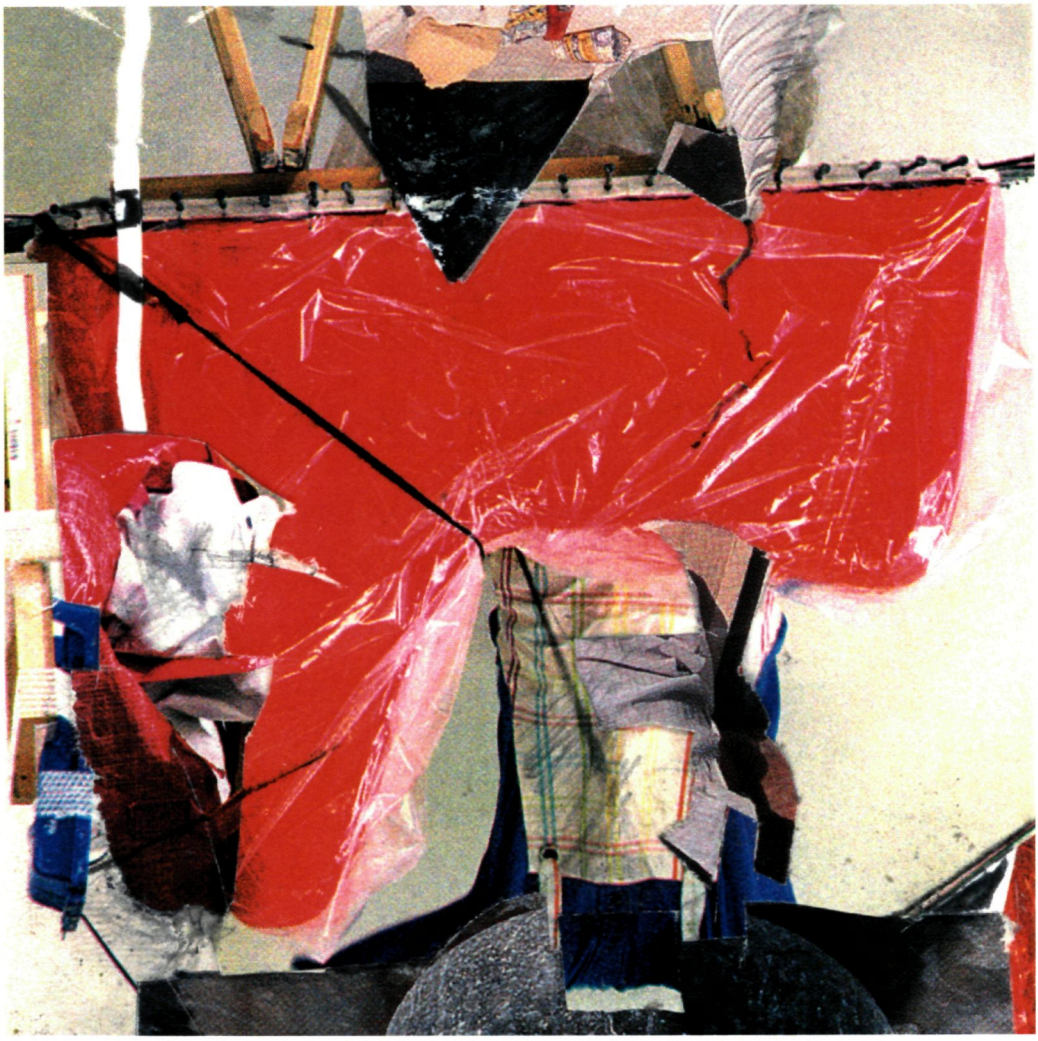
The interplay between innate immunity and Th17 responses in *Candida* infection

Frank Leo van de Veerdonk



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Colofon

The research presented in this thesis was performed at the Department of Medicine and Nijmegen Institute for Infection, Inflammation and immunity (N4i), Radboud University Nijmegen Medical Center, The Netherlands

