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“Type I Interferon Immune Signaling in Murine Systemic
Candidiasis”

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1 Summary

Fungal sepsis is the most frequent cause of death between hospitalized human patients and *Candida* spp. being the most frequent cause among. Disseminated candidiasis is a life-threatening disease in immunocompromised individuals with a mortality rate of around 30-40%. The yeast-like fungus *Candida glabrata* has emerged as the second most frequent cause of systemic candidiasis. Type I interferons (IFNs) are important modulators of the innate host immunity. Although our understanding about role of type I IFNs in bacterial and viral infections has improved, their role in fungal infections are recently being revealed. Our group has previously reported that *Candida* spp. induce type I interferon (IFN) response in conventional dendritic cells via recognition of *Candida* nucleic acids the TLR7 pattern recognition receptor. Moreover, type I IFNs also contribute to fungal persistence in a murine model of systemic candidiasis.

Here, we have further investigated the role of type I IFNs in murine systemic *C. glabrata* infections. We show that type I IFNs influence pathogen clearance in an organ- and timely-dependent manner, where type I IFN signaling contributes to persistence at later stages of infection. Spleens were more enlarged in the absence of type I IFN signaling at day 7. Additionally, the transcriptional response was also higher at day of IFNAR1^{-/-} spleens. Also our data suggest a delay or absence in resolution of immune responses. Using clustering techniques and with NodeFinder, novel software tool we have created to group genes into co-regulated network, identified several important pathways involved. Immunity-related GTPases were higher expressed in IFNAR1^{-/-} at day 3 of the infection. Noteworthy, these GTPases were also IFN- γ inducible. Interestingly, another group of genes under type II IFN (IFN- γ) regulation was also higher expressed in IFNAR1^{-/-} spleens. Importantly, genes with antimicrobial inflammatory properties were also higher expressed in IFNAR1^{-/-}. Moreover wildtype (WT) spleens were recruiting more inflammatory monocytes upon infection and more macrophages in steady state. Yet macrophage phagocytosis and killing were not affected by type I IFNs *in vitro*. Taken together, our work demonstrates on important role of type I IFN signaling in a murine model of invasive Candidemia.

2 Zusammenfassung

Pilzartige Sepsis ist die häufigste Todesursache bei Krankenhausaufnahmen und *Candida* ist dabei das häufigst isolierte Pathogen. Systemische *Candida* Infektionen zeigen eine Mortalitätsrate von etwa 30-40% bei immunkomprimierten Patienten. *Candida glabrata* ist ein hefeartiger pathogener Pilz, der sich als die zweit-häufigste Ursache der systemischen Candidiasis entwickelt hat. Typ I Interferone (Typ I IFN) sind wichtige Regulatoren des angeborenen Immunsystems. Obwohl unser Verständnis über die Rolle von den Typ I IFN bei viralen und bakteriellen Erkrankungen sich verbessert hat, ihre Rolle bei pilzerkrankungen wird erst neu ausführlich beschrieben. Unsere Gruppe hat schon publiziert, dass die *Candida* Spezies bei konventionellen dendritischen Zellen durch die Erkennung der fungalen Nukleinsäuren von TLR7 (Mustererkennungsrezeptor) die Typ I IFN induzieren. Außerdem tragen die Typ I IFN bei der Ausdauer der systemischen Infektion mit *C. glabrata* in Mäusen bei.

Hier haben wir die Rolle von Typ I IFN in murinen systemischen *C. glabrata* Infektionen in Milz weiter untersucht. Wir haben gezeigt, dass die Rolle von Typ I IFN bei der Entfernung des Pathogens organ- und zeitabhängig ist, wobei die Typ I IFN die Ausdauer der Infektion bei späteren Stadien fördern. Am 7. Tag der Infektion waren die Milze vergrößert von IFNAR1^{-/-} Mäusen und zusätzlich war die transkriptionelle Antwort stärker. Durch die Anwendung von Clustering-Methoden und mit dem NodeFinder (ein neues Computer-Programm, womit wir die Gene in koregulierten Netzwerken gruppiert haben) haben wir mehrere wichtige beteiligte Mechanismen entdeckt. Immunität-bezogene GTPasen waren höher exprimiert in IFNAR1^{-/-}-Milz am 3. Tag. Merkwürdigerweise stehen diese GTPasen unter Typ II IFN (IFN- γ) Regulation. Zusätzlich waren andere von Typ II IFN-regulierten Gene zum selben Zeitpunkt der Infektion (und 1. Tag) in der IFNAR1^{-/-}-Milz höher exprimiert. Interessanterweise waren antimikrobielle und inflammatorische Gene höher exprimiert am 7. Tag. Zudem waren mehr inflammatorische Monozyten während der Infektion und auch mehr Makrophagen im Stillstand in WT-Milz rekrutiert. Die Phagozytose und tötende Eigenschaften von Makrophagen waren nicht betroffen *in vitro*. Unsere Befunde stellen die wichtige Rolle der Typ I IFN bei der systemischen Candidiasis in Mäusen dar.

3 Introduction

3.1 Fungal Infections

Fungal infections affect about 25% of the human population worldwide making them the most prevalent microbial infections(Brown et al., 2012). Fungal sepsis is a major cause of death between hospitalized human patients with immunodeficiencies and *Candida* spp. being the most frequent cause among(Pfaller and Diekema, 2007). In United States from 1979 to 2000, the number of sepsis cases by fungal pathogens has almost doubled(Martin et al., 2003). Also, a dramatic increase in multiple-cause mortality due to systemic mycoses was recorded from 1557 deaths in 1980 to 6534 deaths in 1997(McNeil et al., 2001). Invasive fungal infections occur less often and mostly affect immunocompromised patients such as AIDS patients, cancer patients undergoing chemotherapy, organ transplant recipients, and immunosuppressed patients with autoimmune diseases. Nonetheless, fungal diseases remain a relatively neglected subject worldwide(Armstrong-James et al., 2014; Brown et al., 2012). Main factors involved in opportunistic mycoses are disruption of epithelial and mucosal barriers, neutrophil dysfunction, defects in cell-mediated immunity, metabolic disorders, and extremes of ages(Pfaller and Diekema, 2007).

3.2 Systemic Candidiasis

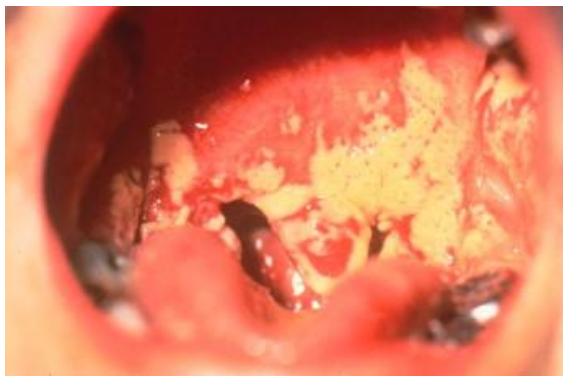


Figure 1: Pseudomembranous form of oropharyngeal candidiasis. (Taken from Thompson III et al., 2010).

Candida spp. are emerged pathogens and most frequent cause for fungal infections(Pfaller and Diekema, 2007) and initially thought to be non-pathogenic(Stenderup and Pedersen, 1962), as these were isolated from healthy individuals. In immunocompetent individuals they are mostly associated with oral/vaginal thrush, skin rashes and mucosal inflammation(Bondaryk et al., 2013; Stoopler and Sollecito, 2014). Systemic candidiasis

is a life-threatening disease with a mortality rate up to 40%(Pfaller and Diekema, 2010).

Beginning with a mucosal non-lethal infection, *Candida* spp. can subsequently disseminate and cause nosocomial blood stream infections (BSI) in immunocompromised patients (Armstrong-James et al., 2014). In HIV/AIDS patients for instance, the primary course of infection is oropharyngeal infection (Figure 1) (Patel et al., 2012).

3.3 *Candida glabrata*

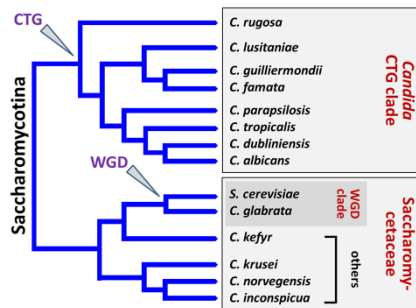


Figure 2: Phylogeny of sequenced *Candida* and *Saccharomyces* clade species.
Taken from Papon et al., 2013.

Candida glabrata (formerly; *Torulopsis glabrata*, is unable to form hyphae (Patel et al., 2012; Pfaller and Diekema, 2010)) and a haploid asexual yeast in the WGD (named after “whole genome duplication” event occurred at the common ancestor) clade (Bennett, 2010; Papon et al., 2013). The term *Candida* was historically attributed to (pseudo)hyphae forming yeasts. Some other species

of *Candida* spp. belong to the CTG clade, where the CTG codon is interpreted as serine instead of leucine (Papon et al., 2013). Unlike other *Candida* sp. *C. glabrata* was never observed in a hyphal form, but only as pseudohyphae (Papon et al., 2013). Mostly growing in the yeast form, recent microevolution experiment indicate that *C. glabrata* is able to form pseudohyphae and become more virulent through a single mutation in chitin synthase-encoding CHS2 gene (Brunke et al., 2014).

First thought to be a non-pathogenic commensal isolated from healthy individuals (Stenderup and Pedersen, 1962), the usage of immunosuppressive drugs promoted *C. glabrata* to emerge as a serious human pathogen (Fidel et al., 1999; Knoke et al., 1997; Pfaller, 1996; Schwab et al., 1997; Wingard et al., 1993). Infections with *C. glabrata* were also often associated with marked fluconazole resistance (Hitchcock et al., 1993; Pfaller, 1996; Schwab et al., 1997; Wingard et al., 1993). Despite its pathogenicity, relatively little is known about the underlying mechanisms of *C. glabrata* pathogenicity.

Azoles are being used since 60s and they are the most successful antifungal compounds in the clinic. A member of the azoles family fluconazole is widely used to treat *Candida*

infections. However, fluconazole resistance in *C. glabrata* has been a serious issue in the clinic(Hitchcock et al., 1993; Ruan et al., 2008). A very recent study published by our group revealed novel antifungal tolerance genes with systemic phenotyping of a large-scale deletion library(Schwarz Müller et al., 2014). The usage of fluconazole might be the reason for the increase in number of *C. glabrata* incidences. This is also in accordance with the shift observed to non—*albicans* candidiasis cases from *C. tropicalis* to *C. glabrata* as the second most frequent cause after introduction of fluconazole as antifungal therapy(Pfaller et al., 2001). However, a newer drug – Caspofungin – has been available since 2001, which is more effective in treating *C. glabrata* infections(Posteraro et al., 2006).

3.4 Innate and Adaptive Immune Systems

Hosts provide niches for colonizing microbes and multicellular parasites and are therefore under constant pressure of being invaded and exploited. This pressure led to development of one of the most complex systems in biology, the innate immune system, which needs to respond to a variety of intruders in a well-regulated appropriate manner in order to survive. Throughout the evolution distinct mechanisms were developed. The immune system can be divided into two subsystems; the phylogenetically oldest mechanism, beginning with first multicellular organisms is the innate immune system, and a newer mechanism appeared in jawed-vertebrates, known as the adaptive immune system.

3.4.1 Innate Immune System

Innate immune system constitute the first defence barrier against pathogens(Murphy et al., 2008), with ability to fine tune the type and intensity of the response. Deregulation of such responses often results in hyper-inflammation, autoimmune disorders and other diseases(Waldner, 2009).

The innate immune system involves many distinct professional cell types especially phagocytes but in broader sense also mucosal barriers, anti-microbial peptides, epithelial tissues, all of which are taking part in host defence(Gallo et al., 2002) (Figure 3). In contrast, professional innate immune cells include phagocytic cells such as dendritic cells, macrophages and neutrophils, natural killer cells and granulocytes including mast cells, basophils and

neutrophils. Innate immune system functions by detecting pathogen associated molecular patterns (PAMPs) with so called pattern recognition receptors (PRRs), such as toll-like receptors (TLRs), nucleotide-binding oligomerization domain receptors (NOD)-like receptors (NLRs), C-type and S-type lectins. Notably, this view suggests that innate immune system recognizes foreign patterns and reacts to those only. However, this view has changed in last two decades, when it was reported that components of innate immune system can also recognize danger signals from the host itself so called “danger associated molecular patterns” (DAMPs)(Matzinger, 1994; Seong and Matzinger, 2004).

	Skin	Gut	Lungs	Eyes/nose
Mechanical	Epithelial cells joined by tight junctions			
	Longitudinal flow of air or fluid		Movement of mucus by cilia	Tears Nasal cilia
Chemical	Fatty acids	Low pH Enzymes (pepsin)		Enzymes in tears (lysozyme)
	Antibacterial peptides			
	Normal flora			
Microbiological				

Figure 2-7 Immunobiology, 7ed. (© Garland Science 2008)

Figure 3: Generic defence barriers against invading pathogens.
Taken from Murphy et al., 2008

Fungal PAMPs and PRRs

Fungi like other classes of microbial pathogens expose molecular patterns, which are not known to the host. In particular, the outer layer of the fungal cells are providing the primary molecules activating host PRRs, including β -glucans, mannans and chitins(Bourgeois et al., 2010). Endosomal and cell surface TLRs, and C-type lectin receptor family are allowing host innate immune cells to recognize “fungal” molecular patterns. C-type lectins among them are relevant to fungal pathogens in particular(Bourgeois and Kuchler, 2012). However, they are not specialized fungal recognizers, their roles in viral and mycobacterial immunity were also described(Kerrigan and Brown, 2011).

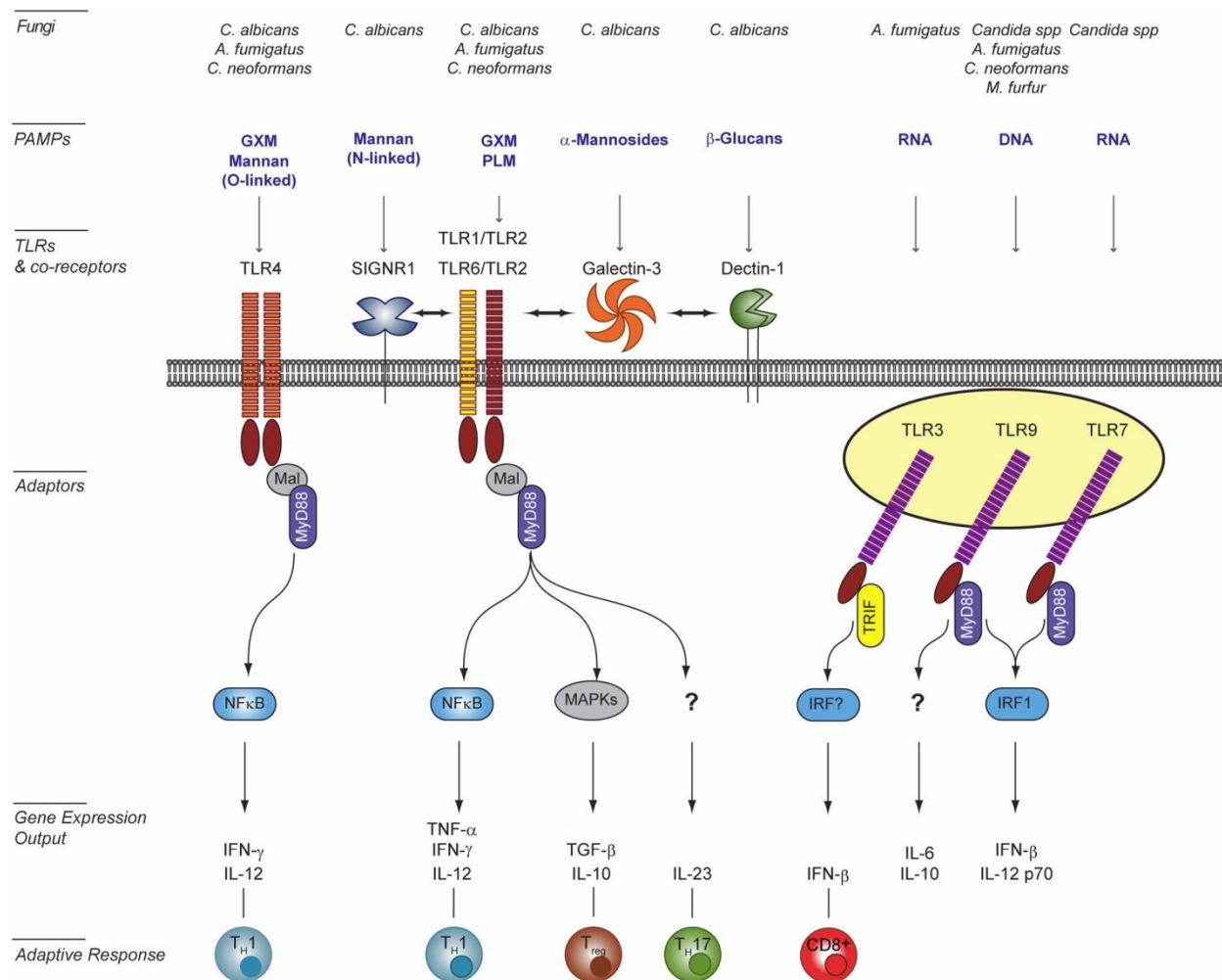


Figure 4: Fungal PAMPs, host PRRs and signaling pathways.

Fungal pathogens expose several molecular patterns such as O-linked and N-linked mannans, β-mannosides, phospholipomannans and β-glucans. Their binding to cell surface TLRs and C-type lectins activates several signaling pathways, resulting in canonical and non-canonical NFκB activation, ROS-response, cytokine response and regulation of adaptive immunity. Notably, fungal nucleic acids are recognized by the phagosomal TLRs, triggering type I IFN responses through MyD88 and TRIF adaptor proteins (taken from Bourgeois and Kuchler, 2012).

Inflammasomes

Inflammasomes are intracellular protein complex required for IL-1β and IL18 activation during inflammation. In addition to cytokine and adaptive immune responses, C-type lectin Dectin-1 is also an activators of noncanonical inflammasome through Syk(Gringhuis et al., 2012). Very recently our group was able to describe the underlying signaling pathways of *C. albicans* triggered noncanonical inflammasome activation and related host survival because of the absence of hyperinflammation(Zwolanek et al., 2014).

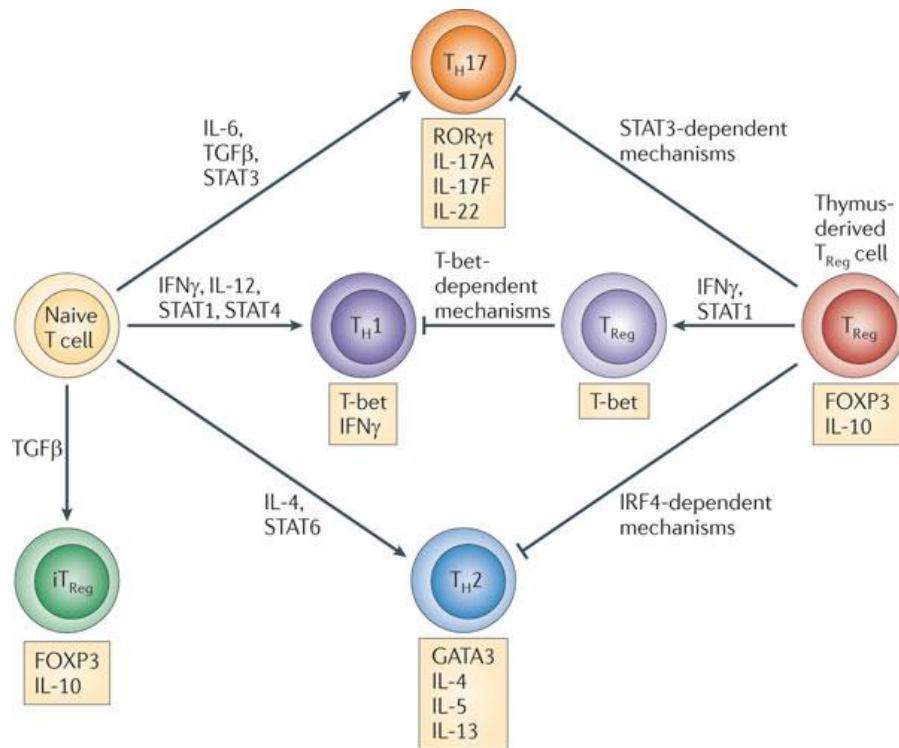
3.4.2 Adaptive Immune System

Innate immune system works only immediately after infections. Most of the infections can be cleared by the innate immune system. However, some agents can overcome innate immune responses or hosts can be exposed to pathogens repeatedly. Adaptive immunity protects the host more efficiently against repeated exposures and also in cases innate immune system fails. Notably, the adaptive immune system requires activation and is directed by the innate immune system (Murphy et al., 2008). Therefore adaptive immunity requires several days to weeks to develop. Adaptive immune cells (T and B cells) harbour receptors and antibodies in great varieties and number in a repertoire generated by V(D)J recombination, somatic hypermutation and clonal selection, in great specificity. Therefore novel pathogens can also be targeted by host defence machinery after instructions of adaptive immunity.

3.4.3 B and T Cell Immunity

Humoral adaptive immunity is established by B cells through production of immunoglobulins (Ig), which can opsonize and target antigens for clearance through the humoral complement system as well as cellular mechanisms such as phagocytosis or degranulation.

