished). To test the hypothesis that TYK2 signalling in innate immune cells acts detrimental during *C. albicans* skin infection, we used mice on a *Rag2*$^{-/-}$ background, which are devoid of T cells and B cells.

Rag2$^{-/-}$, *Rag2*$^{-/-}$*Tyk2*^{K923E} and *Rag2*$^{-/-}$*Tyk2*$^{-/-}$ mice were intradermally infected with *C.albicans* and the local fungal load was determined. On day 4 *post infection* (*p.i.*), the fungal load did not differ between *Rag2*$^{-/-}$, *Rag2*$^{-/-}$*Tyk2*^{K923E} and *Rag2*$^{-/-}$*Tyk2*$^{-/-}$ mice (Figure 1A). On day 7 *p.i.*, a clear decrease in the fungal load was observed as compared to day 4 *p.i.* (Figure 7A, 7B). Again, there was no difference between mice of the 3 genotypes, albeit the variation between mice was higher on day 7 *p.i.* than on day 4 *p.i.* (Figure 7A, 7B). Collectively, these results indicate that in the absence of T cells and B cells, TYK2 does not impact the local fungal control within the first week of infection.

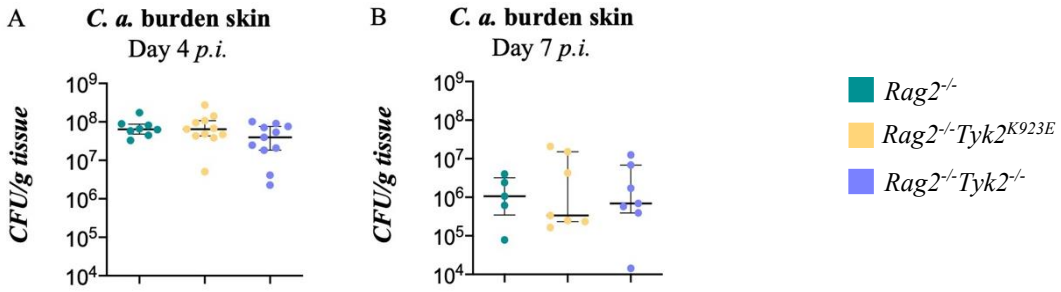

Figure 7: Rag2$^{-/-}$, *Rag2*$^{-/-}$*Tyk2*^{K923E} *and Rag2*$^{-/-}$*Tyk2*$^{-/-}$ *animals were intradermally infected with 1x10*^{8} *CFU of C. albicans. The skin was harvested and CFU assays were performed 4 days (**A**), and 7 days (**B**) p.i. (A-B) n=5-11, pooled from 2 independent experiments; Median ± interquartile range is given.*

3.2 *C. albicans* barely spreads to distal organs in mice devoid of T cells and B cells, irrespective of the presence or absence of TYK2 or TYK2^{K923E}.

Next, we investigated fungal dissemination into peripheral organs. Fungal load in spleen and kidneys was evaluated by CFU assay and, in addition, changes in spleen and kidney weight in response to *C. albicans* infection were assessed.

On day 4 *p.i.*, 1 out of 8 *Rag2*$^{-/-}$ mice, 1 out of 11 *Rag2*$^{-/-}$*Tyk2*^{K923E} mice and 2 out of 11 *Rag2*$^{-/-}$*Tyk2*$^{-/-}$ mice presented fungal colonies in the spleen. However, in all these cases, the *C. albicans* load was just above the detection limit (Figure 8A). *C. albicans* was not detectable in spleens from mice of all 3 genotypes on day 7 *p.i.* (Figure 8B). Spleen weight slightly increased in response to the infection in all animals on day 4 and day 7 *p.i.,* albeit it was only statistically significant for *Rag2*$^{-/-}$*Tyk2*$^{-/-}$ mice at day 7 p.i. Spleen weight did not differ between *Rag2*$^{-/-}$, *Rag2*$^{-/-}$*Tyk2*^{K923E} and *Rag2*$^{-/-}$*Tyk2*$^{-/-}$ mice before and after the infection (Figure 8C, 8D).

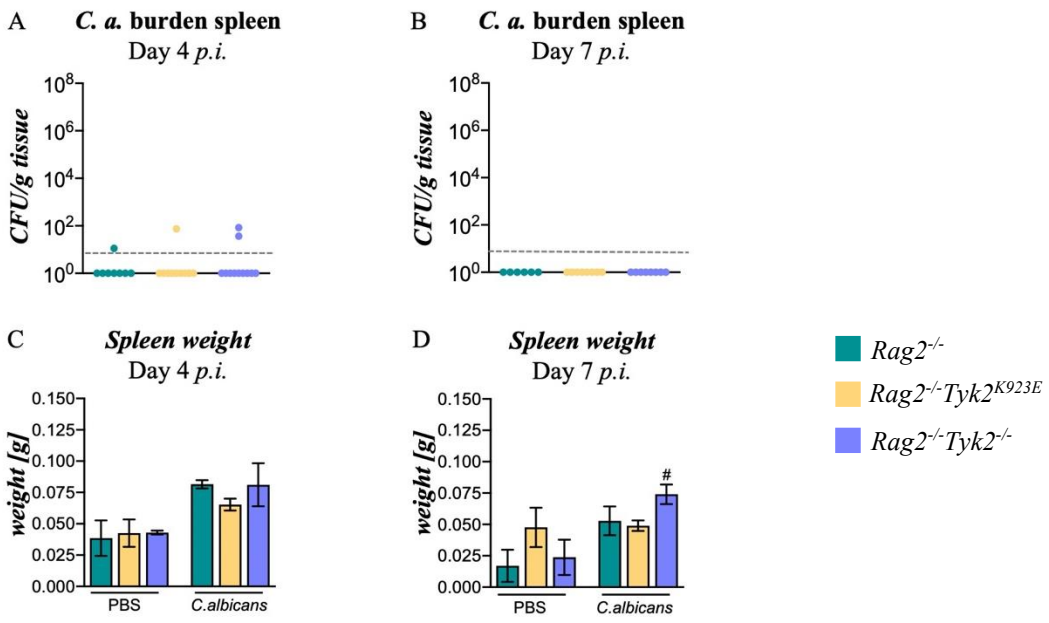

Figure 8: Rag2$^{-/-}$*, Rag2*$^{-/-}$*Tyk2*^{K923E} *and Rag2*$^{-/-}$*Tyk2*$^{-/-}$ *animals were intradermally infected with 1x10*^{8} *CFU of C. albicans. The spleen was harvested and CFU assays were performed 4 days (**A**), and 7 days (**B**) p.i. (A-B) n=6-11, pooled from 2 independent experiments; Median ± interquartile range is given. Spleens were weighed on day 4 p.i. (**C**) and on day 7 p.i.*