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The interplay between innate immunity and Th17 responses in *Candida* infection

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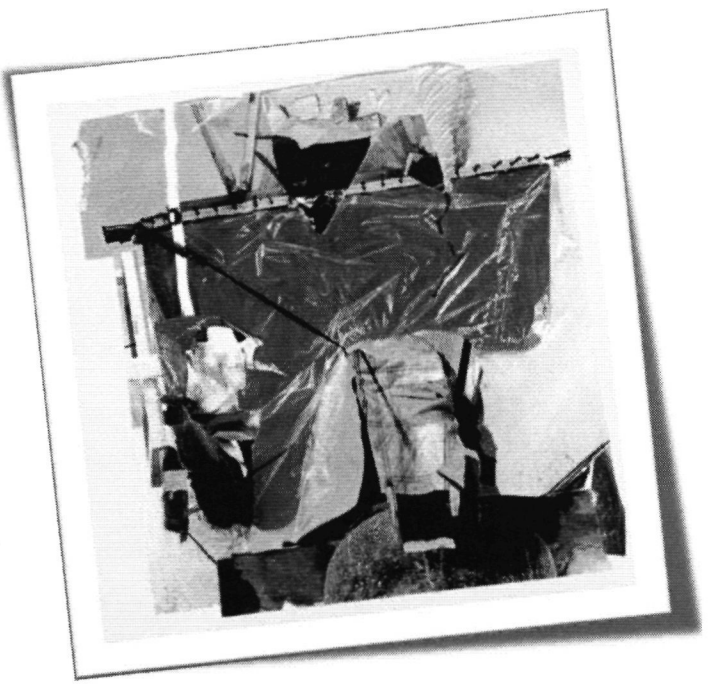
Chapter 1

Introduction and outline of the thesis



General Introduction

Microorganisms constantly challenge us When mechanical barriers such as the skin and mucosa fail in their role of keeping microorganisms outside the body, pathogenic microorganisms have a chance to invade the tissue and multiply When this occurs, host defense mechanisms that recognize the microorganisms are activated and these subsequently induce an optimal immune response to eliminate or control the pathogens Tailored immune responses are to be initiated, since infections with various microbes require different host defense strategies for defeating the invaders At the tissue level, resident macrophages are among the first types of cells that have to accomplish this task When they come in contact with pathogens and recognize them as dangerous, several actions are triggered they ingest and kill the microorganisms, they release activating cytokines, but probably most important, they will attract more cells of the innate immune system such as neutrophils and monocytes (which become exsudate macrophages) to the site of infection Together these cells orchestrate an effective response that will ultimately be able to clear the invading microorganisms If the innate immune response is not capable to control the microbiological threat, the adaptive immune response will come into play Adaptive immunity consists of humoral (B lymphocytes) and cellular (T lymphocytes) responses These responses are activated during a complex process of antigen presentation by immune cells (especially dendritic cells) When this stage is entered, there will be an intensive interplay between lymphocytes and other immune cells This interplay is heavily dependent on cytokines that shape and direct the immune response (Figure 1)

Candida albicans is a commensal fungal microorganism that is often part of the flora residing in the human oral cavity and genital tract Under normal conditions *Candida albicans* will not cause disease However, when mucosal or skin barriers are damaged and/or the immune system is compromised, *Candida albicans* is able to cause disseminated infections that are associated with a high mortality, despite the availability of novel and effective antifungal drugs (1)

In addition to these disseminated infections, a large number of patients suffer from mucosal *Candida* infections such as oropharyngeal or vaginal candidiasis In order to develop new strategies in the fight against *Candida* infection, a better understanding of the anti-*Candida* host defense is needed The aim of the present thesis is to elucidate the innate and adaptive defense mechanisms against *Candida* in order to provide insights that might contribute to the development of adjunctive immunomodulatory treatment options in patients with candidiasis