By contrast, T cells play a more central role in adaptive immunity and mainly can be divided into CD8⁺ (cytotoxic T cells or CTLs) and CD4⁺ T cells (T helper cells). CTLs are associated with cellular effector responses. T helper cells, however, assist other cell types to orchestrate the immune responses. Depending on the costimulatory factors presented by the innate immune cells, T helper cells can differentiate into distinct subtypes; T_H1, T_H2, T_H17, T_H3, T_H9, or T_{FH} (partially covered in Figure 5).



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Figure 5: Functional differentiation of T_H cell subsets and regulatory T cells. (Taken from Campbell and Koch, 2011).

After phagocytosis, so-called antigen presenting cells (APCs) migrate to lymph nodes and present antigens along with co-stimulatory factors and cytokines (Campbell and Koch, 2011; Gutcher and Becher, 2007; Walsh and Mills, 2013). While naïve T cells are differentiating into T helper cells, they are under the influence of these cytokines secreted by APCs and themselves in an autocrine manner, including other costimulatory factors. Cytokines directing a particular T_H subset can be inhibitory for other T_H subsets, thereby guiding the T helper cell response into the “appropriate” T helper cell subset. The balance of the type of T helper cell responses is associated with disease outcome. Although T_H1/T_H2 paradigm was first suggested, recent findings (such as newly characterized T helper subsets, T cell plasticity, T_H1+T_H2 cells) demonstrate that the T helper cell balance is more complex than it was initially thought and not yet to fully understood.

3.5 Type I and Type II Interferons

Interferons are a family of cytokines discovered 5 decades ago and named after their ability to *interfere* with viral replication (Isaacs and Lindenmann, 1957). Interferons were initially distinguished into 2 groups: the acid-stable type I interferons (referred as type I IFNs hereafter),

and the acid-labile type II interferon (referred as type II IFN hereafter)(de Weerd and Nguyen, 2012). Recently, another type of interferon was discovered and designated as type III interferons (referred as type III IFNs hereafter)(Kotenko et al., 2003).

The type I IFNs consist of IFN- α , IFN- β , IFN- κ , IFN- ω , IFN- τ and IFN- δ (first 4 exist only in humans) and share a common receptor, type I IFN receptor, a heterodimer of the IFNAR1 and IFNAR2 transmembrane proteins(de Weerd and Nguyen, 2012). Type II IFN consist of only IFN- γ and activate a distinct receptor, type II IFN receptor, a heterodimer of the IFNGR1 and IFNGR2 transmembrane proteins(de Weerd and Nguyen, 2012). Although the adapters on the cytosolic appear distinct (Figure 6), there is much co-reliance on STAT recruitment to the signaling machinery, albeit type I IFN signaling primarily relies on a STAT1-STAT2 heterodimer. While type I IFN activates interferon-stimulated gene factor 3 (ISGF3), type II IFN signaling on STAT1 homodimer, gamma-activated factor (GAF)(de Weerd and Nguyen, 2012). Nonetheless, all IFNs seems to activate STAT1 but the formation of ISGF3 can be responsible for regulation of different set of target genes. In addition, a recent report clarified the cooperative binding of GAFs through STAT1-STAT1 interaction distinguished between type I and type II IFN signaling(Begitt et al., 2014). Moreover, disturbance of this cooperative binding by a point-mutation abolishes the antibacterial properties of type II IFN signaling.

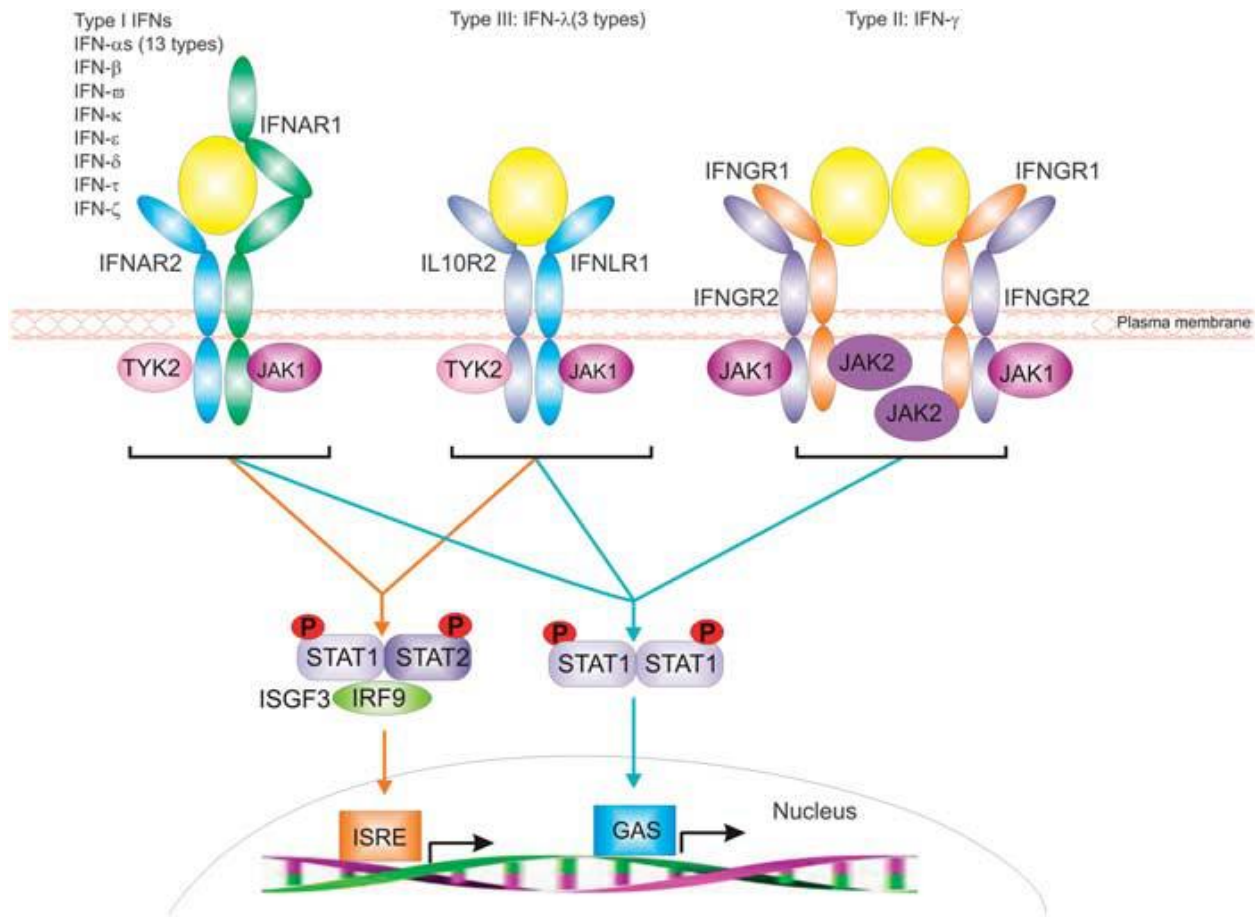


Figure 6: IFNs: receptors, adapters, transcription factors and promoters (taken from de Weerd and Nguyen, 2012).

Type I IFNs, beside their most prominent antiviral effector functions, were discovered as potent regulators of immune responses against distinct classes of pathogens, including bacteria, viruses and fungi (Biondo et al., 2011; Carrero, 2013; Gafa et al., 2010; Majer et al., 2012; Rai et al., 2012; Stockinger et al., 2002; Stockinger and Decker, 2008). However, there seems to exist a distinction concerning the protective and detrimental roles of type I IFNs between intracellular and extracellular bacteria (Carrero, 2013).

Induction of type I IFNs occurs through cytosolic and transmembrane receptors and mostly by the recognition of nucleic acids (except TLR4). The latter one involves TLR4 and endosomal TLRs such as TLR3, TLR7, TLR8 and TLR9 (Stetson and Medzhitov, 2006). Additionally, on the cytosolic side, RIG-I and MDA5 are other associated coreceptors. Hence, the localization of PAMPs can play an important role triggering type I IFN release. The type II IFN, IFN- γ is produced by a limited number of cells, mainly; T helper cells (T_H1), natural killer cells, natural killer T cells and CD8 T cells (Schoenborn and Wilson, 2007).

3.6 Spleen and Other Target Organs

Spleen is major organ of the both innate and adaptive immune system. Lymphocytes can traffic to and from the spleen from virtually all body sites via blood vessels(Murphy et al., 2008). Spleen is compartmentalized in several areas; the red pulp is the region, where red senescent blood cells are disposed, whereas lymphocytes reside in the white pulp, forming a sheath so called periarteriolar lymphoid sheath (PALS)(Murphy et al., 2008). Organs without primary immunological function such as liver, brain and kidneys can be targets in systemic infections. Infection and inflammation in these organs can have devastating effects on host survival. Importantly, brain is protected by blood-brain-barrier, of which one important function is to protect nervous system from pathogen invasion(Banks, 2015). Some organs have developed resident local macrophage populations, including microglia in the brain and Kupffer cells in the liver(Bilzer et al., 2006; Nayak et al., 2014).

3.7 Interaction between Host Immune System and *C. glabrata*

C. glabrata is less virulent and less inflammatory when compared to its distinct cousin *C. albicans*, which remains as the most frequent cause of deaths by systemic candidiasis. Their different infection strategies can be explained by different interactions with the innate immune system. As *C. albicans* induced hyphae formation upon engulfment by macrophages and killing them, *C. glabrata* on the other side resists phagosome starvation, maturation and acidification without or less efficiently killing the macrophages(Brunke and Hube, 2013; Seider et al., 2011). These distinct virulence traits also correlate with host tissue damage, which is the main driver of hyper-virulence(Brunke and Hube, 2013).

3.7.1 Adhesion and Invasion

Adhesion of *C. glabrata* to host epithelial cells *in vitro* and *in vivo* is mediated by a group of genes known as the EPA genes(Cormack et al., 1999; Maestre-Reyna et al., 2012, p. 1999). *C. glabrata* is very often isolated from patients with *C. albicans* oropharyngeal candidiasis, making it difficult to identify the initial invader(Maenza et al., 1997; Thompson III et al., 2010).

The invasion of deeper host tissues by *C. glabrata* is not well understood. Injuries, contaminated medical equipment and medical operations, or other routes of entrance may play

a role. However, there are no active mechanisms observed for *C. glabrata* invasion so far. Furthermore, although a report demonstrates an interaction of *C. glabrata* with epithelial cells, it fails to cause inflammatory responses(Li et al., 2007).

3.7.2 Immune Response against *C. glabrata*

Fungal pathogens are cleared via different host mechanisms, such as reactive nitrogen species (RNS) and reactive oxygen species (ROS) released mainly by innate phagocytic cells(Nicola et al., 2008). Although these mechanisms efficiently clear fungal pathogen, they are also primary cause of host-driven host tissue damage. Phagocytosis alone, however, minimizes self-damage of the host, at the same time limiting nutrition, acidifying the microenvironment and iron-depletion to the pathogen. Most importantly phagocytosis itself also contributes to regulate immune response pathways by delivering essential information to signaling pathways(Flannagan et al., 2012; Geisow et al., 1981; Moretti and Blander, 2014; Nicola et al., 2008; Underhill and Goodridge, 2012). Fungal pathogens under this pressure developed mechanisms to resist host weaponry. For instance, *Cryptococcus neoformans* survives and multiplies in human macrophages leading to host cell death(Levitz et al., 1999). Similarly *C. glabrata* also survives and multiplies in phagosomes of macrophages(Seider et al., 2011). *C. glabrata* resists oxidative stress with a single catalase Cta1p – but it is not necessary for virulence(Cuéllar-Cruz et al., 2008, p. 1). Similarly *C. neoformans* resists ROS with thiol peroxidases, and the absence is associated with less-virulence and survival(Miller and Britigan, 1997; Missall et al., 2004).

C. glabrata can be engulfed by phagocytic cells such as macrophages, thereby limiting their resources (e.g. nutrients and iron), but fungi can persist within phagosomes. Interestingly, *C. glabrata* growth is inhibited by human serum due to depletion of iron through transferrin binding(Otto and Howard, 1976). Although this study from 1976 could not observe any intracellular growth, in contrast, a more recent study could demonstrate *C. glabrata* replicating inside phagosome of human monocyte-derived macrophages(Seider et al., 2011). Importantly, pro-inflammatory cytokine production, ROS response and phagosome maturation were inhibited by *C. glabrata*(Seider et al., 2011). These results are in accordance with mouse systemic infection models(Arendrup et al., 2002).

A recent study demonstrates important insights into mechanisms underlying *C. glabrata* persistence *in vivo* (Fukuda et al., 2013). Briefly, authors have demonstrated that the transcriptional profile of *C. glabrata* in spleens of infected mice resembles the response to carbohydrate deprivation and phagocytosis by neutrophils. Fungal cells responded by activating alternative energy sources through gluconeogenesis glyoxylate cycle, and long-chain fatty acid metabolism. Moreover, genes associated with peroxisomes and autophagic pathways were upregulated in fungi interacting with neutrophils. Surprisingly genes associated with ergosterol synthesis were downregulated. However, the sterol transport gene AUS1 was upregulated and it was speculated that *C. glabrata* may replace ergosterol with host cholesterol to maintain viability.

3.7.3 Induction of Type I Interferons

Our group has previously demonstrated that *Candida* spp. induce type I IFNs upon in conventional dendritic cells via a novel mechanism of fungal recognition via TLR7. Notably, this response required phagocytosis, phagosome acidification and maturation as well as TLR7-MyD88 signaling. Ultimately, the transcription factor IRF1 induces the transcription of IFN β (Figure 7). Interestingly, type I IFNs also promote *C. glabrata* persistence in mice.

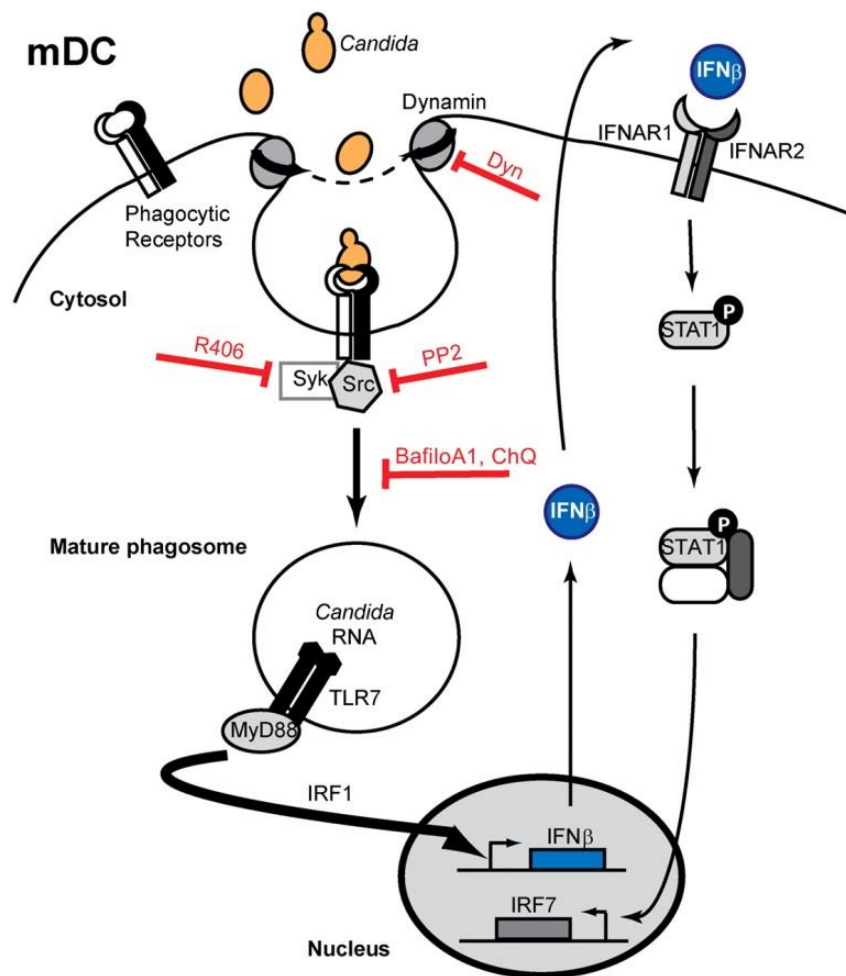


Figure 7: *Candida* spp. induce type I IFN response in conventional DCs.
An integrated model of type I IFN response upon *C. glabrata* challenge in BM-DCs *in vitro* (taken from (Bourgeois et al., 2011)).

Aims of this Master Thesis

The main aim of this thesis was to investigate the biological role of type I interferons in systemic candidiasis and to elucidate mechanisms behind the beneficial/detrimental effects of type I interferons and how type I interferon signaling interplays with other immune pathways.

Our group has previously shown a novel mechanism inducing type I interferon response against *Candida* spp. by conventional dendritic cells. Especially *C. glabrata* was subject of the study, as this opportunistic pathogen induced strongest response and most importantly type I IFN signaling, was associated with persistence of the pathogen and weaker immune response.

We therefore wanted to further investigate and answer following questions:

- I. Do type I IFNs change the course of infection upon systemic *C. glabrata* challenge?
- II. Which immune signaling mechanisms are involved *in vivo*?
- III. What cell types are affected by type I IFN signaling?
- IV. How can type I interferons regulate other immunity-related mechanisms *in vivo* and *in vitro*?

4 Results

4.1 Kinetics of Fungal Persistence are Different between Wild-Type (WT) and IFNAR1^{-/-} Mice

Since our group has reported a novel type I IFN signaling pathway against *Candida* spp. (*C. glabrata* as the strongest trigger among them), and the increased persistence of *C. glabrata* under type I IFN signaling, we were interested in the kinetics of fungal persistence in several organs. WT and IFNAR1^{-/-} mice were injected with *C. glabrata* (or PBS as uninfected control) with 5×10^7 CFUs per 25g mouse through the lateral tail vein. Each group was sacrificed at the corresponding day of infection (day 14 for the PBS control), organs were cut in half and kept for the assessment of fungal burden and RNA isolation. Fungal burden was higher in brain and the liver of the IFNAR1^{-/-} at day 1. Although in average the CFU counts appeared higher in these organs of IFNAR1^{-/-} at day 3, the results failed to reach insignificance (Figure 8). In contrast, at day 7 CFU counts were significantly higher in WT liver and spleens when compared to IFNAR1^{-/-}. Additionally, this trend was also obvious at day 14 for all organs tested except for kidneys.

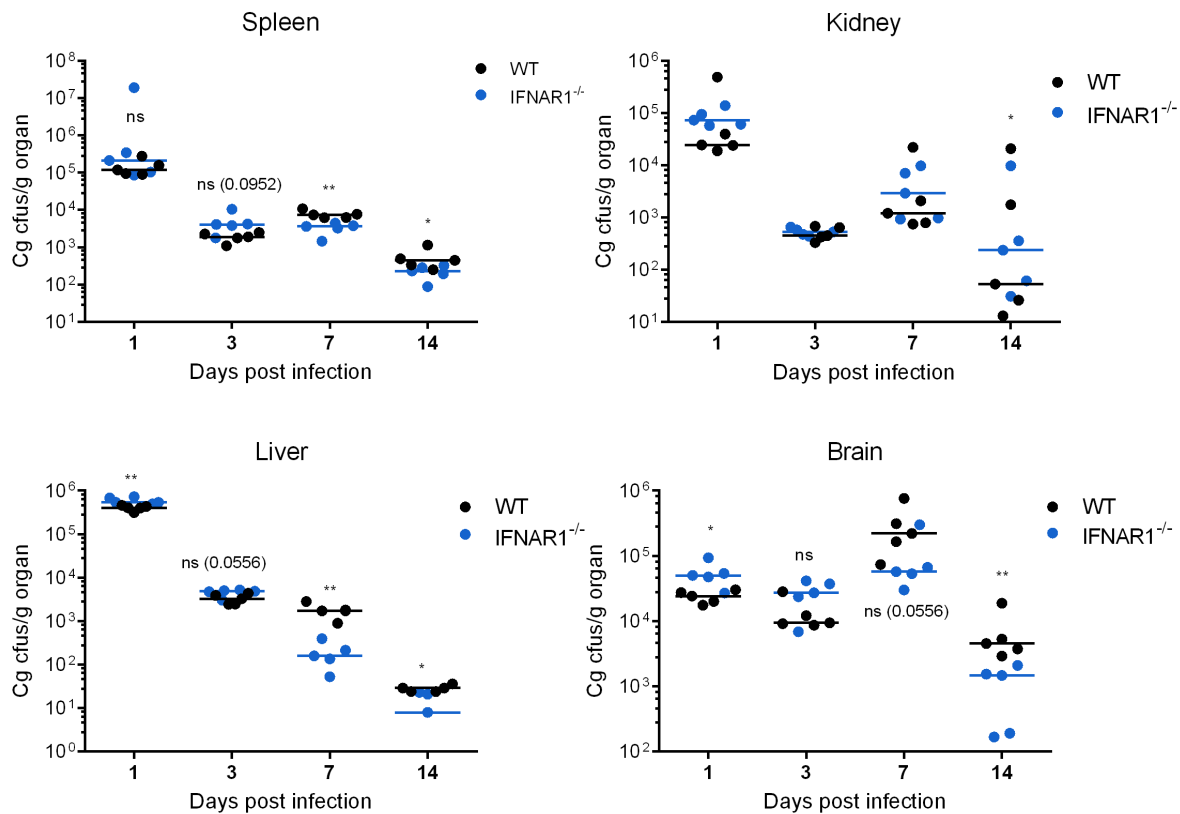


Figure 8: Time course of fungal burden in organs after *C. glabrata* i.v. injection.