*(**D**). (C-D) n=6-11, pooled from 2 independent experiments; Mean ± SEM is given. # show significant difference towards the PBS control, # p ≤ 0.05, ## p ≤ 0.01, ### p ≤ 0.001, #### p ≤ 0.0001.*

In the kidneys, fungal dissemination was traced in 2 out of 8 *Rag2*$^{-/-}$ mice, 3 out of 11 *Rag2*$^{-/-}$*Tyk2*^{K923E} mice and none of the *Rag2*$^{-/-}$*Tyk2*$^{-/-}$ mice on day 4 *p.i.* (Figure 9A). Wherever detectable, *C. albicans* load was close to the detection limit (Figure 9A). *C. albicans* was not detectable in kidneys on day 7 *p.i.* (Figure 9B). Kidney weight did not change upon infection and was not different between mice of the 3 genotypes (Figure 9C, 9D). Overall, these results lead to the conclusion that fungal dissemination into spleen and kidneys is very low in *Rag2*$^{-/-}$ mice and that dissemination is not affected by the absence of TYK2 or the presence of TYK2^{K923E}.

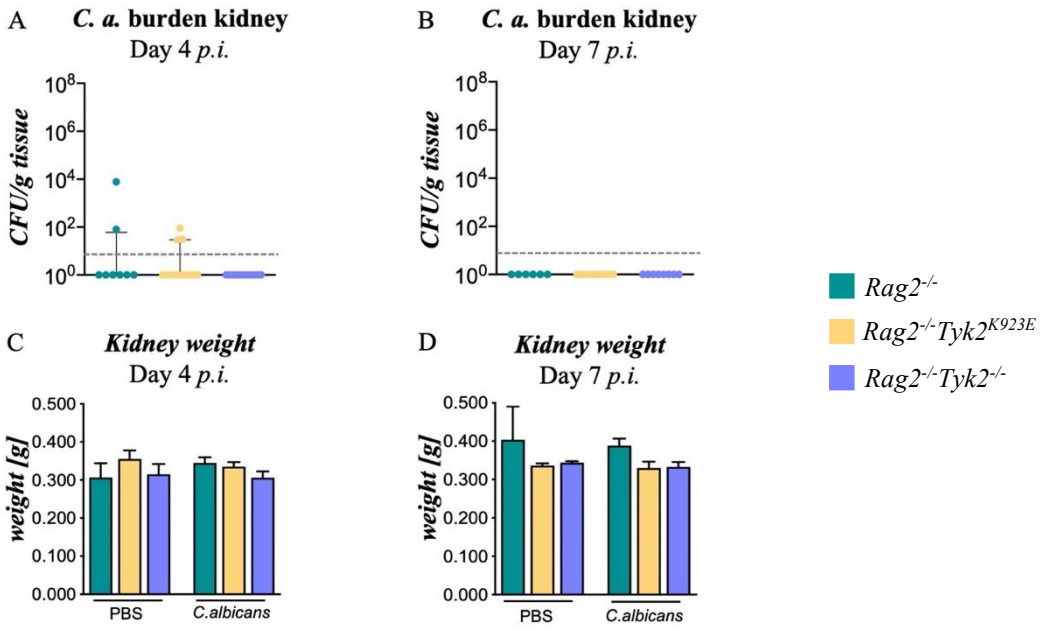

Figure 9: Rag2$^{-/-}$*, Rag2*$^{-/-}$*Tyk2*^{K923E} *and Rag2*$^{-/-}$*Tyk2*$^{-/-}$ *animals were intradermally infected with 1x10*^{8} *CFU of C. albicans. The kidneys were harvested and CFU assays were performed 4 days (**A**), and 7 days (**B**) p.i. (A-B) n=6-11; pooled from 2 independent experiments; Median ± interquartile range is given. Kidneys were weighed on day 4 p.i. (**C**) and on day 7 p.i. (**D**). (C-D) n=6-11, pooled from 2 independent experiments; Mean ± SEM is given.*

3.3 *C. albicans* does not invade into deeper skin tissue in mice that lack T cells and B cells, irrespective of the presence or absence of TYK2 and TYK2^{K923E}.

In previous experiments, invasion of yeast cells into deep skin was observed in *WT* but not *Tyk2*$^{-/-}$ and *Tyk2*^{K923E} mice (Miranda et al. unpublished). Thus, infected *Rag2*$^{-/-}$, *Rag2*$^{-/-}$ *Tyk2*^{K923E} and *Rag2*$^{-/-}$*Tyk2*$^{-/-}$ mice were also examined for fungal dissemination.

To investigate the fungal penetration into the skin, Gomori methenamine-silver nitrate (GMS) staining for fungi was used. A dense black area could be observed in *Rag2*$^{-/-}$ mice, where the fungi seem to be contained and mainly present in the yeast form (Figure 10A). With increased magnification (Figure 10B), tissue intruding hyphae could be seen but no tissue invasion of the yeast form was found (Figure 10C). A similar picture was observed in *Rag2*$^{-/-}$*Tyk2*^{K923E} and *Rag2*$^{-/-}$*Tyk2*$^{-/-}$ animals: *C. albicans* was mainly contained to the injection site and mostly present in its yeast form with only few hyphae and barely any invasion into deep skin (Figure 10D-F and data not shown). No differences between mice of the 3 genotypes were detectable, indicating that fungal invasion into the deep skin is not affected by TYK2 in mice that lack T and B cells.