Outline of the thesis

The first section of the thesis focuses on "Innate pattern recognition of *Candida albicans*" and comprises the Chapters 2-4 In **Chapter 2** we provide a general overview of host and fungal pathogen interactions and try to answer the question how innate immunity has achieved a certain degree of specificity and delivers a tailored response to various pathogens In 1989, the late Charles Janeway Jr argued that the early phases of host defense are regulated by receptors and ligands that in evolution preceded the development of clonally distributed receptors, encoded by rearrangement of genes (e g T- cell receptors and

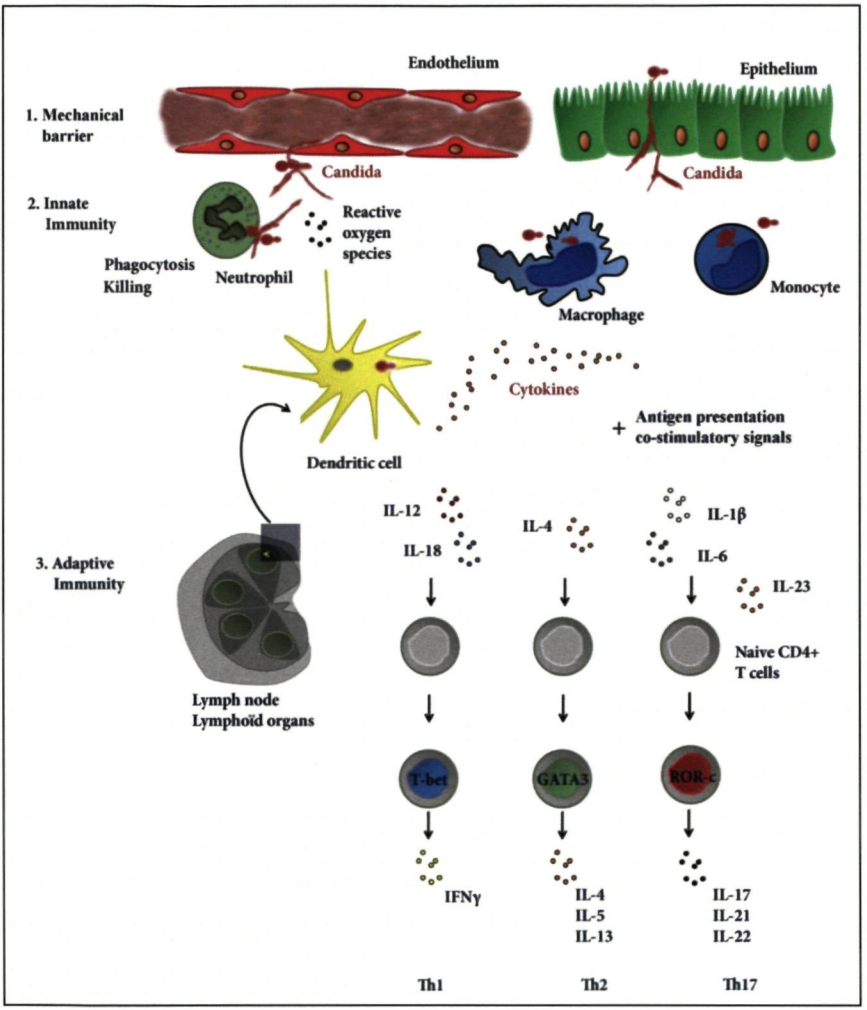


Figure 1. Pathogenesis of host defense against Candida.
The epithelia of the skin and mucous membranes form the first line of defense by posing primarily a mechanical barrier against the fungus. When *Candida* invades the tissues, professional phagocytic cells (neutrophils and mononuclear phagocytes) will phagocytose and kill the fungus. This innate immune response can be followed by an adaptive response, which is initiated by cells (dendritic cells, macrophages and B cells) that present antigen of the fungus. Memory T cells and naive T cells will be recruited and activated, and the cytokine profile that is present in the micro-environment will polarize the T helper lymphocyte response towards a predominant Th1, Th2 or Th17 response. IL=interleukin; IFN=interferon.