Mice were infected intravenously (tail-vein) with 5×10^7 *C. glabrata* CFU per 25g mouse. Organs were isolated, weighed and cut in half, where one half was kept in ice-cold MPO-Buffer (for CFUs) and the other half in RNeasy lysis buffer (for gene expression analysis). Organs in MPO-Buffer were homogenized and plated on YPD plates with antibiotics to assess CFUs. CFUs were normalized per gram tissue. Experimental design by Christelle Bourgeois. Mice were injected, sacrificed and organs were collected by Florian Zwolanek. Homogenisation and plating was done by me, Christelle Bourgeois and Florian Zwolanek (Mann-Whitney U Test).

4.2 Spleens in IFNAR1^{-/-} Mice were Enlarged

The spleen is a major organ of the immune system and therefore was chosen to as a system to understand underlying mechanisms under type I IFN signaling, promoting *C. glabrata* persistence. Notably, kinetics of fungal burden was comparable for liver and brain. Mice were injected with the same amount of *C. glabrata* and spleens were weighed at several days following infection (Figure 9). Spleens of WT and IFNAR1^{-/-} mice were already enlarged at day 3. However, spleens of IFNAR1^{-/-} mice were much larger than the WT spleens at day 7 of infection, suggesting a strong immune response or a delayed resolution of immune signaling in the absence of type I IFN signaling. These findings suggested notion that whether spleen is a major immune organ involved type I IFN-mediated *C. glabrata* persistence.

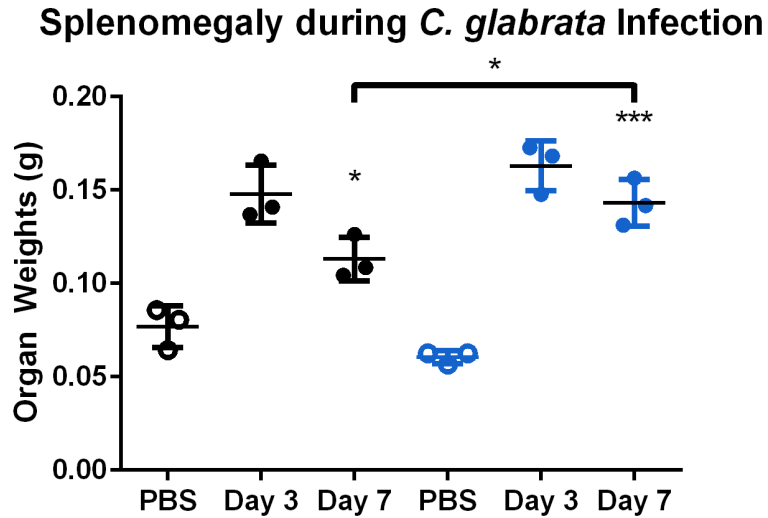


Figure 9: Splénomegaly in infected IFNAR1^{-/-} mice.

Mice were injected with *C. glabrata* and sacrificed as described before. Organs from infected and control mice were collected in PBS and weighed (n=3; unpaired student's t-test).

4.3 Microarray and Principal Component Analysis

To investigate mechanisms underlying role of type I IFN signaling, RNA was isolated from the other halves of spleens, from which the fungal burden was quantified. RNA was tested for integrity with Agilent Bioanalyzer 2100. 3 samples with highest integrity of each infected spleen RNA and 4 of each uninfected controls were coupled with fluorescent molecule (Cy3) to prepare cRNA library for microarray hybridisation. Hybridised mouse genome microarray chips were detected with Agilent Microarray Scanner (performed by Andriy Petryshyn). Raw data was analysed and differentially expressed genes were calculated (performed by Walter Glaser).

To check the quality of the microarray data, we examined genes downstream of type I IFN signaling. Ideally, we would expect upregulation of type I IFN-regulated genes in WT spleens during infection but not in IFNAR1^{-/-} spleens. Type I IFNs are also released and sensed in basal levels, therefore difference in uninfected mice was also expected. Indeed, *Irf7*, *Oasl1*, *Oasl2*, *Ifit1* and *Ifit2* were all lower expressed both in PBS injected mice and during the course of infection (Figure 10). Of note, the upregulation of downstream targets for type I IFN signaling was primarily restricted to day 1.

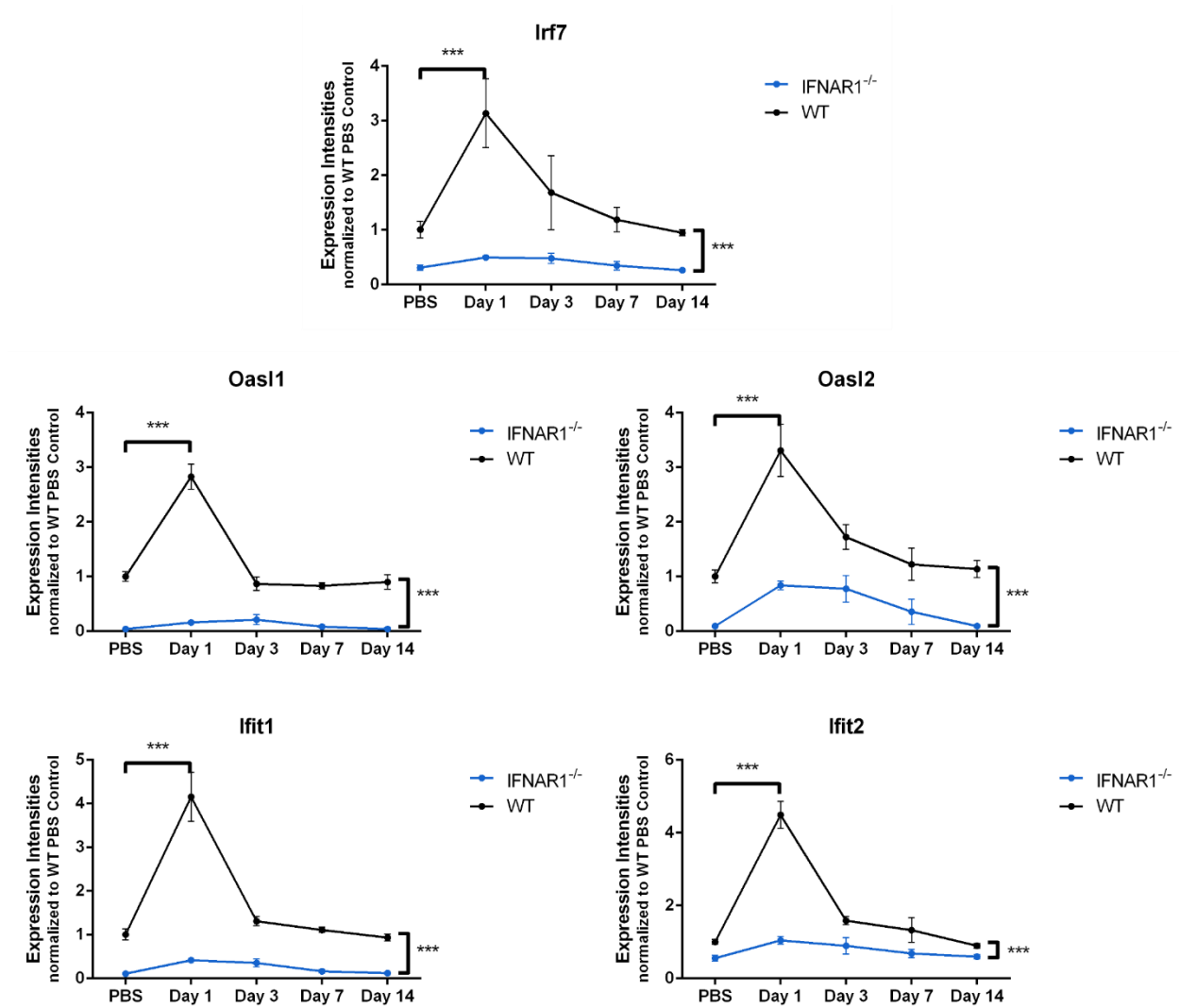


Figure 10: Type I IFN signaling is active in spleens and is upregulated during infection.

RNA was isolated from other halves of the spleens, from which the fungal burden was determined. Gene expression was quantified with mouse genome microarrays and analysed (n = 4 for PBS controls, n = 3 for infected).

Principal component analysis (referred as PCA hereafter) is a an eigenvector-based multivariate analysis tool(Pearson, 1901), which can be used to reduce high-dimensional datasets, including microarray data to low-dimensional representations. PCA calculates so called principal components (PC) consisting of variables with high covariance, where first PC with the highest variance, second with less variance etc. Therefore, a “shadow” of a multidimensional data can be illustrated.

PCA analysis of the microarray data delivered some insights about the gene expression patterns after infection of spleens in WT and IFNAR1^{-/-} mice (Figure 11). Most importantly, IFNAR1^{-/-} spleens follow a different course when compared to WT. The first PC (PC1 on the x-

axis) of the WT dataset accounts for the 48% of the variance and the second PC (PC2 on the y-axis) accounts for 28% of the variance (Figure 11 A). On the IFNAR1^{-/-} side PC1 accounts for the 52,3% of total variance and PC2 for the 22,8% of the variance (Figure 11 B). Strikingly, transcriptional differences were higher between the control and day 14 of the infection in IFNAR1^{-/-} spleens, when compared to differences between the control and day 14 of the infection in WT spleens. Otherwise, the transcriptional differences were following more or less similar patterns.

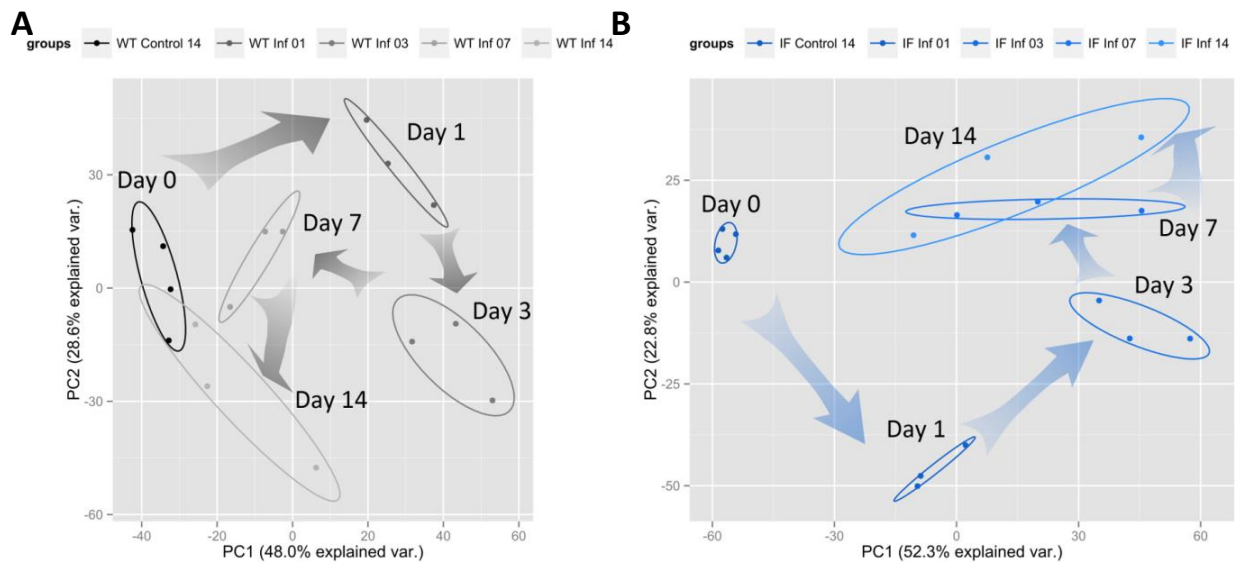


Figure 11: Principal component analysis (PCA) analysis of WT and IFNAR1^{-/-} microarrays.

PCA analysis was performed on normalized log-intensities in R statistics language (release 3.1.1 (2014-07-10)) using R-Studio(R Core Team, 2014; *RStudio*, 2012). Analysis was performed with *stats* package and illustration was performed with *ggbiplot* package, which is based on the *ggplot2* package (R Core Team, 2014; “vqv/ggbiplot,” n.d.; Wickham and Chang, 2014).

4.4 General Transcriptional Amplitude was Higher in IFNAR1^{-/-} Mice

Differential gene expression (differentially expressed genes, DEGs) data was calculated between WT and IFNAR1^{-/-} datasets and the results were plotted as a dot plot. Initial results for spleen at day 7 of infection have shown a higher transcriptional response in general in IFNAR1^{-/-} mice compared to WT spleens (Figure 12). Among these genes, those involved as effector immune responses and cell proliferation were detected, including many genes marked with GO terms associated with host-symbiont interaction.

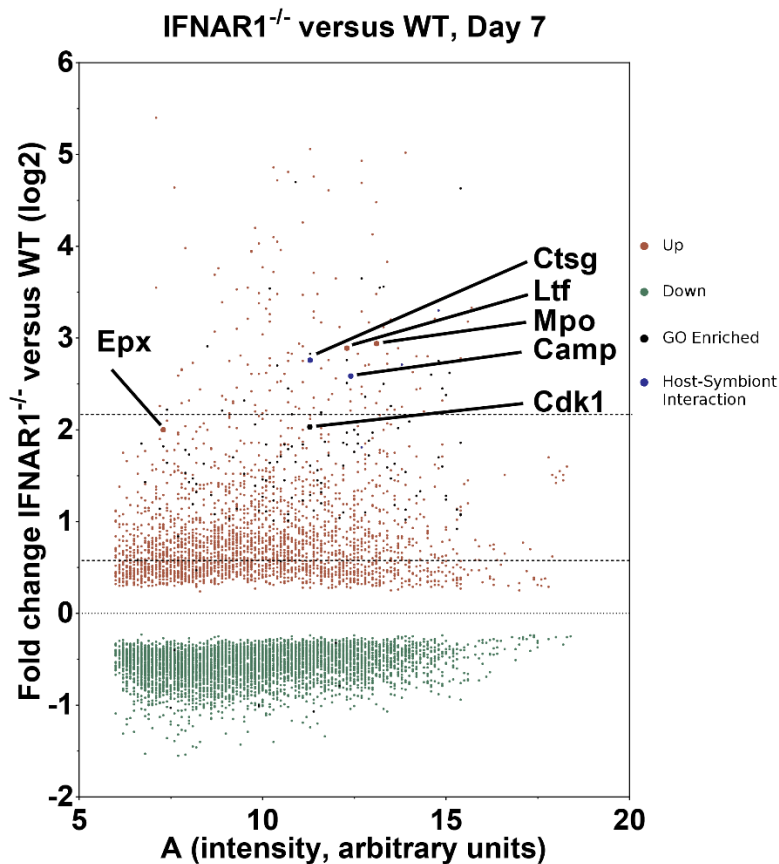


Figure 12: General transcriptional response was higher in IFNAR1^{-/-} spleens.

Differentially expressed genes were calculated with a cut-off of FDR < 0,05 (Benjamini & Hochberg method) using a fold-induction of $\geq 1,5$ and illustrated in log₂ scale. Some genes with effector functions were marked.

4.5 Clustering Revealed Distinct Pathways Kinetics

The initial data showing the time-dependent gene expression patterns subjected to clustering analysis. First, microarray results were filtered for DEGs at least at one time point between WT PBS control and WT infected (WT dataset). The IFNAR1^{-/-} dataset was selected for all DEGs between IFNAR1^{-/-} and WT at least at one time point. However, the gene expression data was normalized to the IFNAR1^{-/-} uninfected control. To investigate the time-dependent gene expression patterns, microarray results were hierarchically clustered using uncentered Pearson correlation with average linkage. Furthermore to obtain unbiased clusters, hierarchical clusters were split (mean silhouette split) using the NodeFinder program, we have recently developed (see section 6.5.3 p. 65). Mean silhouette split (MSS) joins elements in a tree structure, such as hierarchical clustering, until the group is still considered homogenous. For this purpose, the initial goal was to find an appropriate threshold for the silhouette split, since strict thresholds would lead to distinction between gene expression patterns. Alternatively, too low

thresholds would result in heterogeneously large clusters. Therefore, we ran NodeFinder with thresholds ranging from 0 (no correlation) to 1 (perfect correlation) (Figure 13).

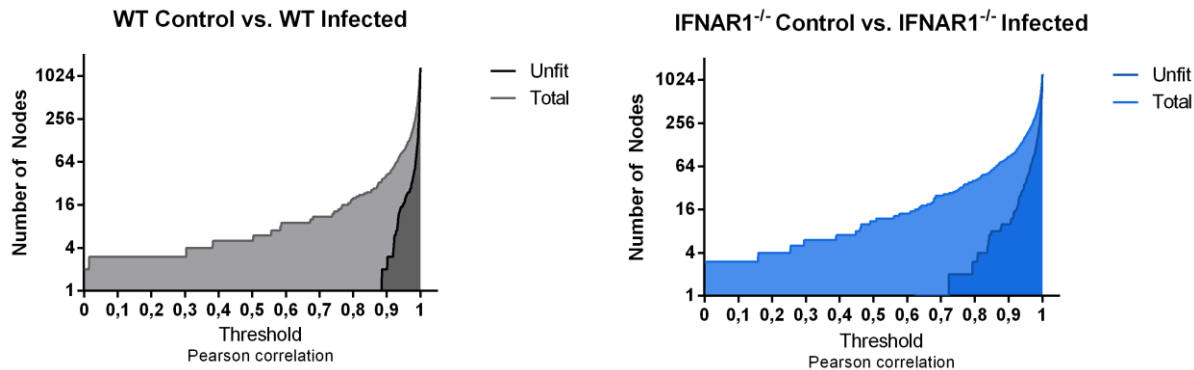


Figure 13: Threshold simulation of WT and IFNAR1^{-/-} datasets with the NodeFinder.

Significantly changed genes from WT and IFNAR1^{-/-} spleen microarray results were clustered with Cluster 3.0, using hierarchical clustering technique with uncentered Pearson correlation. These clusters were split with NodeFinder with thresholds ranging from 0 to 1 with 0,001 steps. Light lines depict total number of clusters (fit (above the threshold) and unfit (below the threshold)) and dark lines depict unfit number of clusters.

According to these results a threshold value of 0,9 was chosen for silhouette splitting, which was performed with WT and IFNAR1^{-/-} clustering results (Figure 14 & Figure 15). Furthermore, NodeFinder gathered information on the genes from Ensembl databases, such as synonyms, GO terms and full names. Finally, key terms were provided to the software to mark genes of interest referred to “genes of interest” hereafter:

defence	immune	neutrophil	natural killer cell
interleukin	T cell	dendritic cell	macrophage
interferon	B cell	autophag ¹	defense
inflammatory			

Additionally NodeFinder automatically marks transcription factors based upon 3 GO terms, including:

GO:0003700 sequence-specific DNA binding transcription factor activity
GO:0000987 core promoter proximal region sequence-specific DNA binding
GO:0003705 RNA polymerase II distal enhancer sequence-specific DNA binding transcription factor activity

¹ NodeFinder does not look for whole words in GO terms but occurrence of any character sequence. “autophag” is therefore selected for words such as “autophagic” or “autophagy”.

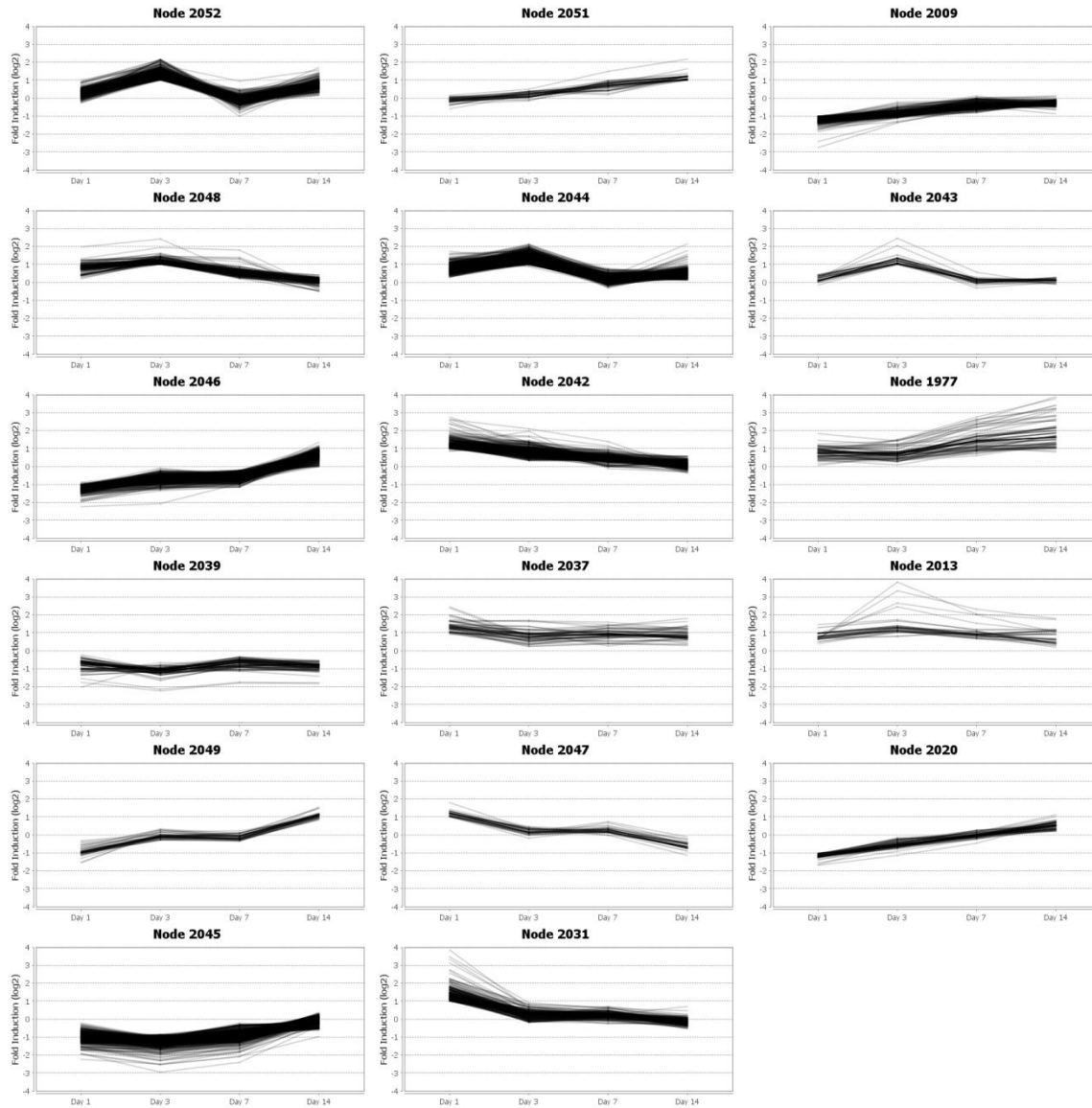


Figure 14: WT spleen gene expression patterns upon systemic *C. glabrata* challenge.