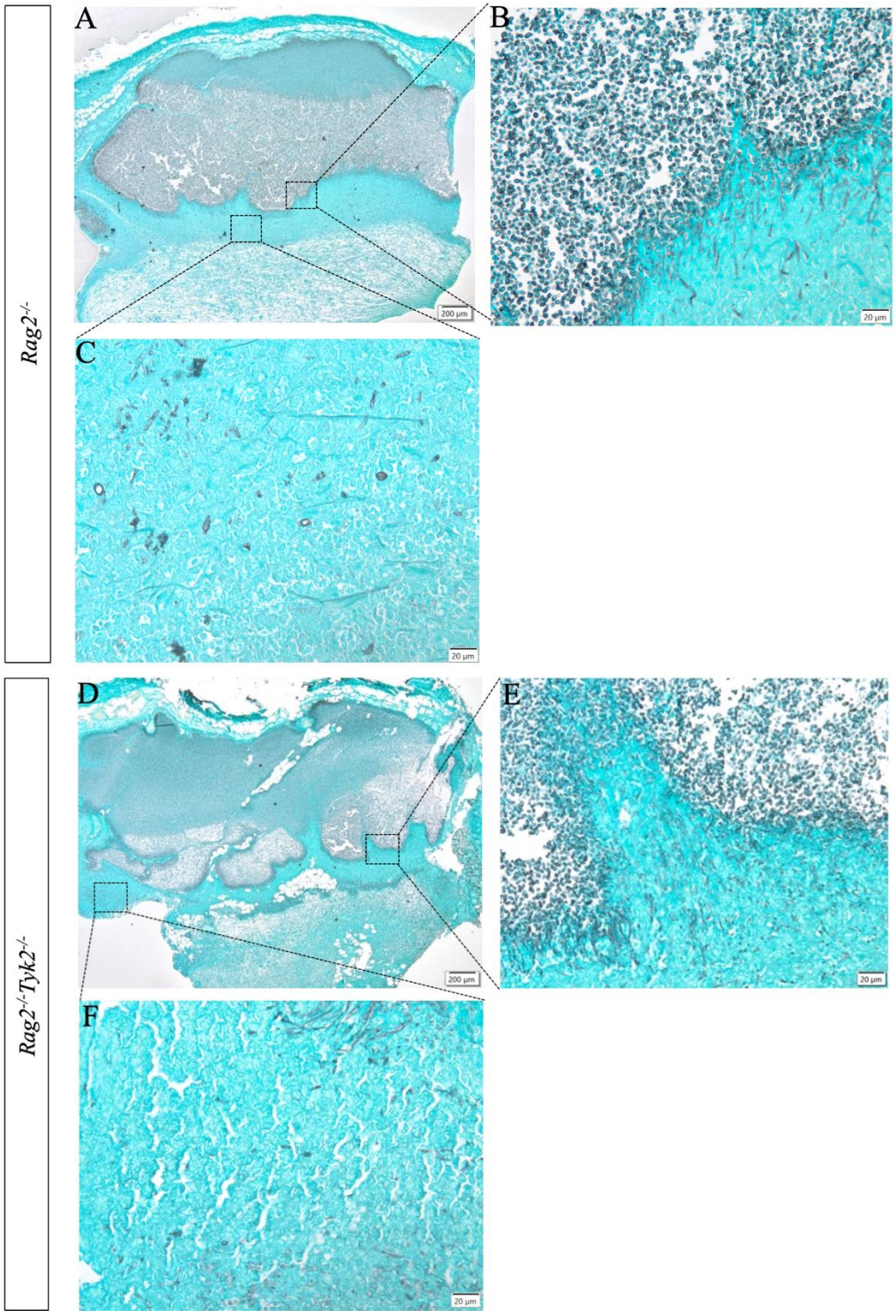

*Figure 10: Rag2$^{-/-}$ and Rag2$^{-/-}$Tyk2$^{-/-}$ animals were intradermally infected with 1x10^{8} CFU of C. albicans. The skin was harvested and stained with GMS at day 4 p.i. Skin sections from Rag2$^{-/-}$ mice are shown at 4x (**A**) and 40x magnification (**B**), (**C**). Skin sections stained with GMS at day 4 p.i. from Rag2$^{-/-}$Tyk2$^{-/-}$ mice are shown at 4x (**D**) and 40x magnification (**E**), (**F**). Representative pictures are shown.*

3.4 Intradermal *C. albicans* infection induces massive neutrophil infiltration, independently of the presence of T cell and B cells and TYK2.

Immune responses at the injection site were investigated by staining for infiltrating neutrophils. In *Rag2*$^{-/-}$, *Rag2*$^{-/-}$*Tyk2*^{K923E} and *Rag2*$^{-/-}$*Tyk2*$^{-/-}$ animals, neutrophils (shown in brown) accumulate surrounding the injected fungi, which appear in light blue in our staining protocol (Figure 11A-D). We did not detect gross differences in the amount and distribution of neutrophils between mice of the 3 genotypes (Figure 11A-D and data not shown), indicating that neutrophil recruitment upon *C. albicans* skin infection is independent of TYK2 in *Rag2*$^{-/-}$ mice.