B-cell receptors), and that engagement of these receptors by microbial ligands would trigger innate non-clonal responses (2). The first molecules that were identified as such were the Toll-like receptors (TLRs), which now belong to the most studied classes of pattern recognition receptors (PRRs).They are named Toll-like, because of their homology to Toll, a receptor identified in the fruit fly *Drosophila melanogaster*. Lemaitre et al. discovered in 1996 that Toll had an essential role in the fly's immunity to fungal infection (3). There are 10 Toll-like receptors expressed in humans and each of them has its own specificity of

recognition of components of microorganisms. In the host defense against *Candida*, the role of TLR2,and TLR4 is well established, also based on research performed in our laboratory (4). In **Chapter 3** we investigated the importance of TLR9 in disseminated candidiasis, asking the question whether TLR9-/- mice would be more susceptible to invasive candidiasis, and if TLR9 plays a key role in cytokine responses against *C. albicans*. Similar questions were tackled in **Chapter 4,** in which we investigated the role of TLR1 and TLR6 in antifungal host defense in a murine model of invasive candidiasis.

The second part of the thesis "The role of the inflammasome in host defense against *Candida albicans*" includes the Chapters 5-8. Activation of many TLRs results in de-novo production and subsequent intracellular accumulation of the inactive pro-form of the potent pro-inflammatory cytokine IL-1β. A second signal through a nucleotide-binding domain and leucine-rich repeat-containing protein (NLR) receptor activates a cascade of proteins (a 'protein platform') called the inflammasome, which results in caspase-1 activation and subsequently processing of the TLR-induced pro-interleukin (IL)-1β into mature, bioactive IL-1β. Dual triggering of TLR and NLR is believed to be necessary for the tight regulation of proinflammatory cytokines such as IL-1β (5). In **Chapter 5** we give an introduction on the inflammasome and describe the differences in the regulation of IL-1β processing between monocytes and macrophages. Since IL-1β plays an important role in anti-*Candida* host defense (6), we wanted to dissect the pathways that are important for IL-1β production in response to *Candida albicans,* and we hypothesized that the inflammasome would play an important role in antifungal host defense. We addressed these issues in human monocytes in **Chapter 6** and in a murine model of disseminated candidiasis in **Chapter 7** and **Chapter 8**.

The third part of the thesis "T helper17 lymphocytes and antifungal host defense" consists of the Chapters 8-13. T helper (Th) 17 lymphocytes have recently been described as a third subset of T helper lymphocytes, and these cells are important for both antimicrobial host defense and for the development of autoimmune diseases (7). In general, Th17 responses are critical for mucosal production of defensins in response to *Candida* and for epithelial host defense against extracellular bacteria and fungi. This is discussed in detail in **Chapter 9**. In **Chapter 10** we have addressed the question which components of *Candida albicans* and which receptors are important for the induction of a Th17 response. Since it had been described that prostaglandins have synergistic effects together with IL-23 to induce Th17 responses (8), we investigated in **Chapter 11** whether prostaglandin E2 also played an important role in the induction of the Th17 response induced by a fungal pathogen such as *C. albicans*. Both protective and deleterious effects have been attributed to the Th17 response during fungal infection (9, 10). We have explored whether this is true for the Th17 response in a murine model of disseminated candidiasis and in a murine model of inflammation-driven multi-organ failure in **Chapter 12**. In **Chapter 13**, we investigated the question to what extent the host defense against *Aspergillus fumigatus,* another important fungal pathogen, relies on the Th17 response.