Significantly changed genes from WT spleen microarray results were clustered with Cluster 3.0, using hierarchical clustering technique with uncentered Pearson correlation. Hierarchical clustering tree for WT dataset was split using NodeFinder (Pearson correlation ≥ 0.9). Clusters with 20 or more genes were chosen for the figure.

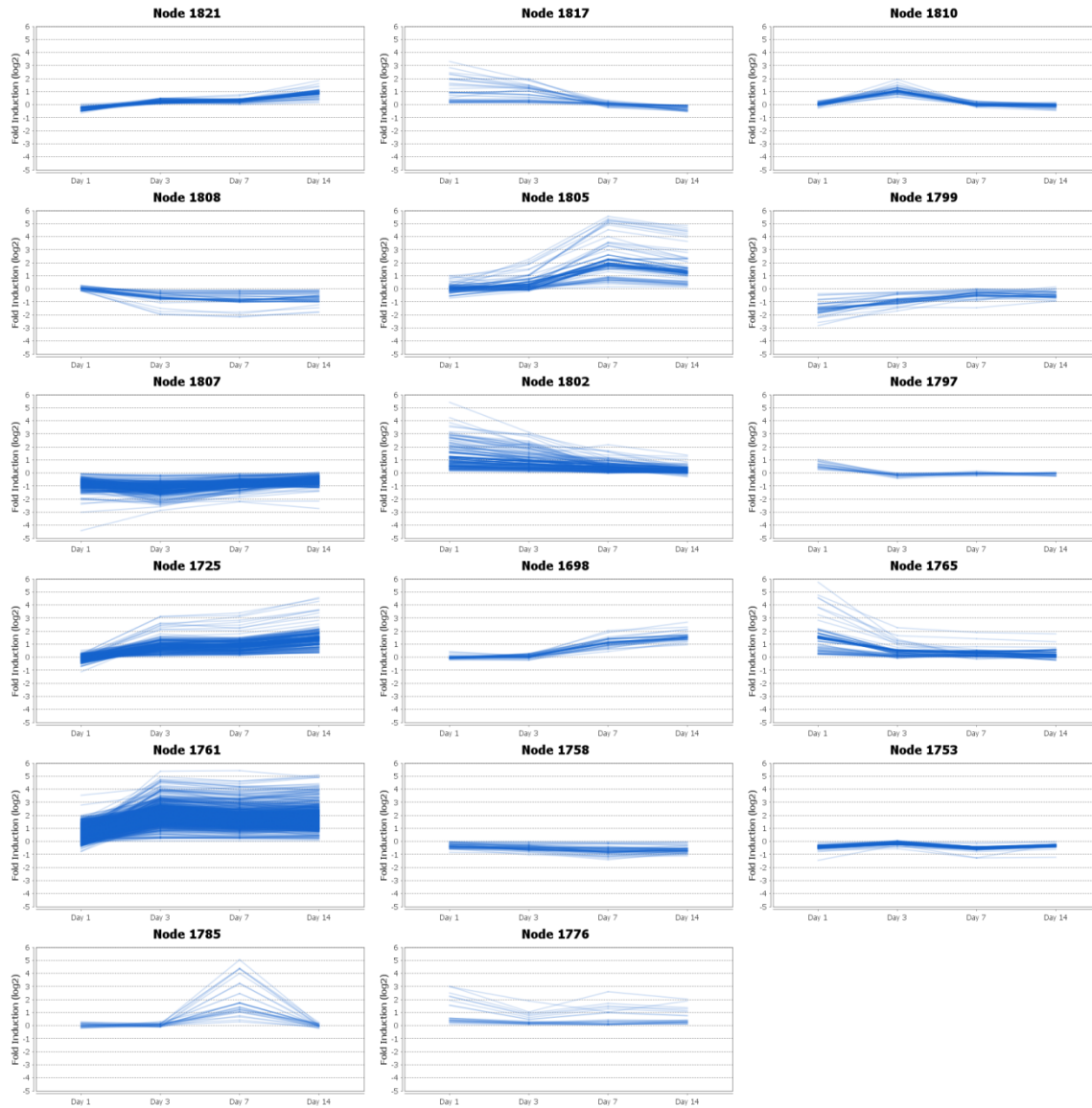


Figure 15: IFNAR1^{-/-} spleen gene expression patterns upon systemic *C. glabrata* challenge.

Significantly changed genes from spleen microarray results were clustered with Cluster 3.0, using hierarchical clustering technique with uncentered Pearson correlation. Hierarchical clustering tree for IFNAR1^{-/-} dataset was split using NodeFinder (Pearson correlation ≥ 0.9). Clusters with 20 or more genes were chosen for the figure.

4.5.1 Immunity-Related GTPases

NodeFinder maps clusters between datasets for common genes. Briefly, if two clusters from WT and IFNAR1^{-/-} datasets have many genes in common, these genes might be co-regulated or have common functions in related pathways. Therefore, we mapped WT and IFNAR1^{-/-} datasets and filtered for mappings with high number of genes of interest. The first mapping revealed 12 genes in common between clusters “2031” (n = 129) and “1817” (n = 34). Among those, 10 genes were of particular interest (Figure 16 A). Interestingly, 7 of these genes

were immunity-related, including the GTPases *Irgm1*, *Irgm2*, *Iigp1*, *Gbp2*, *Gbp4*, *Tgtp1* and *Tgtp2*, all of which were higher expressed in *IFNAR1*^{-/-} spleens at day 3 (Figure 16 B-H). These genes belong two different families of GTPases known as the 65kDa and 47kDa families. All were shown to be inducible by IFN- γ (Boehm et al., 1998). The 65kDa family consists of guanylate binding proteins (GBP)(Degrandi et al., 2007; Olszewski et al., 2006), some of which are protective against Listerial and Mycobacterial infections(Kim et al., 2011). The second family of, 47kDa (IRG) GTPases consists of many members, including *Irgm1*, *Irgm2*, *Iigp1*, *Tgtp1* and *Tgtp2*(Bekpen et al., 2005). Of note, almost all members of this family share IFN-stimulated response elements (ISRE) in their promoters(Bekpen et al., 2005). *Irgm1*, *Irgm2*, *Iigp1*, *Gbp2*, *Gbp4*, *Tgtp1* and *Tgtp2* were all higher expressed in *IFNAR1*^{-/-}-deficient spleens, despite the fact that the type I IFN signaling was absent (Figure 16 B-H). The difference in gene expression was also significantly higher at day 3, except for *Tgtp1* although the difference was only for *Gbp2* two-fold or higher between *IFNAR1*^{-/-} infected and WT infected spleens (Figure 16 E, G).

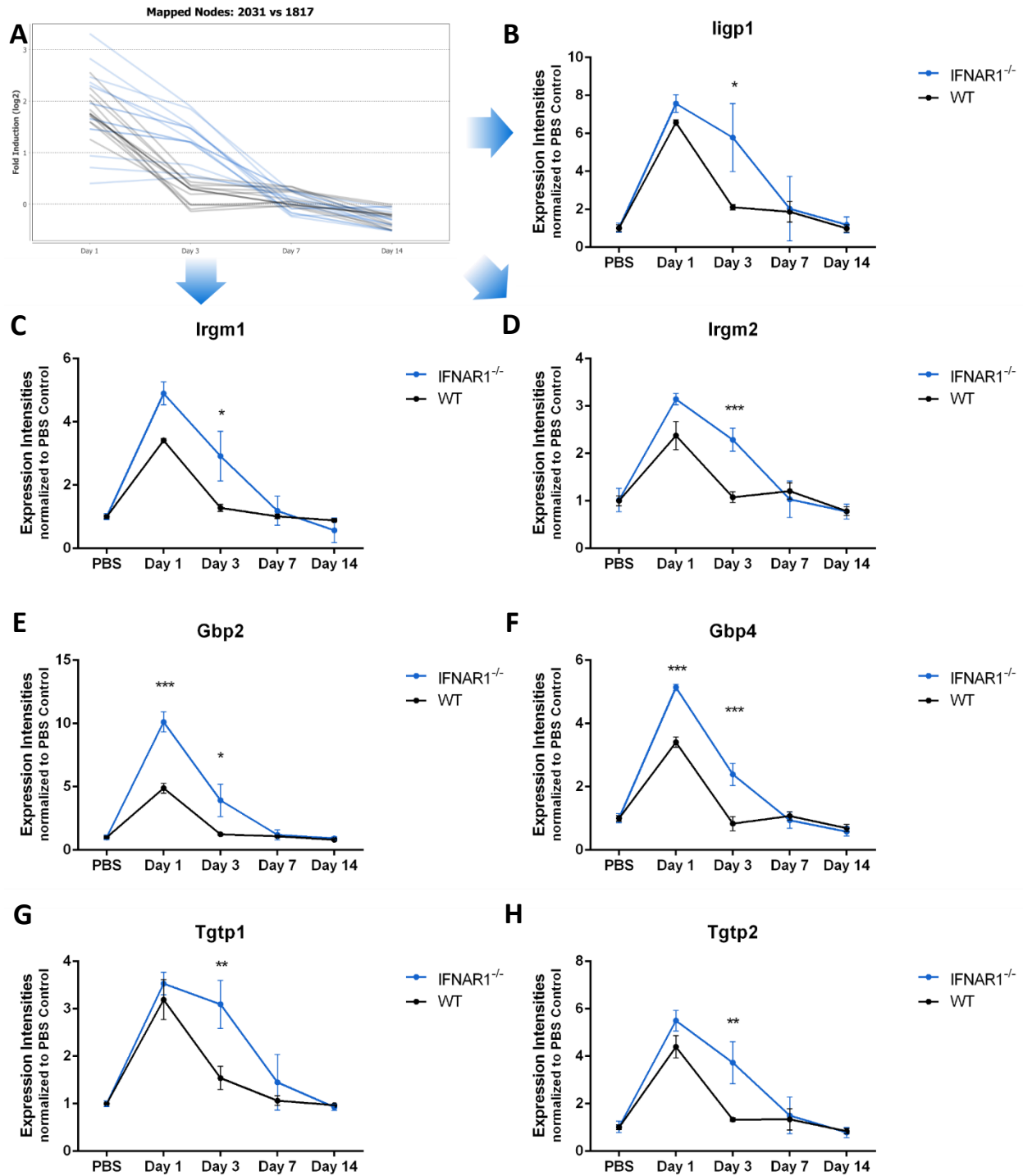


Figure 16: Immunity-related GTPases are higher expressed in IFNAR1^{-/-} spleens.

(A) Clusters “2031” and “1817” were mapped by NodeFinder. (B - D) Single gene figures were created with Prism (from GraphPad) with the microarray expression data (Benjamini Hochberg False Discovery Rate (FDR) $\geq 0,05$).

4.5.2 Proinflammatory Responses

Another cluster-mapping of particular interest was detected between clusters “1977” (n = 54) and “1805” (n = 58). The analysis revealed 16 common genes, 10 of which were genes of interest and one was a transcription factor (Figure 17 A). Most importantly, these genes were associated with pro-inflammatory, antimicrobial and neutrophil-associated effector functions (Figure 17 B and D). Proteinase 3 (Prtn3), Elastase (Elane) and Cathepsin G (Ctsg) are neutrophil serine proteases and major components of neutrophilic azurophilic granules (Korkmaz et al., 2010). These genes mediate tissue remodelling and non-oxidative pathogen killing via protein degradation. Notably, they are also implicated in NET (neutrophilic extracellular traps). NETs are released by the neutrophils as chromatin fibres and proteins to trap pathogens and limit their spreading (Brinkmann et al., 2004). In this cluster-mapping, we identified Prtn3 and Ctsg, both of which were higher expressed in IFNAR1^{-/-} spleens (Figure 17 B and D). However, Elane was placed in another cluster (“2051”), and therefore did not appear in this mapping, although showing the same basic pattern (Figure 17 C).

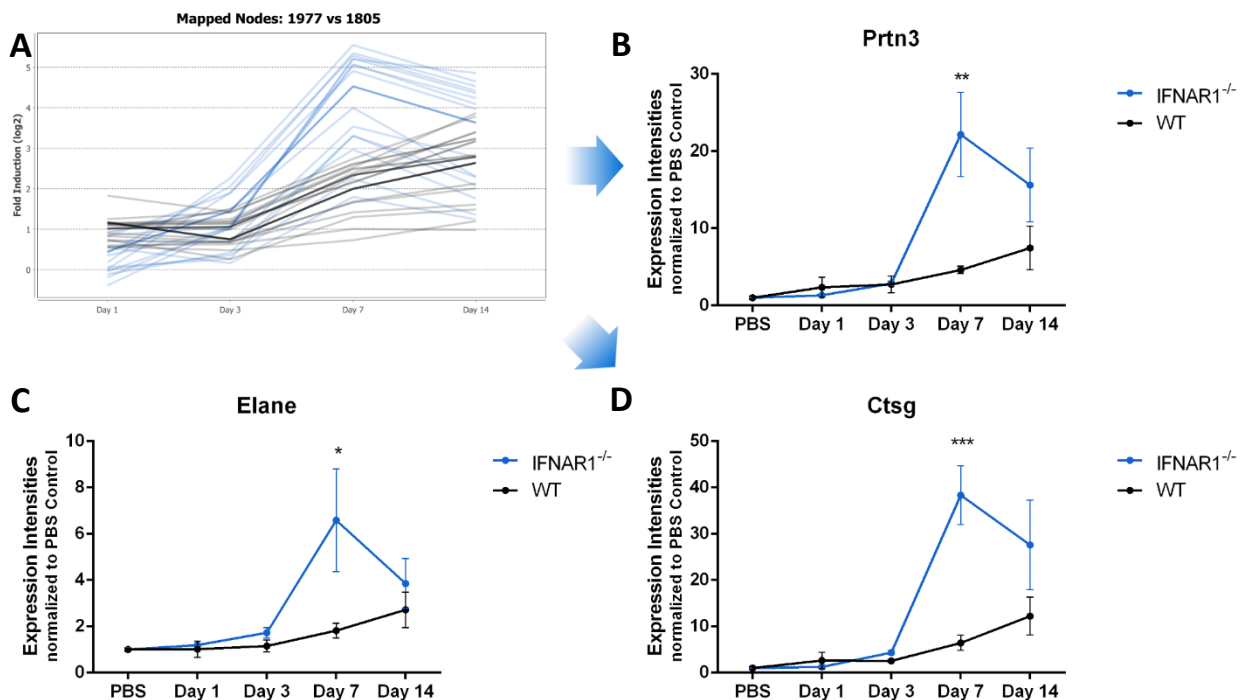


Figure 17: Neutrophilic serine proteases were higher expressed in IFNAR1^{-/-} spleens at day 7 of the systemic *C. glabrata* challenge.

(A) Clusters were mapped with NodeFinder. (B – D) Single gene figures were created with Prism (from GraphPad) with the microarray expression data (Benjamini Hochberg False Discovery Rate (FDR) $\geq 0,05$).

Additionally, the same cluster-mapping revealed effector genes involved in oxidative killing, including Mpo (myeloperoxidase) and Epx (eosinophil peroxidase). These data hint on involvement of eosinophils and higher oxidative killing in IFNAR1^{-/-} spleens (Figure 18). Mpo was higher expressed in IFNAR1^{-/-} spleens both at day 7 and day 14 (≥ 2 fold). Epx was only higher expressed at day 7 of infection.

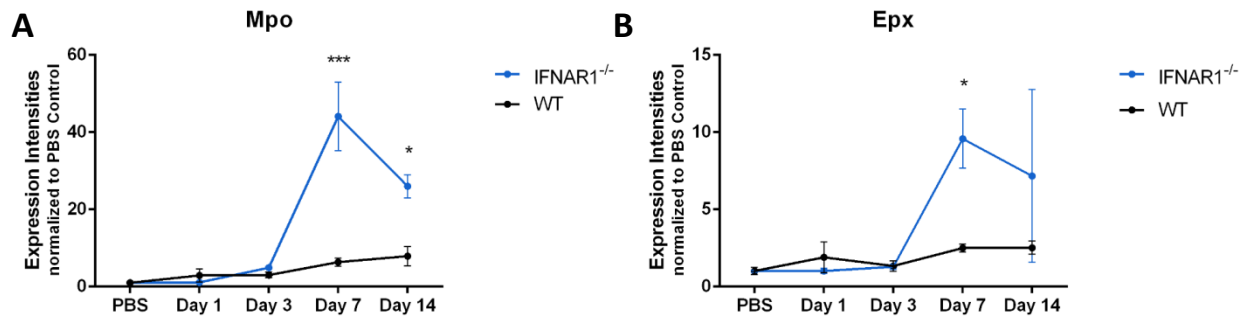


Figure 18: Oxidative killing effector genes were higher expressed in IFNAR1^{-/-} spleens.

(A B) Single gene figures were created with Prism (from GraphPad) with the microarray expression data (Benjamini Hochberg False Discovery Rate (FDR) $\geq 0,05$).

Another set of effector genes occurring in this mapping were Ngp (neutrophilic granule protein), Camp (cathelicidin antimicrobial peptide), Ltf (lactoferrin) and Lcn2 (lipocalin 2) (Figure 19). The latter two, Lcn2 and Ltf, sequester environmental iron, limiting pathogen growth (Brock, 2012; Bullen, 1981; Farnaud and Evans, 2003; Flo et al., 2004). Of note, iron is a scarce but essential resource for microbes in tissues (Bullen, 1981). Thereby, the antifungal effects of Ltf against *Candida* spp. was already reported many years ago (Kirkpatrick et al., 1971; Nikawa et al., 1993). For *Candida* spp., including *C. glabrata*, the combination of lactoferrin and fluconazole has an enhanced antifungal effect (Kuipers et al., 1999). In contrast, very little is known about Ngp. Camp, on the other side, is better studied. Beside its broad antibacterial and antiviral effects, the antifungal effect was reported for *Candida albicans* (Murakami et al., 2004; Vandamme et al., 2012; Wong et al., 2011).

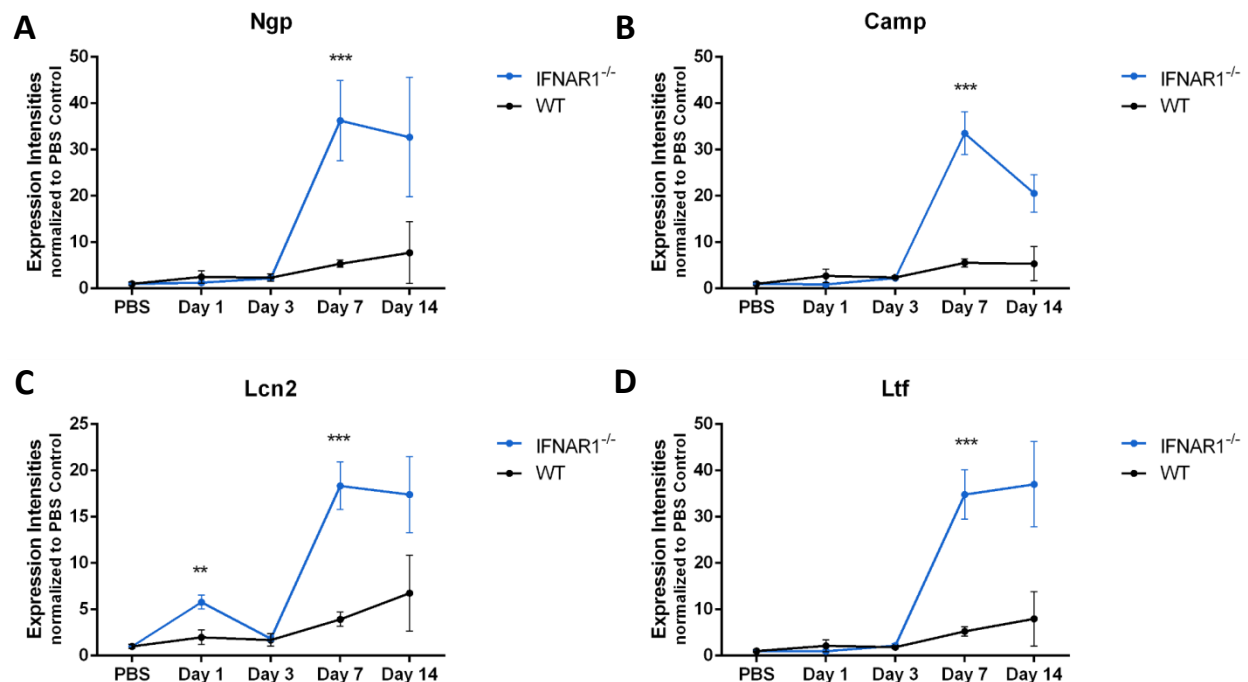


Figure 19: Genes with anti-microbial functions were higher expressed in IFNAR1^{-/-} spleens.