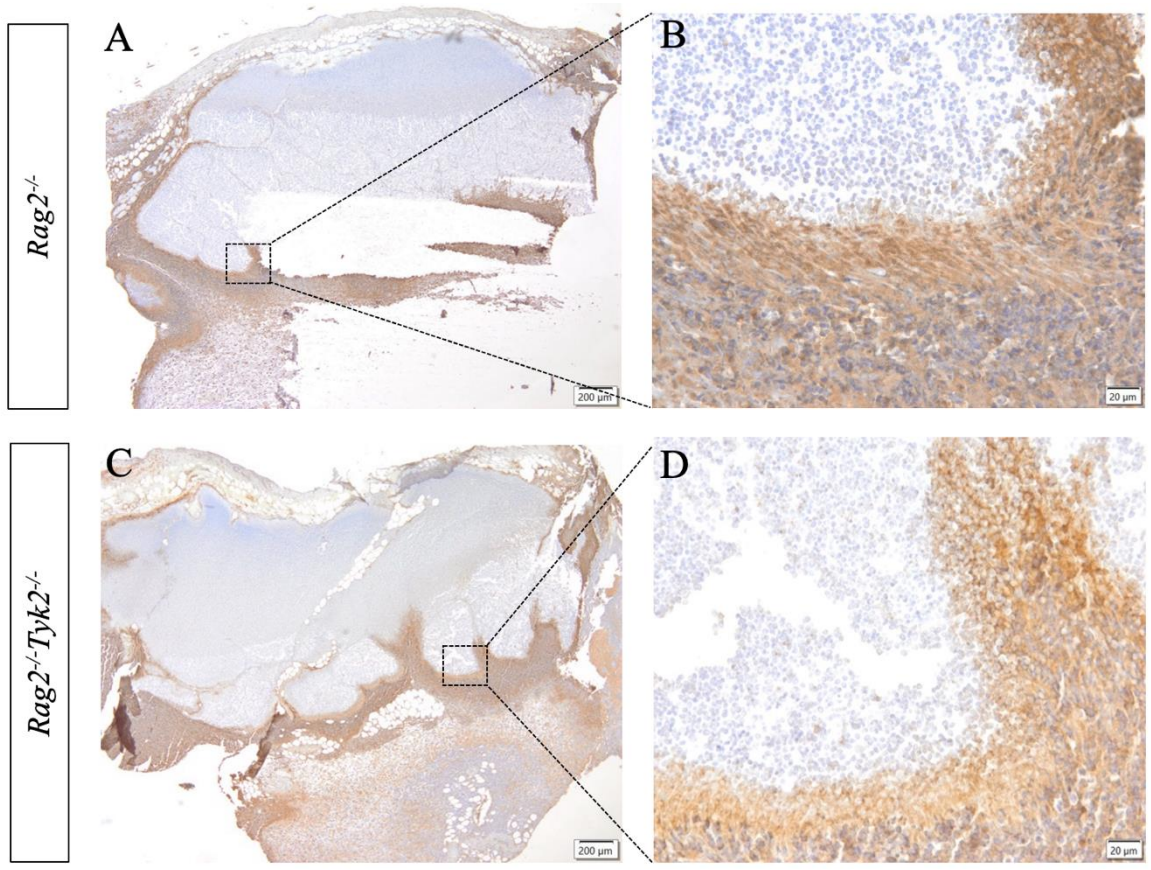

Figure 11: Rag2$^{-/-}$ *and Rag2*$^{-/-}$*Tyk2*$^{-/-}$ *animals were intradermally infected with 1x10*^{8} *CFU of C. albicans. The skin was harvested and stained with NIMP-R14 at day 4 p.i. Skin sections from Rag2*$^{-/-}$ *mice are shown at 4x (**A**) and 40x magnification (**B**). Skin sections stained with GMS at day 4 p.i. from Rag2*$^{-/-}$*Tyk2*$^{-/-}$ *mice are shown at 4x (**D**) and 40x magnification (**E**). Representative pictures are shown.*

3.5 Absence of TYK2 or its kinase activity impairs the upregulation of *Ifng* mRNA in the skin of *Rag2*$^{-/-}$ mice, whereas it does not affect the upregulation of *Il22* mRNA.

In *Rag2*-proficient mice, enzymatically active TYK2 was needed for the upregulation of *Ifng* mRNA in the skin at day 4 *p.i.*, whereas it did not affect the upregulation of *Il22* mRNA at this time point *p.i.* (Miranda et al. unpublished). Therefore, we investigated *Ifng* and *Il22* gene expression in skin biopsies from *Rag2*$^{-/-}$, *Rag2*$^{-/-}$*Tyk2*^{K923E} and *Rag2*$^{-/-}$*Tyk2*$^{-/-}$ mice.

Il22 expression was significantly upregulated upon infection in *Rag2*$^{-/-}$*Tyk2*^{K923E} and *Rag2*$^{-/-}$*Tyk2*$^{-/-}$ mice at day 4 *p.i.* compared to PBS samples (Figure 12A). *Ifng* mRNA was barely upregulated in the skin of *Rag2*$^{-/-}$ mice in response to intradermal *C. albicans* infection and the variation between mice was high compared to *Il22* (Figure 12A). *Ifng* mRNA was not detectable in skin homogenates from infected *Rag2*$^{-/-}$*Tyk2*^{K923E} and *Rag2*$^{-/-}$*Tyk2*$^{-/-}$ mice (Figure 12B). Neither *Ifng* nor *Il22* mRNA was detectable on day 7 *p.i.* in mice of the 3 genotypes (data not shown).

These findings suggest that the expression of *Il22* in response to intradermal *C. albicans* infection occurs independent of TYK2, whereas the low levels of *Ifng* mRNA induction observed in the absence of T cells and B cells may be produced in a TYK2-dependent manner.

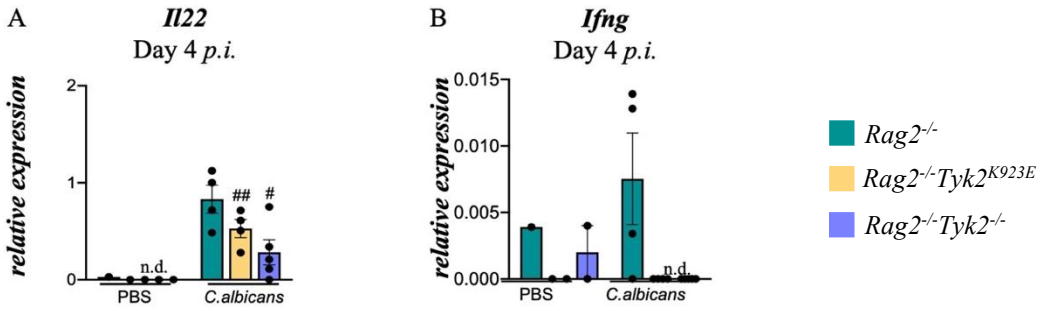

Figure 12: Rag2$^{-/-}$*, Rag2*$^{-/-}$*Tyk2*^{K923E} *and Rag2*$^{-/-}$*Tyk2*$^{-/-}$ *animals were intradermally infected with 1x10*^{8} *CFU of C. albicans. Il22 (**A**) and Ifng (**B**) mRNA expression was determined in skin homogenates by qPCR at day 4 p.i.. Gene expression is given relative to the house keeping gene Ube2d2. (A-B) n=1-5, pooled from 2 independent experiments; Mean values ± SEM are given. # show significant difference compared to the PBS control, # p ≤ 0.05, ## p ≤ 0.01, ### p ≤ 0.001, #### p ≤ 0.0001.*

3.6 Absence of TYK2 does not grossly affect shifts in blood cell composition in response to intradermal *C. albicans* infection in *Rag2*$^{-/-}$ mice.

We next performed differential blood analysis to better understand the systemic immune response to the local fungal infection.