The final part of the thesis "The inflammasome/Th17 axis in clinical syndromes" is outlined in the Chapters 14-19. Patients with chronic granulomatous disease have a defect in NADPH-oxidase of phagocytic cells and therefore are unable to generate reactive oxygen species (ROS) in these cells (11). It had been claimed that NADPH-dependent ROS are critical for inflammasome activation (12). In **Chapter 14,** we challenged this claim and investigated the

activation of the inflammasome in patients with chronic granulomatous disease that are deficient in NADPH-dependent ROS
The Th17 response is associated with autoimmune diseases, such as multiple sclerosis (MS) and rheumatoid arthritis (RA) (7) Studies demonstrated a beneficial effect of rituximab, an anti-CD20 antibody that depletes B cells Such an effect was completely unanticipated in these two Th17 mediated diseases in which B lymphocytes are not considered to play a role (13, 14) We hypothesized however that B cells do have a key role in Th17 responses and investigated this hypothesis in **Chapter 15.**

In **Chapter 16,** we investigated the role of the Th17 response in patients with the immunodefciency disorder hyper-IgE syndrome, since these patients are especially susceptible to mucocutaneous *Candida* infection and *S aureus* skin abscesses pneumonia (15), and these two pathogens are potent inducers of the Th17 response in-vitro In **Chapter 17** we investigated five families that suffered from severe forms of autosomal chronic mucocutaneous candidiasis (CMC), a clinical syndrome in which the genetic background has been enigmatic We used a systematic functional approach to pinpoint the defects in the immunological pathways in CMC by integrating the knowledge that was obtained from the experiments performed during this thesis and from earlier work from our group

As outlined in the introduction, new approaches are needed to improve the outcome of patients suffering from *Candida* infections In **Chapter 18,** I present an overview of novel strategies for the prevention and treatment of infections caused by *Candida*, with emphasis on the potential of novel immunotherapeutic approaches

A summary of the findings and conclusions of this thesis is presented in **Chapter 19.**

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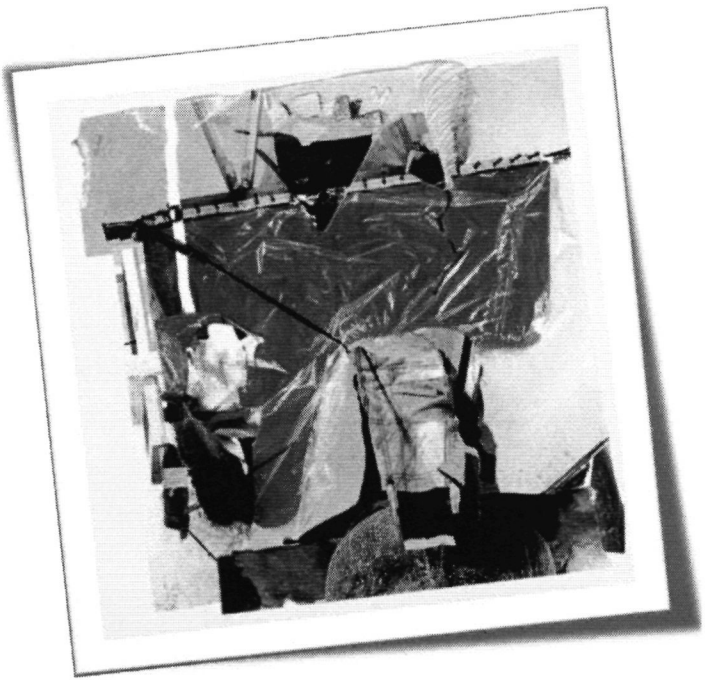
Pattern recognition of *Candida albicans*