(A – D) Single gene figures were created with Prism (from GraphPad) with the microarray expression data (Benjamini Hochberg False Discovery Rate (FDR) $\geq 0,05$).

We have evaluated some of these genes with real-time PCR in an independent experiment (Figure 20). Although all genes followed the same pattern, we could only demonstrate significant differential expression for *Mpo* and *Ctsg* (Figure 20 B and D), due to the high amplitude (and thus higher variance) of gene expression in IFNAR1^{-/-} infected spleens.

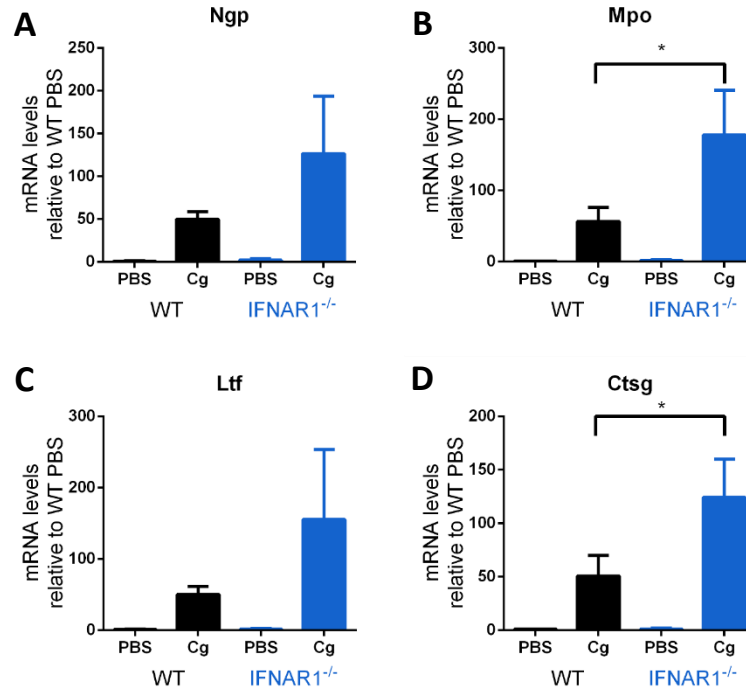


Figure 20: Real-time PCR validation of mapped genes.

Mice were i.v. injected with *C. glabrata* (5×10^7 CFUs/25g mouse) by Florian Zwolanek. At day 7, splenocytes were isolated and red blood cells were lysed. RNA was isolated and cDNA was prepared from the isolated splenocytes. Real-time PCR was run and analysed. Expression values were normalized to WT PBS control (n=3; unpaired Student's t-test).

4.5.3 Type-II Interferon Signaling

Mapping between the clusters “2031” (n=129) and “1802” (n=116) unravelled 21 common genes, and 14 genes of interest and a transcription factor. Remarkably, almost all genes were either regulated by type I or II IFNs (Figure 21 A). More importantly, several genes (Cxcl9, Cxcl10, Irg1, Igtp, Batf2 and Ubd), under type II IFN regulation were higher expressed IFNAR1^{-/-} spleens (Figure 21 C - H), although type I IFN signaling pathway is absent. While Ifn- γ expression pattern is similar to the mapped genes (Figure 21 B), it did not come up in the mapping.

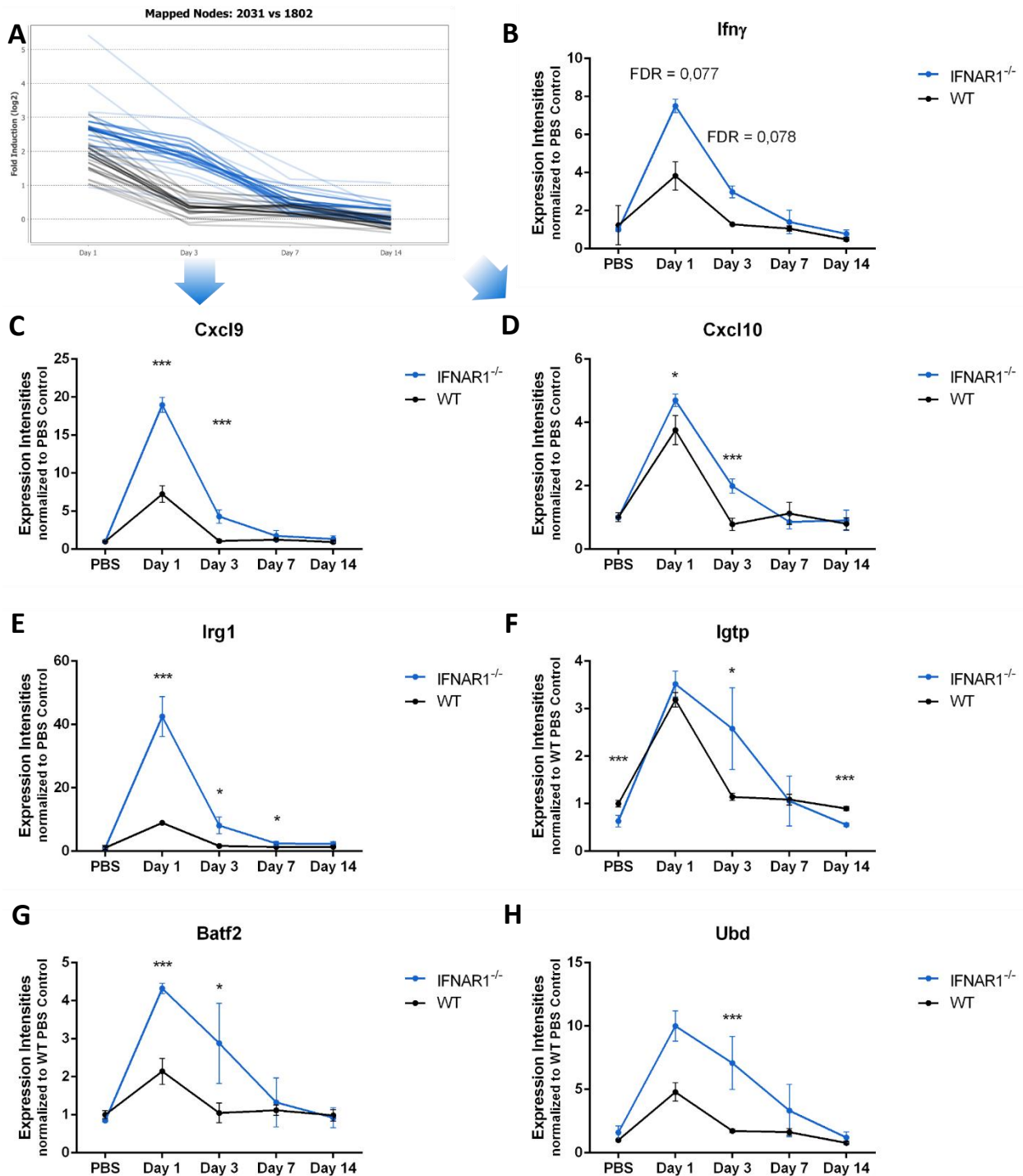


Figure 21: Type II IFN regulated genes were higher expressed in IFNAR1^{-/-} spleens at day 3 of infection.

(A) Clusters were mapped with NodeFinder. (B – H) Single gene figures were created with Prism (from GraphPad) with the microarray expression data (Benjamini Hochberg False Discovery Rate (FDR) $\geq 0,05$).

IFN- γ is a hall of T_H1-mediated immunity and it is also secreted by T_H1 cells(Schoenborn and Wilson, 2007). If *Ifn- γ* is higher expressed in infected spleens of IFNAR1^{-/-} mice at day 1 and day 3, this might be due to an increased T_H1 responses. Therefore, we wanted to examine the

adaptive immune responses in the spleen. We isolated splenocytes from infected and non-infected mice and restimulated them with either live or UV-killed *C. glabrata* for 24 hours. Next, we isolated RNA from cells and determined *Irf7* expression levels as control for type I IFN signaling (Figure 22). Then we have quantified *Ifn γ* expression. Overall, *Ifn γ* expression was higher in IFNAR1^{-/-}-immunized restimulated splenocytes, although it was not significant.

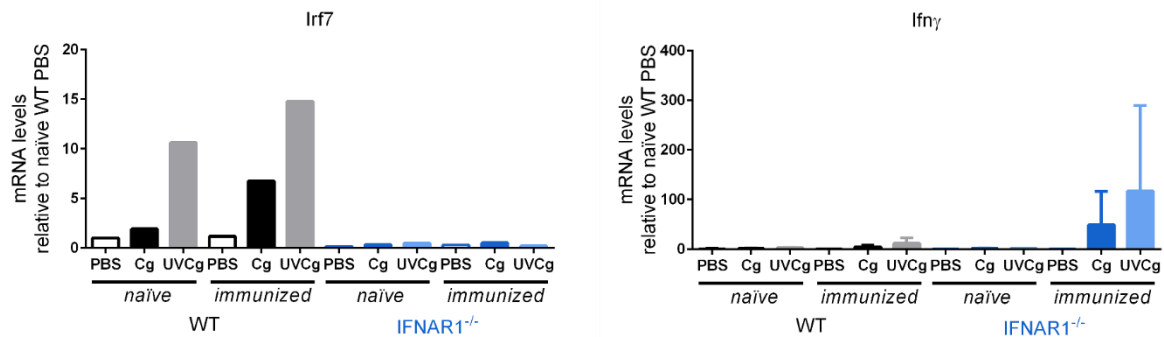


Figure 22: *Irf7* and *Ifn γ* expression in restimulated spleens.

Spleens were isolated from infected (immunized) and PBS-injected (naïve) mice and splenocytes were put into culture. After 1 hour resting, cells are restimulated either with PBS, live *C. glabrata* or UV-killed *C. glabrata* (MOI 1:0,1). (*Irf7*: n = 1; *Ifn γ* : n = 3; unpaired Student's t-Test)

4.6 IFNAR1^{-/-} Spleens and Bone-Marrow Exhibit Different Myeloid Cell Recruitment Patterns

Hematopoietic stem cells are the precursor for the majority of immune cells. Upon infection, cells can be recruited from bone-marrow and cell differentiation can be directed into specific lineages (Murphy et al., 2008). Furthermore the recruitment of different cell subsets into infected spleens, might explain the high inflammatory propensity of IFNAR1^{-/-} spleens upon i.v. *C. glabrata* challenge. More interestingly the relationship between bone-marrow and spleen cell composition might explain source and the destination of cells. Notably, a related study from our group showed reduced inflammatory monocyte and neutrophil recruitment to kidneys of IFNAR1^{-/-} mice upon systemic *C. albicans* challenge (Majer et al., 2012). Here, we investigated neutrophil and inflammatory monocyte (Ly6C⁺) populations in spleen and bone marrow, using the same experimental set-up described previously for day 7 of the infection (Figure 23). Hence, they can be rapidly mobilized from bone-marrow via chemokine-mediated adhesion to sites of inflammation (Lauvau et al., 2014). Neutrophils were much less abundant in bone-marrow of infected IFNAR1^{-/-} mice, whereas in spleen the kinetics appeared to oppose bone-marrow.

However, Ly6C⁺ monocytes were more abundant in bone-marrow and less abundant in spleens of IFNAR1^{-/-} mice, which is contrasting the data about inflammatory properties obtained from microarray data.

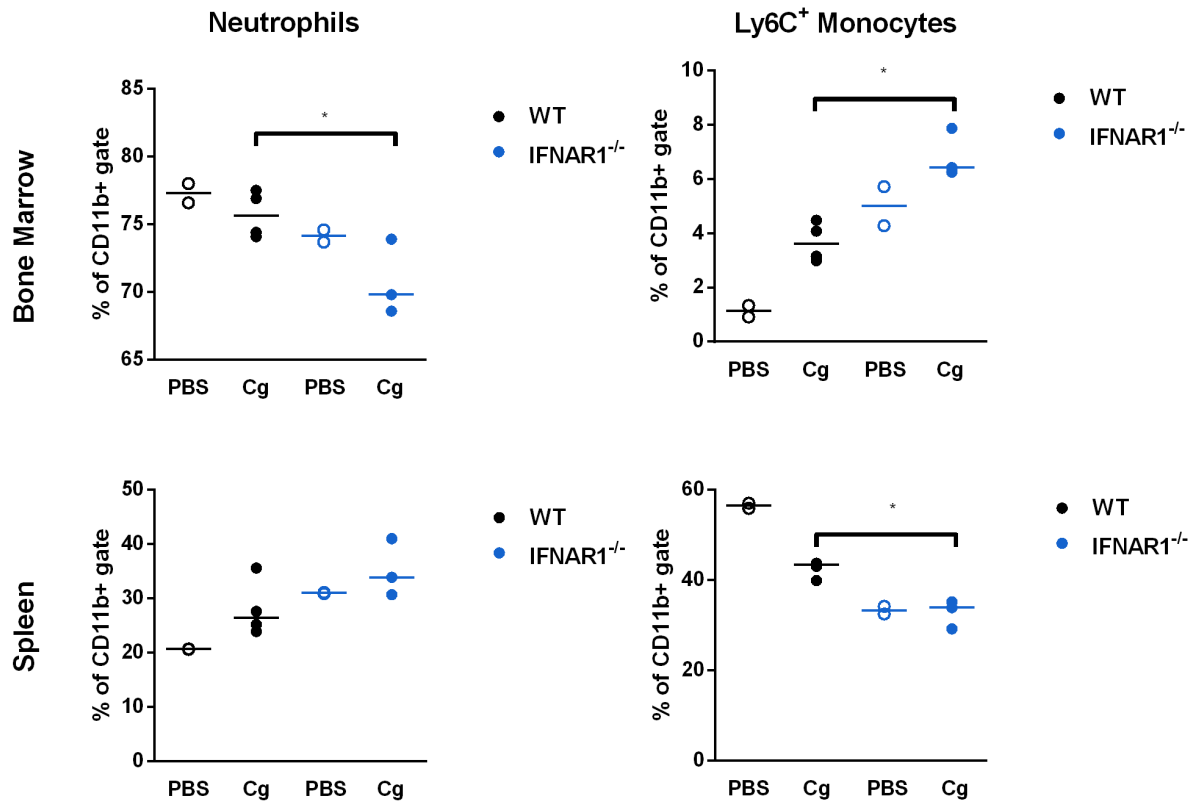


Figure 23: Neutrophils and monocytes in spleen and bone marrow.

Mice were injected either with PBS or *C. glabrata* i.v. from tail vein with 5x10⁷ CFUs per 25g mouse. At day 7 of infection, splenocytes were isolated and stained antibodies against several cell markers. Injections and measurements were carried out by Olivia Majer with fluorescent flow cytometry and analysed by Christelle Bourgeois (PBS controls: n = 2; WT Cg: n = 4; IFNAR1^{-/-} Cg: n = 3; unpaired Student's t-Test).

Spleen is a reservoir for monocytes and the steady-state murine cell composition can determine the outcome of infections (Swirski et al., 2009). It was striking that the basal levels of Ly6C⁺ monocytes were lower in IFNAR1^{-/-} spleens. We were interested to see if this also applies for macrophage populations in the spleen. Therefore, we have quantified macrophage levels in spleens of these mice (Figure 15). Indeed, macrophage viability was reduced in IFNAR1^{-/-} spleens. Limited macrophage numbers in spleen might be relevant, since *C. glabrata* is efficiently phagocytosed by macrophages but can persist in phagosomes.

MHCII⁺/F480⁺ Spleen macrophages

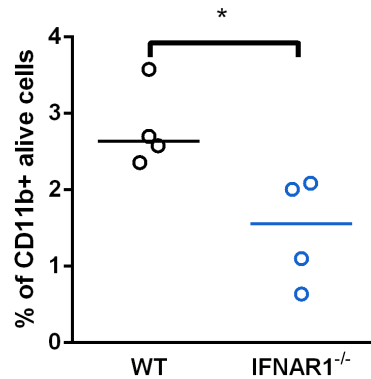


Figure 24: IFNAR1^{-/-} spleens recruit less viable macrophages in the steady state.

Splenocytes were isolated and stained antibodies against several cell markers. Measurements were carried out by Olivia Majer with fluorescent flow cytometry and analysed by me and Christelle Bourgeois using FlowJo software (version 7) (n = 4; unpaired Student's t-Test).

4.7 WT and IFNAR1^{-/-} BM-DMs do not Differ in Phagocytosis and Killing against *C. glabrata* under IFN- β Signaling *in vitro*

As there were fewer viable macrophages in spleens of infected IFNAR1^{-/-}, we wanted to see if the effector functions on these cells are also regulated by type I IFN signaling. Therefore, we have challenged bone-marrow derived macrophages with recombinant IFN- β followed by coincubation with Alexa Fluor 488-labelled *C. glabrata* and then measured phagocytosis by fluorescent flow cytometry (Figure 25 A). In addition, the same macrophages were treated separately with IFN- β only to test activity of the recombinant IFN- β used for this experiment by determining *Irf7* gene expression (Figure 25 B). We did not observe any differences in phagocytosis between WT and IFNAR1^{-/-} BM-DMs at any concentration of IFN- β used (Figure 25 A), although the recombinant IFN- β was potent to trigger type I IFN signaling in these cells (Figure 25 B).

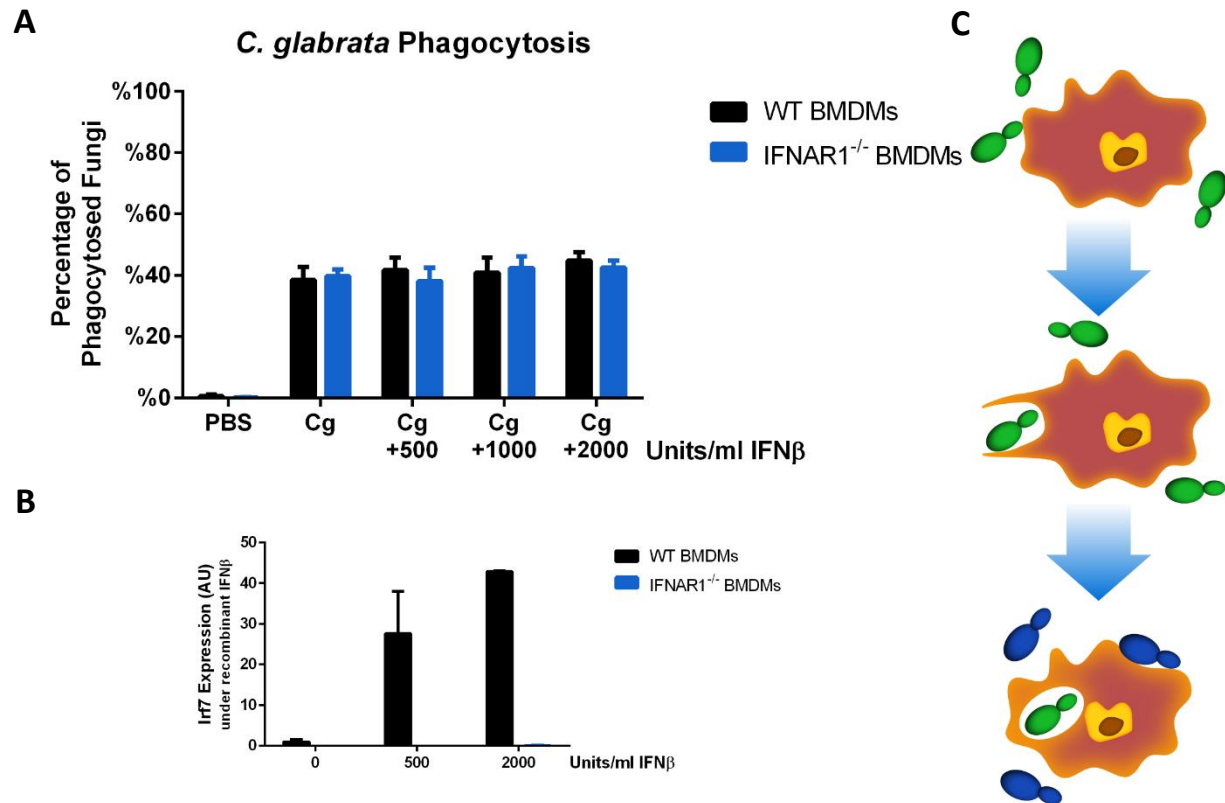


Figure 25: Phagocytosis of *C. glabrata* is not regulated by type I IFNs.

(A) Bone-marrow derived macrophages were treated with different concentrations of recombinant mouse IFN- β for 16 hours. Then Alexa Fluor 488-labelled *C. glabrata* was added to cultures (except for the PBS control). Dishes with macrophages and *C. glabrata* were put on ice as no-phagocytosis controls, whereas other dishes were kept at normal culture conditions for interaction (5% CO₂ at 37°C with humidity). After 45 minutes, dishes were washed with PBS to remove floating fungi and quenched with Trypan Blue for sticking but not phagocytosed fungi. Samples were measured with FACS and gated for macrophages (n=2). (B) To test activity of our recombinant IFN- β stock, macrophages were treated with recombinant IFN- β . After 16h RNA was isolated and cDNA library was prepared. Samples were run on real-time PCR for Irf7 and reference gene (bAct). Data was normalized to reference gene and to untreated WT (n=2). (C) A schematic illustration of phagocytosis assay. Macrophages internalize Alexa Fluor 488 labelled fungi. After 45 min, fluorescence of extracellular fungi is quenched with Trypan Blue.