White blood cell counts increased in all mice upon infection, while red blood cells were not affected (Figure 13A and data not shown). *Rag2*$^{-/-}$*Tyk2*^{K923E} mice showed slightly higher levels of white blood cells than *Rag2*$^{-/-}$ and *Rag2*$^{-/-}$*Tyk2*$^{-/-}$ mice on day 4 *p.i.* (Figure 13A). Within the white blood compartment, the frequency of granulocytes increased upon infection, with a peak on day 4 *p.i.* and no difference between *Rag2*$^{-/-}$, *Rag2*$^{-/-}$*Tyk2*^{K923E} and *Rag2*$^{-/-}$*Tyk2*$^{-/-}$ mice (Figure 13B). The frequency of lymphocytes decreased after infection to similar levels in mice of all 3 genotypes (Figure 13C). The frequency of monocytes decreases at day 4 *p.i.* compared to PBS controls, albeit this was not statistically significant (Figure 13D).

Taken together, these data show that intradermal *C. albicans* infection induces a shift in blood cell composition in *Rag2*$^{-/-}$ mice, indicating the induction systemic immune responses is independent of the presence of T cells and B cells. In addition, data suggest that TYK2 does not grossly impact on these responses.

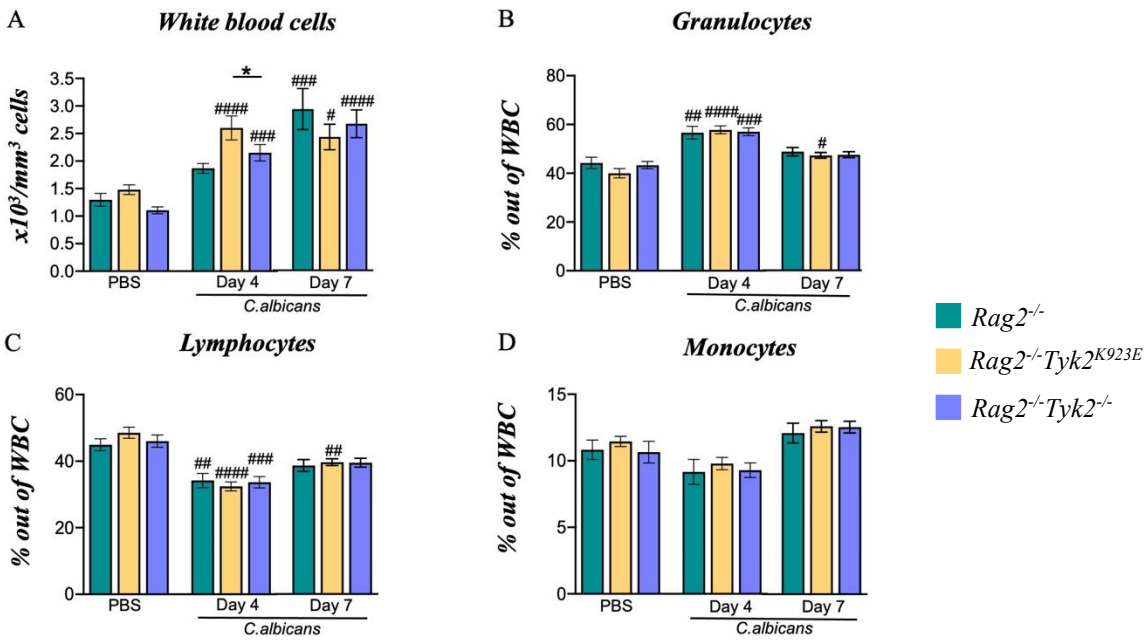

*Figure 13: Rag2$^{-/-}$, Rag2$^{-/-}$Tyk2^{K923E} and Rag2$^{-/-}$Tyk2$^{-/-}$ animals were intradermally infected with 1x10^{8} CFU of C. albicans. Blood was drawn 4 and 7 days p.i. and analyzed with VetABC. Counts of white blood cells (**A**) and percentage of granulocytes (**B**), lymphocytes (**C**) and monocytes (**D**) out of white blood cells (WBC) were measured 4 and 7 days, p.i.. (A-D) n=6-11, pooled from 2 independent experiments; Mean ± SD is given. * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001, **** p ≤ 0.0001; # show significant difference compared to the PBS control, # p ≤ 0.05, ## p ≤ 0.01, ### p ≤ 0.001, #### p ≤ 0.0001.*

3.7 Absence of TYK2 or presence of TYK2^{K923E} accelerates wound healing in *Rag2$^{-/-}$* mice upon intradermal *C. albicans* infection

To evaluate disease progression and healing capacity, *Rag2$^{-/-}$*, *Rag2$^{-/-}$Tyk2^{K923E}* and *Rag2$^{-/-}$Tyk2$^{-/-}$* mice were intradermally infected with *C. albicans* and wounds were monitored until completed healing at the macroscopic level. The wounds were monitored every day and, in addition, body weight was measured every 3 to 4 days. Furthermore, blood was drawn on day 2 and day 34 *p.i.*

Wounds of all *Rag2$^{-/-}$Tyk2^{K923E}* and *Rag2$^{-/-}$Tyk2$^{-/-}$* mice were fully healed approximately 21 days *p.i.,* whereas wounds of *Rag2$^{-/-}$* mice were healed on day 32 *p.i.* (Figure 14A). Mice challenged with *C. albicans* lost 7-13% of their initial body weight within the first 7 days but regained their initial body weight after 11 days. Despite the differences in wound healing,

Rag2$^{-/-}$, *Rag2*$^{-/-}$*Tyk2*^{K923E} and *Rag2*$^{-/-}$*Tyk2*$^{-/-}$ mice showed comparable weight loss and recovery (Figure 14B).

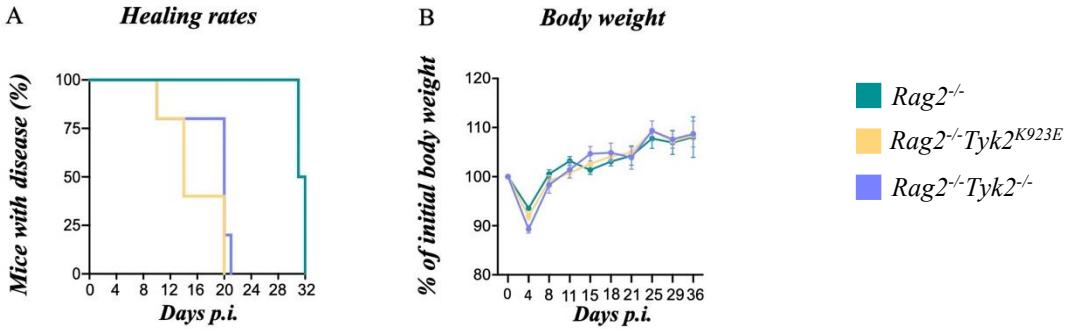

Figure 14: Rag2$^{-/-}$*, Rag2*$^{-/-}$*Tyk2*^{K923E} *and Rag2*$^{-/-}$*Tyk2*$^{-/-}$ *animals were intradermally infected with 1x10*^{8} *CFU of C. albicans. Wound healing was monitored for up to 36 days and data are given as healing rates (**A**). Body weight was monitored in 3 to 4-day intervals (**B**). (A-B) Rag2*$^{-/-}$*, n=2; Rag2*$^{-/-}$*Tyk2*^{K923E}*, n=5; Rag2*$^{-/-}$*Tyk2*$^{-/-}$*, n=5; Data are from 1 experiment.*