Chapter 2

Host-microbe interactions:

innate pattern recognition of fungal pathogens

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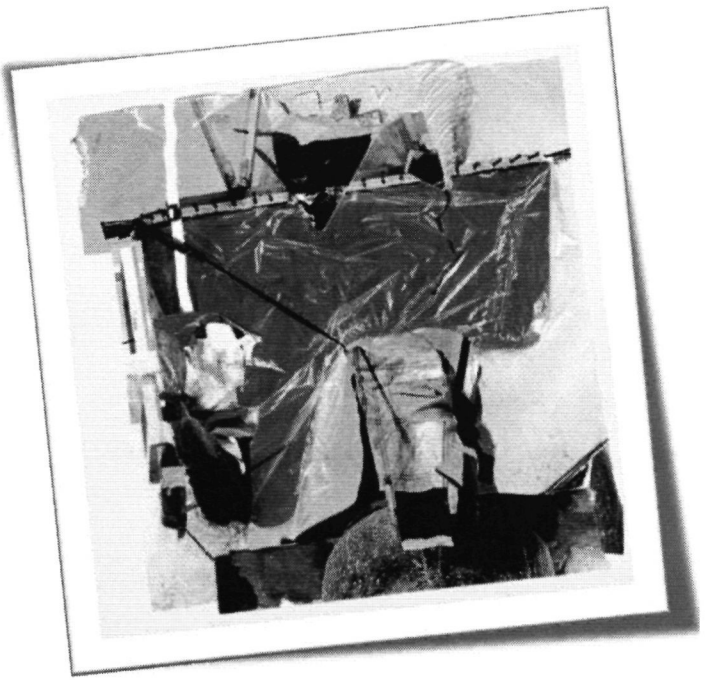

van de Veerdonk FL, Kullberg BJ, van der Meer JW, Gow NA, Netea MG.

Summary

The recognition of fungi is mediated by germline pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) and C-type lectin receptors (CLRs) that interact with conserved structures of the microorganisms, the pathogen-associated molecular patterns (PAMPs). Subsequently, PRRs activate intracellular signals that collaborate for the efficient activation of the host defense. The specificity of these responses is achieved through the activation of a particular mosaic of PRRs, that is determined by the available fungal PAMPs and the innate immune cells involved. This will determine a divergence of the final type of reaction, and in this way the innate host defense has the capability to deliver tailored responses to each pathogen.

Introduction

The essence of the innate defense mechanism is the ability to recognize and eliminate microbial pathogens. This is mediated by a limited arsenal of pattern recognition receptors (PRRs) that are able to recognize conserved structures of microorganisms called pathogen-associated molecular patterns (PAMPs). Several classes of PRRs have been described, among which Toll-like receptors (TLRs), C-type lectin receptors (CLRs), NOD-like receptors, and RigI-helicases. It is especially the first two classes of these receptors that have been suggested to play an important role in antifungal immunity.

Fungal PAMPs

Most components of the fungal cell wall are not found in mammals, and therefore, represent an ideal target for recognition as nonself. The fungal cell wall can be described as a dynamic, highly organized organelle that determines both the shape of the fungus and its viability. In general, the core structure of the fungal cell wall, as exemplified by the structure of *Candida albicans* cell wall, is composed of a skeleton of polysaccharide fibrils com- posed of β-(1,3)-glucan that is covalently linked to β-(1,6)-glucan and chitin (a β-(1,4)-linked polymer of N- acetyl glucosamine), and is designed to function as a scaffold for the external protein layer. This outer layer consists of proteins that are mainly glycosylated through *N*-linked (1) or *O*-linked mannosylation (2) (also called mannans). Although this basic model of the fungal cell wall is shared by many fungi, at the molecular level these structures differ between fungal species. In *Aspergillus* species an important component of the skeleton of the cell wall is galactomannan, while the outermost cell wall layer is composed of rodlet fascicles of hydrophobic proteins (hydrophobins) that contribute to the shielding properties of the cell wall (3). In *Cryptococcus*, a thick capsule of mannoproteins, galactoxylomannan and glucoronoxylomannan plays a crucial role for inhibiting recognition and activation of host defense mechanisms (4). This diversity will result in different qualities of PRR–ligand interactions and the activation of different sets of PRRs, leading to specific host responses.

The role of PRRs in antifungal host defense

When the host encounters live pathogenic fungi, the initial response by the innate immune system will be determined by the recognition of fungal cell wall components. Neutrophils, monocytes, and macrophages represent the first line of defense against fungal pathogens.