Macrophages under type I IFN signaling might also have less killing efficiency, due to induced anti-viral state. To address this question, we compared the fungi killing by WT and IFNAR1^{-/-} macrophages under the type I IFN signaling. Therefore, BM-DMs were primed with recombinant IFN- β 16 hours earlier the interaction with *C. glabrata*. After 4 hours of co-incubation, cells were scratched and appropriate dilutions were plated on YPD plates. Colonies were counted after several days. We failed to observe any difference in killing by WT and IFNAR1^{-/-} BM-DMs under the concentrations of IFN- β tested (Figure 26).

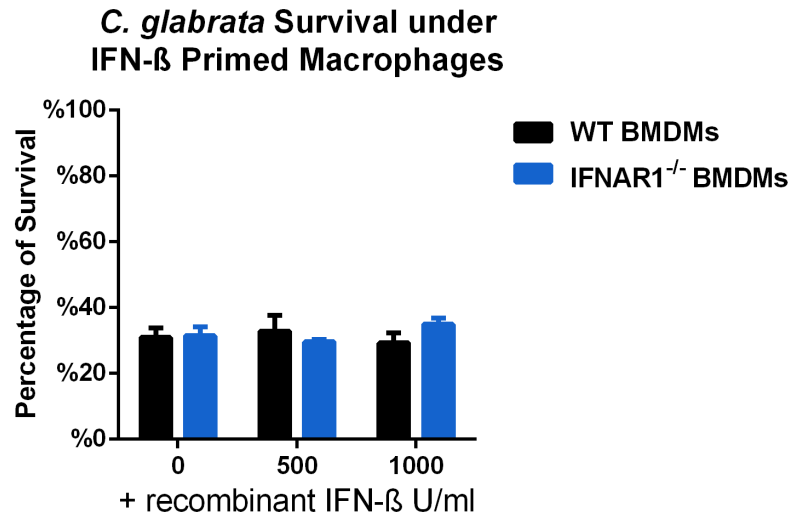


Figure 26: *C. glabrata* killing is not affected by type I IFN signaling.

Macrophages (primed or not-primed with IFN- β) were challenged with *C. glabrata* for 4 hours. Cells and fungi were scratched from the dish and collected into a tube on ice. Dilutions were prepared and plated on YPD plates. After 2 days colonies were counted and this was repeated after another 2 days for late-growing colonies. Results were calculated and normalized with *C. glabrata* growth in media without macrophage killing (n=3, Unpaired Student's t-Test).

Taken together, this work discover detrimental role of type I IFNs in murine systemic candidiasis. Type I IFN signaling dampens the immune response in later stages of infection, therefore, promotes *C. glabrata* persistence in several organs. This trait may rely on inhibition of the type II IFN pathway and increased recruitment of monocytes and macrophages.

5 Discussion

Type I IFNs regulate hundreds of genes and our understanding about its biological function has improved in recent years, xxx that type I IFNs are more than just antiviral-state cytokines. In this study, we have examined its role in a systemic mouse candidiasis model for *C. glabrata*, as this fungus is a potent trigger for type I IFNs in dendritic cells(Bourgeois et al., 2011). With this study, we have demonstrated anti-inflammatory and anti-defence properties of type I IFN signaling on systemic *C. glabrata* infections in mouse.

5.1 Kinetics of Fungal Burden

C. glabrata is less virulent when compared to its fungal relatives such as *C. albicans*(Arendrup et al., 2002; Brieland et al., 2001; Brunke and Hube, 2013; Fidel et al., 1999). Even extremely high inoculum are not deadly for immune-competent mice(Brieland et al., 2001). Moreover although *C. glabrata* organ burden decreases over time, it manages to persist at least 21 days in the host, without killing the host. *C. albicans*, however, with 100x less inoculum is able to damage and kill the host. Other previous studies have emphasized different virulence strategies of *C. albicans* and *C. glabrata*, both species representing the two most frequent causes of lethal nosocomial blood-stream infections in immunocompromised patients(Armstrong-James et al., 2014; Pfaller et al., 2001; Pfaller and Diekema, 2007). At the cellular level, they distinctly respond to host immune surveillance. *C. albicans* escapes phagocytosis with hyphae formation and host cell lysis, whereas *C. glabrata* overcomes phagosome acidification and maturation in macrophages(Brunke and Hube, 2013). Additionally, *C. glabrata* subverts cytokine release and fails to induce hyperinflammatory cytokines(Seider et al., 2011). Both fungal pathogens can be considered a good example for the “dilemma of microbial virulence”; as one uses its spears, the other uses its “shield”, yet both are successful pathogens in their own way.

Type I IFNs regulate hundreds of genes through a highly complex and dynamic system. Its function in bacterial and fungal infections remains ill-defined. Most importantly, the relationships with other parts of the immune system is not as well described as the primary function of type I IFNs – antiviral defence. Its role in fungal infections is even less well

understood. The effects of type I IFNs can, however, determine the outcome of systemic infections. The absence can be either protective or detrimental to the host even for non-infectious diseases such as autoimmune diseases (Majer et al., 2012; O'Connell et al., 2004; Perry et al., 2005; Rauch et al., 2014). It is therefore necessary to have a better understanding of regulatory role of type I IFNs.

Our group has previously reported that *Candida* spp. activate type I IFN signaling in dendritic cells, *C. glabrata* being the strongest trigger. It also promotes higher fungal survival at day 7. A newer study puts type I IFNs as central pathway for host defence using functional genomics (Smeekens et al., 2013). We have also demonstrated a detrimental role of type I IFNs in systemic *C. albicans* infections, through their ability to regulate inflammatory monocytes and neutrophils (Majer et al., 2012). Here, type I IFNs cause increased host death with severe kidney damage due to differential immune cell recruitment to infected organs, yet fungal burden is mainly not affected by type I IFNs. Our findings with *C. glabrata* are quite different, as we demonstrate higher fungal burdens at day 1 in liver and brain of IFNAR1^{-/-} mice, but lower fungal burdens at day 7 in liver and spleen, and at day 14 for liver, brain and spleen. The paradoxical role of type I IFNs may be explained with different degree of response caused by these two different pathogens but also different infections strategies they follow. Combination of other host responses with type I IFNs needs to be investigated for deciphering this paradox.

5.2 Spleen and Systemic Candidiasis

We have chosen to work with spleen, since it is a major immune organ, which may help to shed more light on the underlying mechanisms. However, the spleen is also a very complex model with dynamic cell composition during infections. One needs to keep in mind that due to methodological constraints, gene expression changes, can result from migration of new cell types without any regulation at the transcriptional level. The main methodological pitfall are the difficulties in normalizing of microarray data. We used quantile normalization assuming distribution of general transcription levels would not be different between samples, which is a widely used technique for microarray analysis (Bolstad et al., 2003). Measurements of transcriptional levels or external measurements (such as ELISA for cytokines or characterization of cell populations, *in vitro* models etc.) are essential to verify microarray results. On the other

side, one important caveat of single cell *in vitro* models is that tissue environment is very much different and the cross-talk between different cell types as it occurs *in vivo* is missing *in vitro* with isolated pure cells. Nevertheless, phenotypes we can observe at the organ level are probably biologically more relevant, since it is closer to the organism level.

We had to opt for microarray technology. However, more advanced techniques are available for high-throughput gene expression analysis, such as RNA-Sequencing. RNA-seq allows probe and species independent sequencing, where two species can be sequenced simultaneously(Westermann et al., 2012). Our group has discovered interspecies regulatory networks between *C. albicans* and dendritic cells using dual RNA-seq and systems biology(Tierney et al., 2012). RNA-seq also has a better advantage of identifying possible novel genes. Although, for a complex system such as spleen, microarray still may be suitable, since the RNA-seq might not deliver the required sequencing depths to cover both transcriptional dynamic in host and pathogens in a quantitative manner.

5.3 Time course data, clustering and NodeFinder

The phenotype, we investigate was following a time-course. We hoped to see same pattern also in our gene expression data. Therefore, we used clustering with Pearson correlation, which primarily focuses on “changes” between time-points rather than the absolute value. In other words, the shape of gene expression pattern is more relevant. However Pearson correlation is a hierarchical clustering method, which does not deliver group of genes but a tree structure. k-means clustering on the other hand delivers a defined number of clusters, although one problem is the decision of the number of clusters, which essentially introduces a bias. One possibility is using hierarchical clustering and conducting a meta-analysis on the tree-structure. However, this also introduces a user-bias. Silhouette splitting on hierarchical clusters solves both of these problems, which was first described in 1987(Rousseeuw, 1987). Therefore, we exploited silhouette splitting on our hierarchical clustering data using NodeFinder. Additionally, NodeFinder can also map clusters between datasets to underline co-regulated genes in different experimental groups (such as genotypes). Indeed with the aid of GO term-associated labelling of the genes, we could rapidly identify 3 relevant groups of genes with similar expression patterns and particular interest.

Several methods and tools are available for clustering high-throughput data. A widely used method is k-means clustering. One particular problem with this method is that it introduces user bias, since the user is required to determine the number of clusters. Hierarchical clustering methods are also widely used in the field. However, these methods create tree-structures and do not directly provide groups of genes. (Mean) Silhouette splitting (MSS) can be applied after the hierarchical clustering to obtain stable clusters. However, not many tools are available for this approach. For instance, "*hopach*" package in R provides tools for hierarchical clustering and MSS. We sought to take another approach with mapping of split clusters between two datasets (WT and IFNAR1^{-/-}) after MSS, to find out differences in gene expression patterns between these two genotypes. Therefore, we have developed a novel software - NodeFinder to be used after Cluster 3.0 (a clustering tool with a graphical user interface (GUI) and several techniques including hierarchical clustering) in the pipeline. NodeFinder also provides an interactive GUI and does not require from the user knowledge of any scripting language. Additionally, users can browse their data, export reports, retrieve information (including GO terms and synonyms) from Ensembl databases and most importantly, users can map clusters (groups of genes) between multiple datasets to determine differences and similarities among these datasets. Here we demonstrate, genes with similar functions and gene expression patterns can be identified with NodeFinder. Of note, one gene (Elane) with similar gene expression pattern and function was missed with our data. Care needs to be taken when choosing thresholds for MSS. Too low and too high thresholds need to be avoided.

5.4 Immunity-Related GTPases

Two families of immunity related GTPases emerged from the analysis. In recent years, studies have emerged linking these proteins to autophagy and microbial clearance, especially intracellular pathogens. For instance, *lipg1* protects mice from *Toxoplasma gondii* but not from other intracellular pathogens (Liesenfeld et al., 2011). Four *Gbp* genes in macrophages establish cell-autonomous immunity to *L. monocytogenes* and *Mycobacterium bovis*. *Irgm* regulates macroautophagy by localizing *Gbp2*. However, the absence however leads to increased susceptibility to many organisms (Traver et al., 2011). Remarkably, we identify the same proteins as implicated in fungal host response.

We identify 7 GTPases in one cluster mapping, which are all higher expressed in IFNAR1^{-/-} spleens at the 3rd day of infection. Of note, that these GTPases are primarily transcriptionally upregulated by Ifn γ (Boehm et al., 1998). However, p47-family seems to be absent in humans, and the only full-length orthologous gene is not controlled by IFN γ in humans(Bekpen et al., 2005).

5.5 Antimicrobial and Inflammatory Response at Later Stages

Cluster mapping revealed another interesting group of genes, all of which are higher expressed in IFNAR1^{-/-} spleens. Prtn3, Elane and Ctsg are serine proteases occurring in the neutrophilic granules(Korkmaz et al., 2010). Another neutrophil effector gene is Ngp, which is also following a similar pattern. This indeed confirms our notion about a possible involvement of neutrophils in spleen. Because in the systemic *C. albicans* model, neutrophils were responsible for the type I IFN promoted recruitment to spleen(Majer et al., 2012). Although we did not observe increased neutrophil recruitment to spleen, surprisingly less neutrophils were present in bone-marrow after infection. Either neutrophil production decreased in bone-marrow, neutrophils were mobilized to other tissues or not activated. This remains as an open-question at this point. Interestingly, inflammatory Ly6C⁺ monocytes were less abundant in IFNAR1^{-/-} spleens upon infection. These properties were quite different for the bone-marrow. Furthermore, in steady-state there were less macrophages in IFNAR1^{-/-} spleens.

At this point, we can speculate, based on the available literature, that reduced virulence is probably due to a reduced number of macrophages and monocytes, fungi are exposed to neutrophils. Since *C. glabrata* can survive in macrophages and subvert the cytokine response to attenuated inflammatory response, it might be an advantage for the fungi to be phagocytosed, rather than exposed to neutrophils(Brunke and Hube, 2013; Seider et al., 2011). Co-culture experiments with neutrophils and macrophages with/without type I IFN stimulus might address this open question.

5.6 Type I IFNs vs. Type II IFN

Interestingly a set of IFN- γ regulated genes were higher expressed in IFNAR1^{-/-} spleens at day 3. Ifn- γ expression was similar, but insignificant after Benjamini-Hochberg correction (FDR of

~7,7%). However a set of IFN- γ regulated GTPases were also demonstrating a similar pattern. Therefore we assume that type I IFN signaling was inhibitory on the type II IFN signaling. Notably, this would not be the first report demonstrating inhibitory effect of type I IFN signaling on type II IFN response. For instance type I IFN signaling suppresses type II IFN mediated resistance to mycobacterial (leprosy) infection (Teles et al., 2013). In another report type I IFNs were reported as downregulators of *Ifngr1*, essential component of IFN- γ receptor, via *Egr3* and *Nab1* (Kearney et al., 2013). Moreover in a mouse tuberculosis model authors have suggested that type I IFNs were associated with decreased IFN- γ response and T_H1 phenotype, which was detrimental for the host (Manca et al., 2001). Interestingly *M. tuberculosis* was also shown to trigger type I IFN responses in innate immune cells (BM-DMs) and through delivery of bacterial nucleic acid by a secretion system into cytosol (Manzanillo et al., 2012). However such secretion system is not yet identified for *C. glabrata*. Since, we have similar observations, we hypothesized that spleens of *IFNAR1*^{-/-} mice have increased T_H1 response, which can be followed by *Ifn γ* expression in restimulated splenocytes. Although the average IFN γ response is higher, difference is insignificant. This experiment was performed with 3 biological replicates but needs to be repeated with more to identify outliers. Also performing ELISA on the media would be better idea to pursue the current hypothesis, as transcriptional regulation, might be downregulated at different speed and time.

Yet it is unclear whether type II IFNs are responsible for increased inflammatory and antimicrobial response, however this question can be addressed with mice deficient in type II IFN signaling. Although in average there were more neutrophils in *IFNAR1*^{-/-} spleens, difference was insignificant, suggesting a regulatory role of type I IFNs in neutrophil activation. However more investigation is required to identify if this is a direct regulation.

Here, based on our data we would like to suggest the following model (Figure 27). Fungal burden is higher in *IFNAR1*^{-/-} brains and livers at day 1 of infection. In contrast, at day 7 and day 14 fungal burden is higher in the WT spleens and livers. The induction of immunity related GTPases and type II IFN regulated genes is stronger in *IFNAR1*^{-/-} mice. This suggests a possible inhibitory role of type I IFNs on type II IFN pathway and T_H1 mediated immune response. Moreover, the oxidative, antimicrobial and inflammatory responses are stronger induced in

IFNAR1^{-/-} spleens. This trait may explain better fungal clearance and enlarged spleens in IFNAR1^{-/-} organs at this stage. Interestingly, IFNAR1^{-/-} spleens contain lower levels of live macrophages in steady state and less inflammatory monocytes upon infection, which might affect the kinetics of fungal clearance.

Taken together with NodeFinder we are able to identify coregulated genes. These results are consistent with the biological outcome and give insights about the underlying mechanisms. Additionally, involvement of type II IFNs is also consistent with recent reports. However, further research is necessary to address unanswered questions.

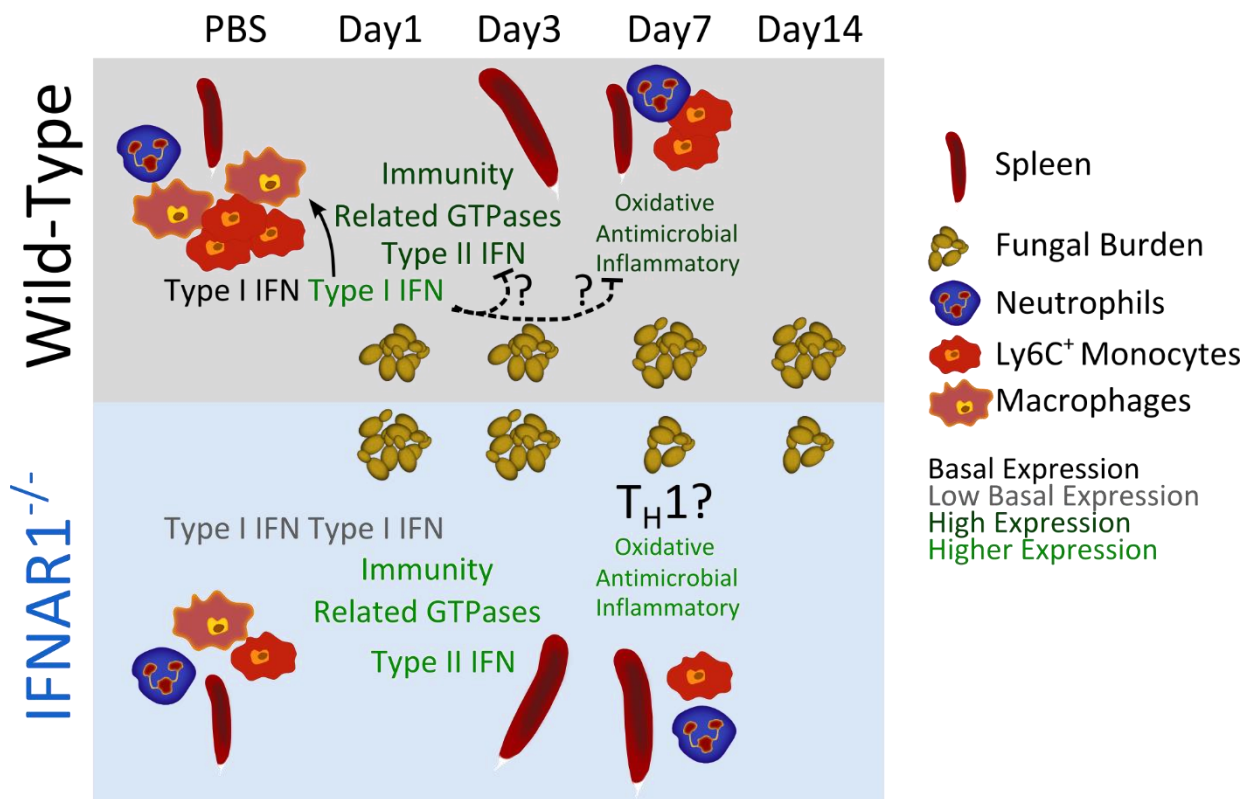


Figure 27: A murine model for the role of type I IFNs during systemic *C. glabrata* infections.

6 Material and Methods

6.1 Fungal Methods

Candida glabrata ATCC 2001 strain (J. Infect. Dis. 1: 98, 1917.) was used in all experiments.

6.1.1 Culture Conditions for *C. glabrata*

Liquid Media

1xYPD	Yeast extract (Difco)	10g/l
	Bacto-Peptone (BD)	20g/l
	Glucose	2%(w/v)

- Yeast extract (Difco) and Bacto-Peptone (BD) were dissolved in ddH₂O to match above mentioned concentrations and autoclaved immediately (1xYP).
- A separate 20% glucose solution was also prepared and autoclaved immediately.
- YPD liquid media was prepared by adding 50ml of glucose to 450ml of 1xYP and stored in room temperature in a sterile environment.

Solid Media

YPD Agar plates	Yeast extract (Difco)	10g/l
	Bacto-Peptone (BD)	20g/l
	Agar	5g/l
	Glucose	2%(w/v)

- Yeast extract (Difco) and Bacto-Peptone (BD) were dissolved in ddH₂O to match twice the above mentioned concentrations and autoclaved immediately (2xYP).
- Separate solutions were prepared for glucose (20%) and agar (twice the above-mentioned concentration) and autoclaved as well. Solutions were stored at room temperature.
- Agar was molten in microwave, mixed with 2xYP and glucose, and poured into plastic plates.

6.1.2 *C. glabrata* Growth and Culture Preparation

- *C. glabrata* was streaked on an YPD plate from frozen -80°C (in 20% glycerol) stocks. After several days a single colony was picked and grown in 5ml YPD at 30°C with shaking.
- After 3-4 hours culture reached an OD_{600nm} around 0,4, which was then diluted to reach an OD_{600nm} of 1,0 next day for the interaction in 20ml YPD.
- ~1,00D_{600nm} of the *C. glabrata* culture was centrifuged at 1200xg for 5min at room temperature and resuspended in 30ml of PBS.
- After another washing step *C. glabrata* pellet was resuspended in 5ml PBS and counted with CASY-cell counter.
 - For *in vitro* experiments a dilution of the fungal suspension was prepared with corresponding medium.
 - For *in vivo* studies the fungal suspension was further diluted with PBS and the fungal concentration was counted again with CASY.
- Viable *C. glabrata* burden was detected as colony forming units (CFUs) by plating on YPD plates.

6.1.3 UV-Killing of *C. glabrata*

- *C. glabrata* culture was prepared as described in 6.1.2
- Cells were transferred into a sterile 10cm round plastic non-treated plate
- Plate with cells was radiated in UV crosslinker 9950μJ/cm²
- Killing was tested by plating aliquots of undiluted suspension on YPD plates
- Cells were centrifuged, resuspended in PBS and finally counted again.