White blood cell counts increased as early as day 2 after infection in mice of all genotypes and were still elevated on day 34 *p.i.* (Figure 15A). In contrast to our findings on day 4 and day 7 *p.i.* (Figure 13A), *Rag2*$^{-/-}$*Tyk2*$^{-/-}$ mice showed lower white blood cell counts than *Rag2*$^{-/-}$ and *Rag2*$^{-/-}$*Tyk2*^{K923E} mice at day 2 and day 34 *p.i.* (Figure 15A). The reason for this discrepancy remained unclear. The frequency of granulocytes and lymphocytes remained grossly unchanged by the infection at day 2 and day 34 *p.i.* (Figure 15B, 15C), whereas the frequency of monocytes slightly decreased in mice of all genotypes, albeit not statistically significant (Figure 15D). Except for a modest difference in the frequency of granulocytes between *Rag2*$^{-/-}$*Tyk2*^{K923E} and *Rag2*$^{-/-}$*Tyk2*$^{-/-}$ mice, we did not observe differences between mice of the 3 genotypes (Figure 15B-D).

Overall, these data suggest that the lack of TYK2 or its enzymatic activity improves wound healing after *C. albicans* infection. Furthermore, blood cell counts indicate reduced systemic response to the local infection in *Rag2*$^{-/-}$*Tyk2*$^{-/-}$ compared to *Rag2*$^{-/-}$ and *Rag2*$^{-/-}$*Tyk2*^{K923E}. However, the latter conclusion is only based on a reduced increase in white blood cells and not consistent with results on day 4 and day 7 *p.i.*

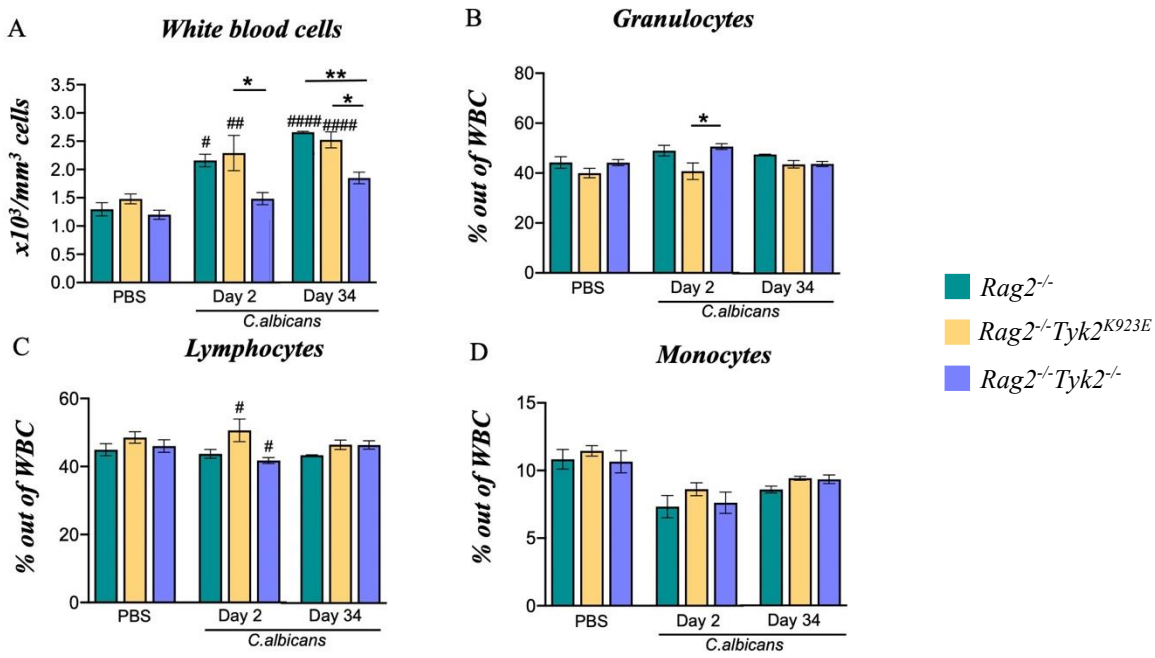

*Figure 15: Rag2$^{-/-}$, Rag2$^{-/-}$Tyk2^{K923E} and Rag2$^{-/-}$Tyk2$^{-/-}$ animals were intradermally infected with 1x10^{8} CFU of C. albicans. Blood was drawn 2 and 36 days p.i. and analyzed with VetABC. Counts of white blood cells (A) and percentage of granulocytes (B), lymphocytes (C) and monocytes (D) out of white blood cells were measured 2 and 36 days, p.i. (A-D); n=6-11; pooled from 2 independent experiments; Mean ± SD is given. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$; # show significant difference towards the PBS control, wherein # $p \leq 0.05$, ## $p \leq 0.01$, ### $p \leq 0.001$, #### $p \leq 0.0001$.*

4 Discussion

Studies in our laboratory revealed that the absence of TYK2 or the presence of a kinase-inactive version of TYK2 in mice results in increased resistance to intradermal *C. albicans* infection (Miranda et al. unpublished). *Tyk2*^{K923E} and *Tyk2*$^{-/-}$ mice had decreased local fungal load in the skin and decreased fungal dissemination into peripheral organs compared to *WT* mice on days 2 and 4 *p.i.* and showed accelerated wound healing. Herein, we used *Rag2*$^{-/-}$, *Rag2*$^{-/-}$*Tyk2*^{K923E} and *Rag2*$^{-/-}$*Tyk2*$^{-/-}$ mice, which all lack T and B cells, to investigate whether TYK2 signalling in innate immune cells acts detrimental during cutaneous candidiasis.

The fungal load in the skin of *Rag2*$^{-/-}$, *Rag2*$^{-/-}$*Tyk2*^{K923E} and *Rag2*$^{-/-}$*Tyk2*$^{-/-}$ mice did not differ on day 4 and day 7 *p.i.*, (Figure 7) suggesting that TYK2 in non-B and non-T cells, such as neutrophils, macrophages and DCs, does not impact on the early control of fungal growth in the skin. The fungal load in *Rag2*$^{-/-}$ mice was 10 times higher than in *Rag2*-proficient mice (Figure 7 and Miranda et al. unpublished), indicating that T cells or B cells play a protective role in the local control of *C. albicans* in the skin. As B cells have a minor role in the early defence against *C. albicans* infections, it seems reasonable to speculate that skin-resident T lymphocytes contribute to the early defence against *C. albicans* (Richmond and Harris, 2014; Sinha et al., 1987). Among these, γδ T cells are the most likely candidates because they are innate-like T lymphocytes with a well-described protective role in cutaneous candidiasis (Castillo-González et al., 2021). To study the contribution of TYK2 signalling in T cells to the defence against cutaneous *C. albicans* infection, conditional deletion of TYK2 in T cells using for example the CD4-Cre system, which deletes in CD4$^+$ and CD8$^+$ T cells (Lee et al., 2001), would be an option.

Ifng mRNA was barely detectable in the skin of *C. albicans* infected *Rag2*$^{-/-}$, *Rag2*$^{-/-}$*Tyk2*^{K923E} and *Rag2*$^{-/-}$*Tyk2*$^{-/-}$ mice, whereas it was strongly induced by the infection in *WT* mice (Figure 12 and Miranda et al. unpublished). As B cells do not produce IFNγ, these data suggests that T cells are the main producers of IFNγ in response to intradermal *C. albicans* infection and that NK cells and ILCs, which are still present in *Rag2*$^{-/-}$ mice, do not significantly contribute to IFNγ production in our experimental model (O'Neill et al., 2020). *IL22* expression was upregulated to similar levels in *C. albicans* infected *Rag2*$^{-/-}$ and *WT* mice (Figure 12A and

Miranda et al. unpublished), indicating that non-B cells and non-T cells are the main producer of IL-22 in our experimental model. Among these cells type 3 ILCs are the most likely candidates, as they are capable of producing IL-22 (Kobayashi et al., 2020). However, this needs confirmation at the protein level and in a direct comparison of *Rag2*$^{-/-}$ and *WT* mice. Importantly, *Il22* mRNA was upregulated at day 4 p.i. with *C. albicans* independent of TYK2 (Figure 12A).

Surprisingly, *Rag2*$^{-/-}$ mice barely showed fungal dissemination into the spleen or the kidneys, irrespective of the presence or absence of TYK2 (Figure 8 and 9). Immunohistochemical staining for *C. albicans* in the skin showed that the fungus is more contained at the infection site and only a few hyphae penetrated the deeper skin of *Rag2*$^{-/-}$ mice (Figure 10). In contrast, substantial yeast penetration into deeper layers of tissue and dissemination into kidneys and spleen was seen in *WT* mice (Miranda et al. unpublished). These results would imply that T cells or B cells promote fungal dissemination. A direct comparison of *Rag2*$^{-/-}$ mice and *WT* mice would be required to confirm this finding.

Another interesting finding of our study is that *Rag2*$^{-/-}$*Tyk2*^{K923E} and *Rag2*$^{-/-}$*Tyk2*$^{-/-}$ mice healed the infection wounds substantially quicker than *Rag2*$^{-/-}$ mice (Figure 14). These results suggest that TYK2 signalling in non-T cells and non-B cells decelerates wound healing. Neutrophils, macrophages, platelets and fibroblasts are vital for wound healing processes (Yussof et al., 2012), raising the possibility that the absence of TYK2 in any of these cell types might facilitate wound healing and recovery. However, the experiment was done only once and with a small number of *Rag2*$^{-/-}$ mice and thus needs to be repeated to allow firm conclusions.

Collectively, our findings suggest that in the absence of T cells and B cells, TYK2 signalling does not affect local *C. albicans* growth within the first seven days after intradermal infection but may delay wound healing.

5 Summary

5.1 English

Candida albicans is a facultative pathogenic fungus. In good health, *C. albicans* is part of the human microflora on mucous membranes such as the mouth, digestive and genital tracts and the skin. A weakened immune system allows the fungus to invade the host´s tissue and cause infections of the skin, mucosal membranes and nails. About 1 billion people worldwide struggle with local fungal infections. More than 150 million people are affected by severe invasive infections as the fungus can disseminate into the kidneys, spleen and other organs, leading to approximately 1.7 million deaths per year. Cases of resistance to common fungicidal drugs are increasing and new therapeutic approaches are still in their infancy. It is thus extremely important to better understand the molecular processes involved in the host immune response against fungal pathogens. TYK2 is a JAK/STAT signalling kinase that is indispensable for the protection against viral and bacterial infections. However, the role of TYK2 in fungal infections is largely unexplored.

Previous studies in our group showed that TYK2 has a detrimental role in cutaneous candidiasis. TYK2 deficient (*Tyk2*$^{-/-}$) mice and mice that express a kinase-inactive version of TYK2 (*Tyk2*^{K923E}) showed decreased fungal load in the skin and decreased fungal dissemination to distal organs at day two and four *post infection* and accelerated wound healing compared to *WT* mice upon intradermal infection with *C. albicans*. The early phenotype raised the hypothesis that TYK2 signalling in innate immune cells has a detrimental role in the immune defence against *C. albicans* skin infections. To test this hypothesis, we used *Tyk2*$^{-/-}$ and *Tyk2*^{K923E} mice on a *Rag2*$^{-/-}$ background, which lack mature T and B cells, and intradermally infected them with *C. albicans*. We show that *Rag2*$^{-/-}$, *Rag2*$^{-/-}$*Tyk2*$^{-/-}$ and *Rag2*$^{-/-}$*Tyk2*^{K923E} mice are equally able to control the local infection, as evidenced by comparable fungal loads in the skin on day four and day seven *post infection*. Interestingly, we discovered that skin wounds in *Rag2*$^{-/-}$*Tyk2*^{K923E} and *Rag2*$^{-/-}$*Tyk2*$^{-/-}$ mice healed faster than in *Rag2*$^{-/-}$ controls. In contrast to what was previously observed in *Rag2*-

proficient mice, *Rag2*$^{-/-}$ animals showed barely any fungal dissemination into the spleen or kidneys within the first week after the infection, irrespective of the presence of TYK2. In line with this, we did not observe fungal invasion into deeper tissues of the skin in *Rag2*$^{-/-}$ mice. This suggests that T cells or B cells promote *C. albicans* dissemination to distal organs.