6.1.4 Alexa Fluor 488-Labeling of *C. glabrata* for Phagocytosis Assay

- *C. glabrata* culture was prepared as described in 6.1.2 but the final pellet was resuspended in 1ml of 100mM HEPES buffer pH7,5 instead of PBS.
- 200μl of the suspension was taken as unstained *C. glabrata*.
- To the remaining 800μl, 200μl of Alexa Fluor 488 from Invitrogen (1mg/ml) was added and cells were incubated at 30°C with shaking for 1 hour in the dark.

- Cells were then washed 3 times with 100mM HEPES buffer pH7,5 3 times by centrifuging at 1200xg for 5min at room temperature.
- Resulting pellet was resuspended in 100mM HEPES buffer pH7,5 and counted for interaction.

6.2 Host & Primary Mouse Cell Culture

All host cell incubations were done at 37°C, with 5% CO₂ and 95% humidity environment/incubator.

6.2.1 Media

DMEM	Dulbecco's modified Eagle's Medium DMEM, high glucose (4.5g/l), with L-Glutamine, without pyruvate (PAA)
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Full-DMEM	DMEM
	FCS <i>10% (v/v)</i>
	Penicillin <i>100U/ml</i>
	Streptomycin <i>100µg/ml</i>

RPMI	RPMI (with stable glutamine) (PAA)
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Full-RPMI	RPMI
	FCS <i>10% (v/v)</i>
	Penicillin <i>100U/ml</i>
	Streptomycin <i>100µg/ml</i>

Macrophage Media (Mφ Media)	Full DMEM
	L-Conditioned Media <i>15%-20% (v/v)</i>

Phosphate Buffered Saline (PBS)	NaCl <i>140mM</i>
	KCl <i>27mM</i>

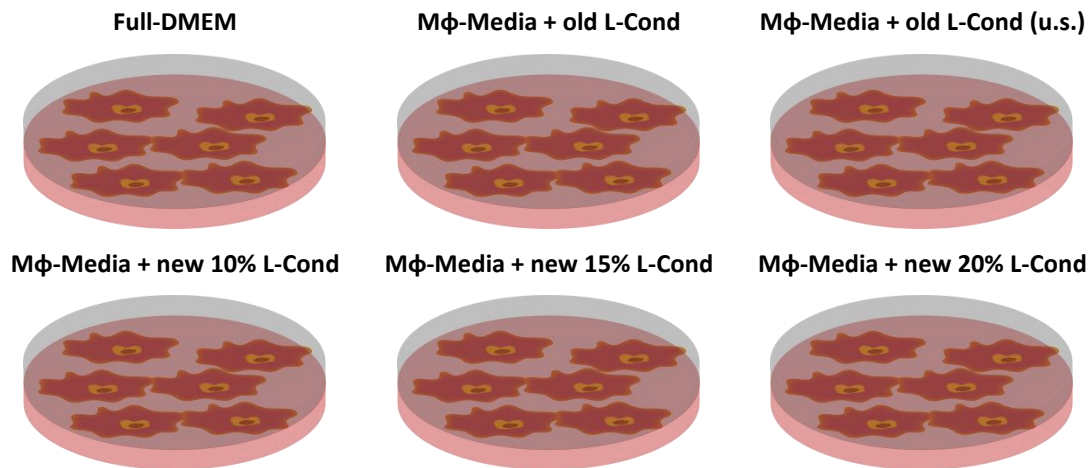
	Na ₂ HPO ₄	90mM
	KH ₂ PO ₄	15mM
FACS Buffer	PBS	
	FCS	10% (v/v)
FACS Fix Buffer	PBS	
	FCS	10% (v/v)
	Paraformaldehyde	4% (v/v)
Freezing Medium	FCS	
	DMSO	10% (v/v)
Red Blood Cell Lysis Buffer (RBC)	Tris/HCl pH7,0	0,01M
	NH ₄ Cl	8,3g/l
MPO Buffer (MPO) pH7	MOPS	20mM
	NaCl	0,1M
	CaCl ₂	1mM

6.2.2 L-Conditioned Media Preparation

- CSF-1 producing confluent culture of L929 cells (ATCC® CCL-1™) was splitted into 175cm² cell culture flasks with 50ml of DMEM (+10% FCS).
- After culture reached 70-80% confluency, media was aspirated and replaced with 100ml of DMEM (without FCS or antibiotics).
- After ten days, conditioned culture (L-Conditioned Media or L-Cond) as collected and filtered with Steritop 0,22µm GP Express PLUS membranes (Millipore, Billerica, MA, USA). Media was stored frozen at -20°C and a small aliquot at +4°C for testing.

L-Condition Testing

- Either frozen or fresh isolated mouse bone marrow (depleted of red blood cells) was resuspended in DMEM and counted with CASY-cell counter.
- 1×10^6 cells were transferred into 6-well plate with **Full-DMEM**, M ϕ -Media with the **new 10% L-Cond**, M ϕ -Media with the **new 15% L-Cond**, M ϕ -Media with the **new 20% L-Cond**, M ϕ -Media with the **old L-Cond**, and M ϕ -Media with the **old L-Cond for unstained control (u.s.)** for fluorescence-based flow cytometry analysis.



- Plate was placed on ice and cells were gently scraped from the dishes at day 10-12 and resuspended in PBS with 1ml 2% FCS and counted on CASY counter to determine the culture with most cells.
- Cells were centrifuged and resuspended in 50 μ l of FACS buffer (PBS with 1% FCS) and subsequently incubated with (+1 μ l of) CD16/32 blocking antibody on ice for 5min to prevent unspecific antibody binding.
- 1 μ l of CD11b-FITC and F4/80-PE antibodies were added to the suspension and incubated further for 30min on ice in the dark.
- Cells were centrifuged at 400xg for 5min at 4°C and washed twice with cold 1ml of FACS buffer.
- Finally cells were resuspended in 400 μ l of FACS fix buffer and stored 4°C in the dark or immediately analyzed with FACS.
- Correct L-Cond concentration was selected based upon most number of macrophages with the purest macrophage culture for the selected markers (mostly 15%).

6.2.3 Isolation of Mouse Bone-Marrow

- 8-10 weeks old mice were dissected; femora and tibiae were prepared, rinsed with 70% ethanol for about 10 seconds and placed in ice-cold PBS.
- Bones were then rinsed again in 70% ethanol shortly and placed in DMEM.
- Femora and tibiae were separated at knee-joints from each other.
- Bones were held with forceps above a 50ml tube, cut at the both extreme edges.
- Bones were then flushed with a 27Gx3/4 needle and a 20ml syringe with 2-3ml full DMEM, until flushed the media was no more turbid.
- Cells were centrifuged at 300xg for 7min at 4°C, media was aspirated.
- Resulting pellet was resuspended in 1ml red cell lysis buffer and incubated for 2 minutes at room temperature to lyse erythrocytes.
- Immediately after the lysis, 9ml of DMEM was added to neutralize the buffer and centrifuged at 300xg for 7min at 4°C.
- Resulting pellet was either frozen in freezing medium (stored for 2-3 days at -80°C and in liquid nitrogen for longer storage), differentiated or for used for FACS analysis.

6.2.4 Differentiation of Bone-Marrow Derived Macrophages

- Either;
 - Bone-marrow was isolated and frozen as described in 6.2.3. Frozen bone-marrow stock (0,5ml) was thawed and immediately placed into DMEM and centrifuged at 300xg for 7min at 4°C. Media was aspirated;
 - OR
 - Bone-marrow was isolated as described in 6.2.3;
- Resulting pellet was resuspended in 3ml of M ϕ media, placed into 100mm square non-treated plastic cell culture dishes with 5ml M ϕ media (8ml in total) and incubated.
- At day 2 5ml M ϕ media was added to the culture.
- At day 6-7, because of high confluency in the cultures, media was aspirated and cells were collected by gently scraping with a soft rubber spatula in fresh media. Cells were splitted into 2 dishes in 8ml M ϕ media.
- After day 10-12, macrophages were collected by gently scraping and counted.

6.2.5 Isolation of Splenocytes

- Spleens were dissected from mice and placed into ice-cold sterile PBS (pre-weighed).
- Tubes with spleens were weighed again to determine the spleen weight.
- Spleens were placed into onto a 70µm cell strainer on a 50ml tube, homogenized and collected in PBS with 2% FCS.
- Cells were centrifuged at 300xg for 10min at 4°C
- Resulting pellet was resuspended in 2ml red blood cell lysis buffer and incubated at room temperature for 2min.
- Lysis was stopped by adding 20ml PBS with 2% FCS and centrifuged again at 300xg for 10min at 4°C.
- Resulting pellet was resuspended in 2ml full-RPMI and cells were counted with CASY cell counter.

6.3 Interactions of Host and Immune Cells with *C. glabrata*

6.3.1 Survival Assays

- BM-DMs were differentiated from mouse bone-marrow as described 6.2.4.
- 5×10^4 BM-DMs were transferred into each well on 96-well plate.
- After 1 hours, recombinant IFN- β was added (except for the controls) to the culture.
- Next day *C. glabrata* culture was prepared as described 6.1.2 and added to the cultures with an MOI of 1:2.
- After 4 hours, plate was placed on ice to stop killing.
- Cells were scratched with yellow tip and media (100µl in total) was placed into a tube with 500µl ice-cold PBS on ice. 200µl of ice-cold PBS was added to the well. This step was performed for all wells.
- Well with PBS were scratched again and placed into the tubes. Another 200µl of PBS was added to the well, scratched and collected in tubes (total 1ml volume for each tube).
- 2 dilutions were prepared (1:100 and from that 1:1000) and 250µl was plated on plates.
- Plates were incubated at 30°C for 1-2 days and colonies were counted.

6.3.2 Restimulation of Splenocytes

- Splenocytes were isolated as described in 6.2.5 and 10^7 splenocytes were distributed in 3,5cm cell culture dishes.
- After 2 hours, cells either PBS, live or UV-killed *C. glabrata* was added to culture.
- 24 hours later dishes were placed on ice and floating cells were collected into tubes by centrifuging at 300xg for 7min at 4°C. Supernatants were kept in -80°C.
- Meanwhile dishes were washed with ice-cold PBS, which was then transferred into the tubes with cell pellets to wash the remaining media.
- Cells were centrifuged again. Meanwhile plates were scraped with RNA lysis buffer for the adherent cells.
- Cell lysates were transferred into the tubes to the non-adherent cell pellet and mixed.
- Lysates were then kept at -80°C for RNA isolation.

6.3.3 Phagocytosis Assays

- 1×10^6 cells were plated into each well on a 6-well plate in 1,5ml media and further incubated over/night.
- Next day, *C. glabrata* culture was prepared and stained as described in 6.1.4 and added to the culture in an MOI of 2:1. Cells were incubated either in normal conditions or on ice as negative control.

Two aliquots of the *C. glabrata* suspension was parallel either incubated at 4°C for 15min or quenched with 0,4% Trypan Blue Solution (from Sigma) at 4°C for 15min as controls for staining and quenching.

- Cells were washed 3 times with PBS.
- Adhered but not phagocytosed *C. glabrata* cells were quenched by adding 0,4% Trypan Blue Solution (from Sigma) at 4°C for 15min.
- Cells were washed again 3 times with PBS and gently scraped from the plates at the last step.
- Cells were collected centrifuged at 300xg for 5min at 4°C in amber tubes and resulting pellet was resuspended in FACS fix buffer.
- Cells were analysed with fluorescence-based flow cytometry.

6.3.4 *in vivo* Experiments

All animal experiments and isolation of primary cells were conducted with mice held in specific pathogen-free (SPF) conditions. C57BL/6 mice and *Ifnar1*^{-/-}-mice were obtained from University of Veterinary Medicine, Vienna.

6.3.5 Systemic *C. glabrata* Infection Model

- *C. glabrata* culture was prepared as described in 6.1.2.
- Male mice between 7-10 weeks were injected through tail vein with 5×10^7 fungi per 25g mice in ~100µl PBS (inoculum was normalized to the body weight of each individual), whereas control mice were injected only with PBS.
- On day 1, day 3, day 7 and day 14 of the infection organs were collected, weighed and cut in half. One half was put in 2ml ice-cold PBS for determining fungal burden and the other half in *RNAlater*[®] (from Ambion).

6.3.5.1 Harvesting Organs for Fungal Burden

- Organs in MPO buffer (+of “protease inhibitor cocktail (complete) without EDTA” from Roche/ 1 tablet per 50ml MPO buffer) were homogenized using “Ultra Turrax” (from IKA) with occasional cooling on ice.
- Serial dilutions were prepared in PBS for organs:
 - 1:10, 1:100 and 1:1000 for organs from day 1 and day 3.
 - 1:10 and 1:100 for organs from day 7
- Dilutions or homogenates were plated in triplicates (per individual, organ, and dilution) on YPD plates with antibiotics (containing: ampicillin (120µg/ml), chloramphenicol (40µg/ml) and tetracycline from Sigma).
 - For day 1 and day 3: all dilutions
 - For day 7 both dilutions and the homogenate
 - For day 14 only the homogenates
- Colonies were counted after 2 days and recounted after 4 days for lately growing colonies.

6.3.5.2 Harvesting Organs for RNA Isolation

- Organs in RNA/later® were washed in PBS in a new tube and then homogenized with Ultra Turrax (from IKA) in 1,5ml RNA lysis buffer (from “SV Total RNA Isolation System” - Promega) with occasional cooling on ice.
- Lysates were frozen at -80°C.

6.3.6 RNA Isolation from Mammalian Cells

- Lysates were thawed for RNA isolation using “SV Total RNA Isolation System” from Promega, described as in the complete protocol by the manufacturer:
- 175µl of lysate in RNA lysis buffer was diluted with RNA dilution buffer, heated to 70°C for 3min and centrifuged at 13000xg for 10min at room temperature.
- Supernatant was mixed 95% ethanol and placed onto columns delivered with the kit and centrifuged.
- Columns were washed with RNA wash solution (with ethanol) and centrifuged.
- Columns were treated with DNase to degrade all DNA remaining in the columns.
- Columns were washed twice again and RNA was eluted from the membrane into nuclease-free tubes by centrifuging with pre-warmed (65°C) nuclease-free water.
- RNA concentration was measured with NanoDrop 2000c UV-Vis Spectrophotometer (ThermoScientific) and RNA quality was checked on Agilent 2100 Bioanalyzer on a RNA 6000 Nano Chip.

6.3.7 Characterizing Cell Population in Spleen or Bone-Marrow

- Spleens were placed in 6-well plates with 2ml collagenase D solution (2mg/ml).
- Spleens were then injected with the same solution (27G needle and 5ml syringe) and cut into pieces.
- Pieces were incubated at 37°C for 30min, then passed through 70µm cell strainer using the plunger of a syringe.
- Cells were washed off from the strainer and the syringe with 6ml buffer and transferred into 15ml tubes.

- Cells were counted on CASY and 1/10th was taken for red cell lysis and the rest was used for sorting without red cell lysis.
- Cells were centrifuged at 300xg for 10min at 4°C and resulting pellet was resuspended with 2ml red cell lysis buffer.
- Cells were incubated for 2min at room temperature and then immediately filled up with 10ml PBS/2%FCS.
- Cells were collected again by centrifuging and resuspended in 250µl PBS/2%FCS for bone-marrow or 1ml PBS/2%FCS for spleen and counted with CASY.
- 106 cells were transferred in FACS tube, centrifuged and resuspended in PBS to wash serum away.
- Live/dead staining was performed by adding 0,2µl of the dye (eBioscience eFluor® 780) into 200µl of the cell suspension and incubating for 30min at 4°C in the dark.
- 1ml PBS was added and cells were centrifuged.
- Cells were resuspended in 20µl FACS buffer with 1µl CD16/32 blocking antibody to prevent unspecific antibody binding to Fc receptors.
- Cells were incubated on ice for 15min and 50µl of antibody mix (1µl of each antibody) was added to the cells:

CD11b	V500
CD11c	PerCP-Cy5.5
Ly6C	V450
CCR5	PE
MHCII	PE-Cy7
F4/80	APC
Source: eBioscience	

- Cells were incubated at 4°C in the dark for 30min and washed with FACS buffer.
- Cells were washed with annexinV binding buffer and 5µl of the annexinV-FITC was added to 100µl cell suspension.
- Cells were washed again with the binding buffer and resuspended in 200µl binding buffer.
- Cells were analyzed within 4 hours with fluorescent flow cytometry.

6.3.8 mRNA Reverse Transcription

- An aliquot (100ng – 2µg) of each RNA sample was diluted up to 19,8µl on a 96-well PCR plate, heated at 70°C for 10min and immediately placed on ice to open secondary structures.
- Reverse transcription mix was prepared from Promega Reverse Transcription Kit and added to each sample:

	per Reaction
MgCl ₂ (25mM)	8µl
Reverse transcription buffer 10x)	4µl
dNTP mixture (10mM each)	4µl
Recombinant RNasin® Ribonuclease Inhibitor	1µl
AMV Reverse transcriptase (30U)	1,2µl
Oligo(dT) ₁₅ Primer	2µl

- Reaction was carried out at 42°C for 70min, followed by 5min of heating at 95°C. Samples were then immediately placed on ice.
- cDNA samples were diluted up to 100-200µl with nuclease-free water.

6.3.9 Quantitative Real-Time PCR

- qPCR mix was prepared and distributed on a white 96-well opaque plate:

	per Reaction
KAPA SYBR Green Mastermix (Peqlab)	10µl
Forward Primer (10µM)	0,2µl
Reverse Primer (10µM)	0,2µl
H ₂ O	7,1µl

- 2,5µl of the prepared cDNA (see 6.3.8) was added to wells.
- Reaction was started immediately on Eppendorf Mastercycler® ep realplex⁴ real-time PCR machine with the following PCR program:

<u>Hot-start</u>	<u>Denat.</u>	<u>Anneal.</u>	<u>Elong.</u>			<u>Melting curve</u>		
<u>& Denat.</u>								
95,0°C	95,0°C	60,0°C	72,0°C	95,0°C	60,0°C	60,0°C→95,0°C	95,0°C	4,0°C

3min 3sec 20sec 8sec 15sec 15sec 20min 15sec ∞

40x cycles

- Each sample was measured at least in duplicates on the same run.
- Results were analyzed either relative to reference gene:
 - Gene expression was calculated relative to reference gene(s) (bAct, HPRT or average of both) according to the following formula:

$$\Delta Ct = \Delta Ct_{GOI} - \Delta Ct_{Ref}$$

$$R = 2^{-\Delta Ct}$$

or as fold induction (relative to untreated control)

- Gene expression was calculated relative to the untreated control and normalized to the reference gene(s) (bAct, HPRT or average of both) according to the following formula:

$$\Delta Ct_{treated} = \Delta Ct_{GOI} - \Delta Ct_{Ref}$$

$$\Delta Ct_{untreated} = \Delta Ct_{GOI} - \Delta Ct_{Ref}$$

Primers used for real-time PCR	Sequence (5'→3')
mLtf-fw	TGAGGCCCTTGGACTCTGT
mLtf-rev	ACCCACTTTTCTCATCTCGTTC
mNgp-fw	AGACCTTTGTATTGGTGGTGGC
mNgp-rev	GGTTGTATGCCTCTATGGCTCTA
mMpo-fw	AGTTGTGCTGAGCTGTATGGA
mMpo-rev	CGGCTGCTTGAAGTAAACAGG
mCtsg-fw	TGCCTTCAGGGGTGATTCTG
mCtsg-rev	TCTTGGTGCAAAGCGTCTCA
mIrf7-fw	CTGGAGCCATGGGTATGCA
mIrf7-rev	AAGCACAAGCCGAGACTGCT
mIfng-fw	TAAGCGTCATTGAATCACAC
mIfng-rev	GTTGTTGACCTCAAACCTTG

6.4 Statistics and Figures

For all statistical analysis (except for microarray results and GO terms analysis) Prism 6 for Windows (Release 6.04) from GraphPad Software Inc. was used. Bars and data points indicate mean values. For all figures: * $p \leq 0,05$; ** $p \leq 0,01$; *** $p \leq 0,005$. GIMP was used to

create final figures as image file. Vector-Image files were edited with Inkscape(Maximilian Albert et al., n.d.; Spencer Kimball et al., n.d.).

6.5 Microarray Analysis and Clustering

6.5.1 Microarray Hybridisation

Microarray hybridisation was carried out by Andriy Petryshyn and the raw data analysis was performed by Walter Glaser. Agilent's SurePrint G3 Mouse GE 8x60K Microarray, (Agilent Microarray Design ID 028005) was used for mouse gene expression analysis.

- Arrays #10-CB26 and #21-CB5 show outlier behaviour due to the air bubble and lower intensities respectively. Removal of the arrays does not show an improvement of statistics. The arrays were therefore retained but down weighted.