Collectively, our data show that in the absence of T and B cells, TYK2 signalling does not affect local *C. albicans* growth, tissue invasion and dissemination of fungi to spleen and kidneys within the first seven days following intradermal infection. However, the data also indicate that TYK2 signalling may delay wound healing, although this awaits confirmation.

5.2 Deutsch

Candida albicans ist ein fakultativ pathogener Pilz. Bei guter Gesundheit ist *C. albicans* Teil der menschlichen Mikroflora auf Schleimhäuten wie im Mund, im Verdauungs- und Genitaltrakt und auf der Haut. Ein geschwächtes Immunsystem ermöglicht es dem Pilz, in das Gewebe des Wirts einzudringen und Infektionen der Haut, der Schleimhäute und der Nägel zu verursachen. Weltweit haben etwa 1 Milliarde Menschen mit lokalen Pilzinfektionen zu kämpfen. Mehr als 150 Millionen Menschen sind von schweren invasiven Infektionen betroffen, da der Pilz in die Nieren, die Milz und andere Organe eindringen kann, was zu etwa 1,7 Millionen Todesfällen pro Jahr führt. Die Fälle von Resistenz gegen gängige pilztötende Medikamente nehmen zu, und neue therapeutische Ansätze stecken noch in den Kinderschuhen. Es ist daher äußerst wichtig, die molekularen Prozesse besser zu verstehen, die an der Immunantwort des Wirts gegen Pilzerreger beteiligt sind. TYK2 ist eine JAK/STAT-Signalkinase, die für den Schutz vor viralen und bakteriellen Infektionen unerlässlich ist. Die Rolle von TYK2 bei Pilzinfektionen ist jedoch noch weitgehend unerforscht.

Frühere Studien unserer Gruppe haben gezeigt, dass TYK2 bei der kutanen Kandidose eine nachteilige Rolle spielt. TYK2-defiziente (*Tyk2*$^{-/-}$) Mäuse und Mäuse, die eine kinase-inaktive Version von TYK2 (*Tyk2*^{K923E}) exprimieren, zeigten am zweiten und vierten Tag nach der Infektion eine geringere Pilzbelastung in der Haut und eine geringere Pilzausbreitung in periphere Organe sowie eine beschleunigte Wundheilung im Vergleich zu wildtyp (*WT)* Mäusen bei intradermaler Infektion mit *C. albicans*. Der frühe Phänotyp warf die Hypothese auf, dass die TYK2-Signalübertragung in angeborenen Immunzellen eine nachteilige Rolle bei der Immunabwehr gegen *C. albicans* Hautinfektionen spielt. Um diese Hypothese zu testen, verwendeten wir *Tyk2*$^{-/-}$ und *Tyk2*^{K923E} Mäuse und kreuzten diese mit einem *Rag2*$^{-/-}$ Mäusen, denen reife T- und B-Zellen fehlen. Die erhaltenen Mäuse wurden intradermal mit *C. albicans* infiziert. Wir konnten zeigen, dass *Rag2*$^{-/-}$, *Rag2*$^{-/-}$*Tyk2*$^{-/-}$ und *Rag2*$^{-/-}$*Tyk2*^{K923E} Mäuse gleichermaßen in der Lage sind, die lokale Infektion zu kontrollieren, was durch eine vergleichbare Pilzbelastung der Haut am vierten und siebten Tag nach der Infektion belegt wird. Überraschenderweise stellten wir fest, dass Hautwunden bei *Rag2*$^{-/-}$*Tyk2*^{K923E} und *Rag2*$^{-/-}$*Tyk2*$^{-/-}$ Mäusen schneller heilten als bei *Rag2*$^{-/-}$ Mäusen. Im

Gegensatz zu dem, was zuvor bei *Rag2* kompetenten Mäusen beobachtet wurde, zeigten *Rag2*$^{-/-}$ Mäuse innerhalb der ersten Woche nach der Infektion kaum eine Pilzausbreitung in die Milz oder die Nieren, unabhängig von der Anwesenheit von TYK2. Im Einklang mit diesen Ergebnissen, konnten wir bei *Rag2*$^{-/-}$ Mäusen keine Invasion der Pilze in tiefere Gewebe der Haut beobachten. Dies deutet darauf hin, dass T-Zellen oder B-Zellen die Verbreitung von *C. albicans* in periphere Organe fördern.

Zusammenfassend zeigen unsere Daten, dass der TYK2-Signalweg in Abwesenheit von T- und B-Zellen das lokale Wachstum von *C. albicans*, die Invasion in das Gewebe und die Verbreitung der Pilze in Milz und Nieren innerhalb der ersten sieben Tage nach der intradermalen Infektion nicht beeinflusst. Weiters deuten die Daten des Wundheilungsexperiments darauf hin, dass der TYK2-Signalweg die Wundheilung verzögern kann, obwohl dies noch nicht bestätigt wurde.

6 Index of abbreviations

APC	Antigen-presenting cells
ATP	Adenosine triphospate
CFU	Colony-forming unit
DC	Dentritic cell
dDC	Dermal Dentritic cells
G-CSF	granulocyte colony-stimulating factor
HKG	Housekeeping gene
IFN	Interferon
IL	Interleukin
JAK	Janus kinase
LC	Langerhans cells
NETs	Neutrophil extracellular traps
NF-κB	Nuclear factor kappa B
NK cell	Natural killer cell
NLR	NOD-like receptor
NLRP3	NLR family pyrin domain containing 3
NOD	Nucleotide-binding oligomerization domain
NTC	No template control
PBS	Phosphate-buffered saline
PRR	Pattern recognition receptor
qPCR	Quantitative PCR
R^2	Correlation coefficient
RAG	Recombination activating gene
ROS	Reactive oxygen species
RT	Reverse transcriptase
STAT	Signal transducer and activator of transcription
TCR	T-cell receptor
Th cell	T helper cell
TLR	Toll-like receptor
TYK2	Tyrosine kinase 2
WBC	White blood cells
WT	Wildtype
YPD	Yeast extract peptone dextrose

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