Number	Label	Sample Description	Strain	Treatment	Day
1	CB17	I1 D1	IF	I	1
2	CB18	I3 D1	IF	I	1
3	CB19	I5 D1	IF	I	1
4	CB20	I6 D3	IF	I	3
5	CB21	I7 D3	IF	I	3
6	CB22	I10 D3	IF	I	3
7	CB23	I12 D7	IF	I	7
8	CB24	I13 D7	IF	I	7
9	CB25	I14 D7	IF	I	7
10	CB26	I17 D14	IF	I	14
11	CB27	I18 D14	IF	I	14
12	CB28	I20 D14	IF	I	14
13	CB29	IC1	IF	PBS	14
14	CB30	IC2	IF	PBS	14
15	CB31	IC4	IF	PBS	14
16	CB32	IC5	IF	PBS	14
17	CB1	W2 D1	WT	I	1
18	CB2	W3 D1	WT	I	1
19	CB3	W5 D1	WT	I	1
20	CB4	W6 D3	WT	I	3
21	CB5	W7 D3	WT	I	3
22	CB6	W10 D3	WT	I	3
23	CB7	W11 D7	WT	I	7
24	CB8	W13 D7	WT	I	7

25	CB9	W14 D7	WT	I	7
26	CB10	W16 D14	WT	I	14
27	CB11	W18 D14	WT	I	14
28	CB12	W19 D14	WT	I	14
29	CB13	WC2	WT	PBS	14
30	CB14	WC3	WT	PBS	14
31	CB15	WC4	WT	PBS	14
32	CB16	WC5	WT	PBS	14

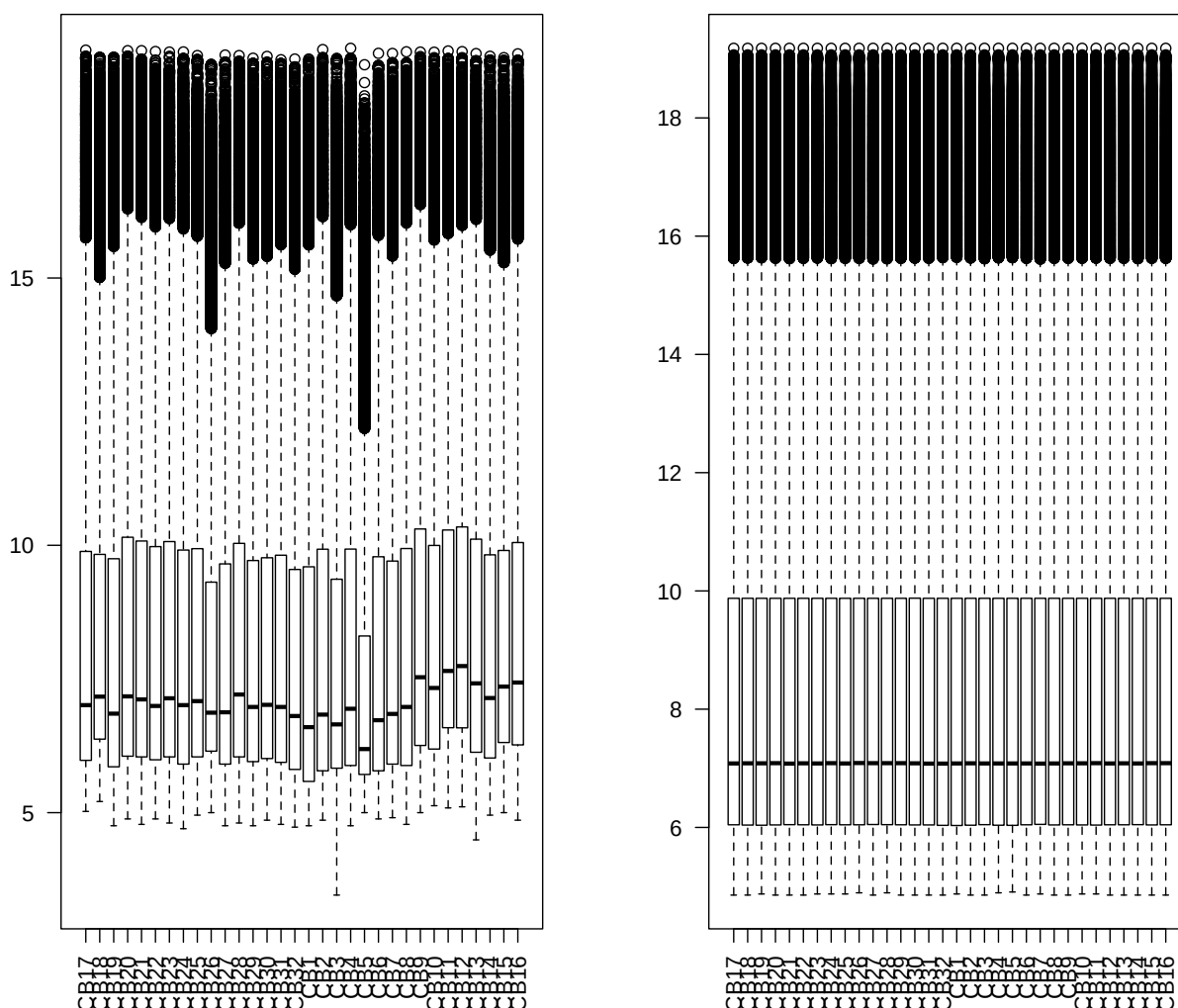


Figure 28: Boxplot of array log₂ intensities before and after quantile normalization.

The array #21-CB5 shows reduced intensities. This effect was reduced by the quantile normalization and in addition the arrays were weighted using the array weights function to reduce outlier effects.

RNA quality was checked on RNA 6000 Nano chips using a Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA, USA). Agilent's Low Input Quick Amp Labelling Kit, one-color was used to generate fluorescent cRNA. The amplified cyanine 3-labeled cRNA samples were then

purified using Promega's SV Total RNA Isolation System and hybridized to Agilent Whole Mouse Genome Microarrays, 8x60K. Microarray slides were washed and scanned with an Agilent Scanner, according to the standard protocol of the manufacturer. Information from probe features was extracted from microarray scan images using the Agilent Feature Extraction software v10.7.3.

Further analyses were performed using Bioconductor(Gentleman et al., 2004), an open source software for the analysis of genomic data rooted in the statistical computing environment R. The raw intensities were imported into Bioconductor using spot weighting and further processed with the limma(Smyth, 2004) package. Quality Controls were performed using the arrayQualityMetrics package(Kauffmann et al., 2009). To reduce the effects of outliers arrays, arrays were weighted using the arrayWeights function of limma. Normalization between arrays was performed using the quantile method, duplicate probes were averaged and a linear model was fitted. P-values were adjusted for multiple testing using the Benjamini & Hochberg method. Initial cut-offs for differential expression were set to a minimum 2-fold up- or down regulation and a maximum adjusted p-value of 0,05.

6.5.2 Clustering

Before clustering significantly changed genes (at least at one time point and 2-fold) were filtered by using following macro in Microsoft Excel with VBA:

```
Function significantlyChanged(Coef As Double, p As Double, CoefCutOff As Double, pCutOff As Double) As Boolean
    If Math.Abs(Coef) >= CoefCutOff Then
        If p < pCutOff Then
            significantlyChanged = True
        Else
            significantlyChanged = False
        End If
    Else
        significantlyChanged = False
    End If
End Function
```

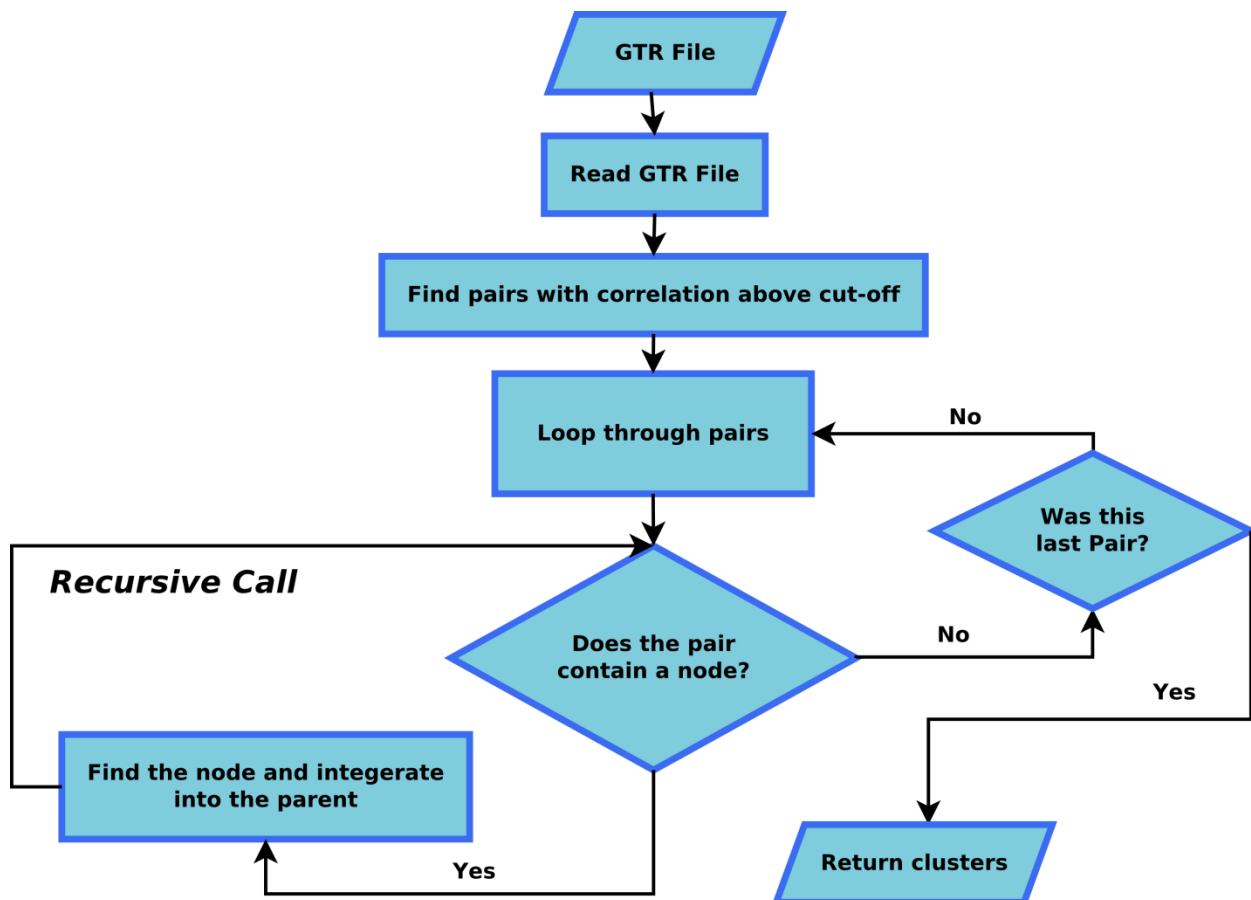
Code Snippet 1 Macro code to select significantly changed genes in visual basic (VBA).

Significantly changed genes were exported as text file and hierarchally clustered in *Cluster 3.0* with Pearson correlation (Uncentered) with average linkage(Hoon et al., 2004).

6.5.3 NodeFinder – Silhouette Splitting and Cluster Mapping

In order to apply silhouette splitting(Rousseeuw, 1987), a novel software tool called “NodeFinder” was programmed in Java programming. NodeFinder is a multi-threaded object oriented software and works as follow:

- Output files (CDT and GTR files) from Cluster 3.0 are read by NodeFinder.
- NodeFinder finds cluster/group of genes with correlation (e.g. Pearson)/similarity (e.g. Euclidean) at the user-set threshold through a recursive algorithm:



Flow chart 1 Schematic overview of the NodeFinder silhouette algorithm

Gene tree (GTR) file (output of Cluster 3.0 software) is loaded as objects with the identifiers provided by the Cluster 3.0 and the calculated correlations for the pairs. Pairs are separated into two object lists (above and below threshold). A recursive function checks whether there are still nodes occurring in a cluster. Nodes are reduced to their children until no more nodes are present in the cluster.

- NodeFinder also gathers information (GO terms, synonyms and identifiers) asynchronously from Ensembl databases via REST API.

- Information gathered from Ensembl databases is also used to mark transcription factors and genes of interest (based on occurrence of user-set keywords in GO terms).
 - Following terms were used to mark genes of interest:

defence	immune	neutrophil
interleukin	T cell	dendritic cell
interferon	B cell	autophag ²
natural killer cell	macrophage	
defense	inflammatory	

- Several datasets can be compared in NodeFinder.
- HTML based and graphic based charts and reports can be generated.
- Genes can be searched in one or multiple datasets.
- Clusters of several datasets can be tested for similarity by mapping them to each other in all combinations.

² NodeFinder does not look for whole words in GO terms but occurrence of any character sequence. “autophag” is therefore selected for words such as “autophagic” or “autophagy”.

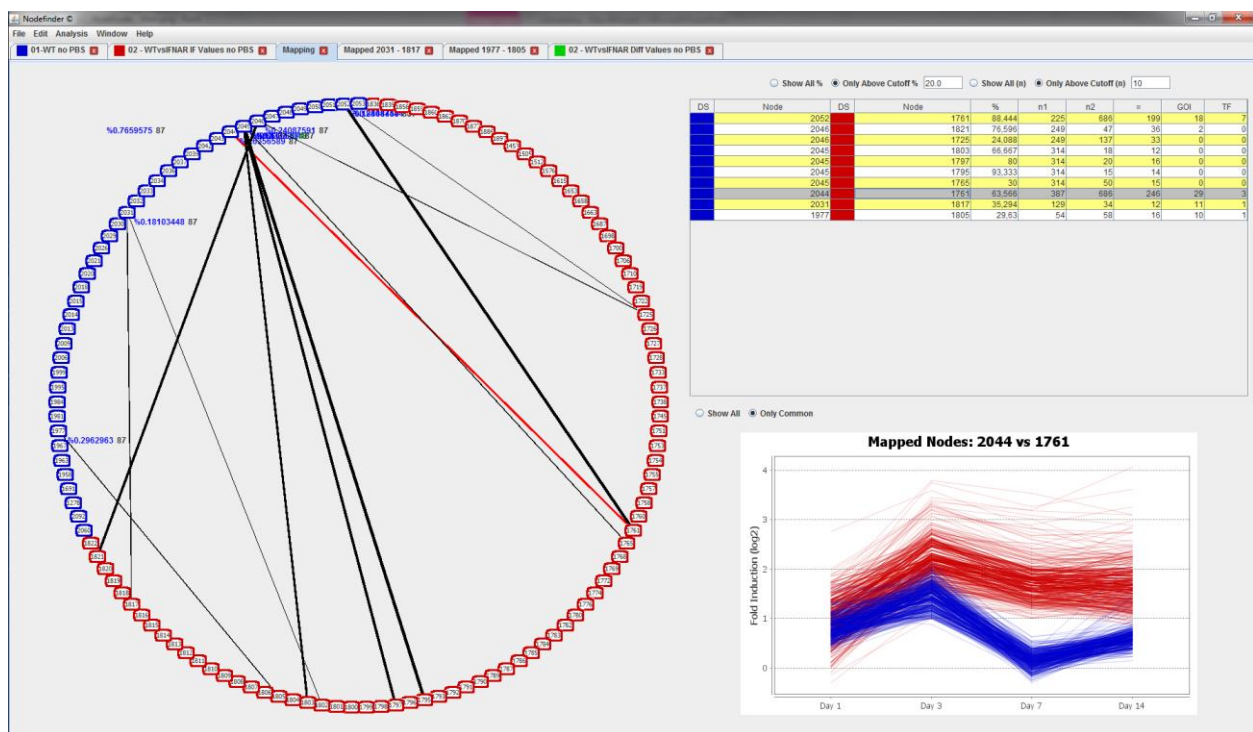
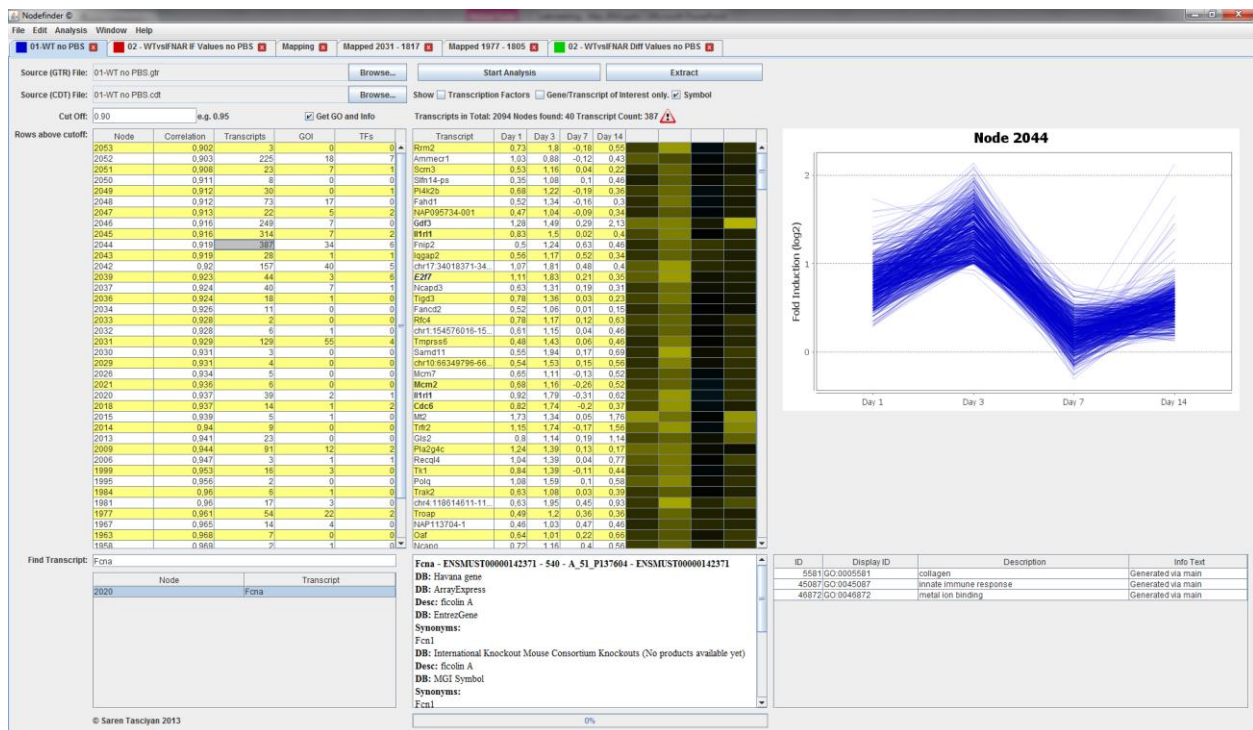


Figure 29: Graphical user-interface of NodeFinder.
Main analysis tab of NodeFinder (upper image). Mapping tab of NodeFinder (lower image).

7 Abbreviations

APC	Antigen presenting cell
FACS	Fluorescence-activated cell sorting
FCS	Fetal Calf Serum
RNA	Ribonucleic acid
DNA	Deoxyribonucleic acid
UV	Ultraviolet
Vis	Visible light
PCR	Polymerase chain reaction
AMV	Avian Myeloblastosis Virus
dNTP	Deoxyribonucleoside triphosphate
Ref	Reference or reference gene
GOI	Gene of interest
Ct	Cycler time
BM-DC	Bone marrow derived dendritic cells
TLR	Toll-like receptor
RIG-I	Retinoic acid-inducible gene I
MDA5	Melanoma differentiation-associated protein 5
API	Application programming interface
REST	Representational state transfer
PC	Principal component
PCA	Principal component analysis
WT	Wild-type
GO	Gene Ontology
DEGs	Differentially expressed genes
ISGF3	interferon-stimulated gene factor 3
GAF	gamma-activated factor
GAS	gamma-activated sequences
ISRE	IFN-stimulated response elements
ROS	Reactive oxygen species

FDR	False Discovery Rate
MSS	Mean Silhouette Split

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Name and type of organisation providing education and training	Österreichisches St. Georgs-Kolleg Istanbul (High School) 2003-2008
Name and type of organisation providing education and training	Global Young Leaders Conference (GYLC) (Conference) 1919 Gallows Road, Suite 700, 22182 Vienna, VA (United States of America) 30 July 2005-11 August 2005
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Teaching/Tutor	University Vienna, Lab courses for undergraduates (Molecular biology and biochemistry)

MEETINGS ATTENDED/ORGANISED

Name and type of organisation	ITU International Molecular Biology and Genetics Student Congress <i>17 August 2009-20 August 2009</i>
Name and type of organisation	4th FEBS Special Meeting on ABC Proteins - ABC2012 <i>March 2 - 9, 2012, Innsbruck, Austria (Organizing Team)</i>
Name and type of organisation	Viennese Symposium on Immunology, Infection biology and Inflammation (VI3) <i>December, 12 2012, Vienna, Austria (Organizing Team)</i>
Name and type of organisation	5th FEBS Special Meeting on ABC Proteins - ABC2014 <i>March 8 - 14, 2014, Innsbruck, Austria (Organizing Team)</i>
Name and type of organisation	Advanced Lecture Course on Systems Biology <i>March 2 - 8, 2014, Innsbruck, Austria (Organizing Team)</i>
Name and type of organisation	10 th YSA PhD Symposium 2014 <i>June 11 – 12, 2014 (Poster Presentation)</i>
Name and type of organisation	6 th ÖGMBT Annual Meeting <i>September 15 – 18, Vienna Austria (Short Talk & Poster Presentation)</i>

SCHOLARSHIPS

Mondi Austrian Student Scholarship
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PROFESSIONAL ACTIVITIES

Name and Type of the activity	Small Business – PhageApps <i>Since 2011 (to finance Master Studies)</i> Information Technologies – Web & Mobile Solutions, Scientific Software, Web Administration and Security
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Foreign Languages

	Understanding		Speaking		Writing
	Listening	Reading	Spoken interaction	Spoken production	
German	C2	C2	C2	C2	C2
English	C2	C2	C2	C2	C2

Technical skills and competences	Bioinformatics, SDS-PAGE, Western Blotting, FACS, Immunoprecipitation, Fluorescence Microscopy, Hybridoma – Antibody Screening, Molecular Biology, Animal Studies, qPCR analysis, Confocal Microscopy etc.
Computer skills and competences	Office Software (Databases, Spreadsheets, Writing etc.), image processing/analysis, Programming (R statistics language, Perl, Python, Visual Basic, VB.Net, C/C++, Java, PHP, HTML and CSS), Linux (server

|administration), 3D modelling and printing.