Later on, recognition of fungal structures by dendritic cells leads to the activation of specific immunity, especially T-cell-mediated. These various cell populations differ in their expression of TLRs and CLRs on the cell membrane, and are therefore capable of initiating different responses.

TLRs

The first suggestion for a role for TLRs in antifungal host defense was made by Lemaitre et al., who observed that Drosophila flies deficient in the Toll receptor rapidly succumbed to *Aspergillus fumigatus* infection, because of defective synthesis of the drosomycin defensin (5). Ligand recognition by the functionally equivalent TLRs induces the activation of kinase cascades in mammalian cells, and the nuclear translocation of transcription factors such as NF-κB, NF-AT, and IRF3, that induce gene expression and production of various chemokines and cytokines (6). Moreover, recently a human homolog of drosomycin called drosomycin-like defensin has been described, that has activity against a variety of filamentous fungi, and is expressed mainly in the skin (7). Shortly after the discovery of TLRs, TLR2 and TLR6 were shown to be involved in the recognition of the fungal structure zymosan derived from *Saccharomyces cerevisiae* (8). Moreover, the adaptor molecule MyD88, that is shared by most TLRs, has proven to be crucial for antifungal defense by several in vivo studies (9–11), strongly suggesting that TLRs play a crucial role in host defense against fungi.

TLR4

TLR4 is one of the best studied PRRs, because of its role as the main receptor of bacterial lipopolysaccharides. In a murine experimental infection model of disseminated *C. albicans* infection, it has been shown that the absence of TLR4-mediated signaling resulted in increased susceptibility to the infection, decreased chemokine production, and impaired neutrophil recruitment (12). The effects on survival were challenged by a later study (13). Wang et al. suggested for the first time a role for TLR4 in the recognition of A. fumigatus (14), and subsequent studies have shown that TLR4 is involved in signaling and cytokine production in response to *Aspergillus* (9,15). These data were supported by a study showing that TLR4-/- mice died significantly sooner when they were infected with *A. fumigatus* conidia (9). TLR4 is also involved in the susceptibility to *Pneumocystis* pneumonia, with mice deficient in TLR4 having a defective cytokine production by alveolar macrophages leading to increased susceptibility to *Pneumocystis* infection (16). By contrast, the *C. neoformans* component glucuronoxylomannan binds to TLR4 and leads to translocation of NF-kB, but not to induction of cytokine production (17), and these findings were supported by the fact that TLR4 does not play a major role in cryptococcal host defense (18).
Only limited knowledge exists regarding the nature of the fungal PAMPs that are recognized by TLR4. In addition to the work described above reporting the recognition of glucuronoxylomannan by TLR4, another study reported that TLR4 recognizes mannans from *S. cerevisiae* and *C. albicans* (19). A subsequent study found that short linear *O*-bound mannans of *C. albicans* are recognized by TLR4 and induce proinflammatory cytokines such as TNF (20). Overall, TLR4 appears to participate in antifungal host defense by recognizing mannan structures and mediating proinflammatory responses.

TLR2

One of the first studies that investigated TLR2 in fungal host defense reported that blocking of TLR2 by specific antibodies resulted in decreased production of TNF and IL-1β after stimulation of monocytes by *C. albicans* (12).
An additional study showed that TLR2-/- mice have a decreased production of TNF and MIP-2, and reduced neutrophil recruitment after a challenge with *Candida* (21). However, two other studies found that TLR2-/-mice showed an increased resistance to disseminated candidiasis that was accompanied by decreased production of IL-10, and increased IL-12 and INFγ production (9,22). In line with this, TLR2-deficient macrophages have shown to have an increased ability to contain *C. albicans* (23). This immunomodulatory effects induced by TLR2 were found to be mediated through the generation of T-regulatory cells with immunosuppressive potential (22,24). Evidence for an anti-inflammatory role for TLR2 in antifungal host defense is further supported by a recent study reporting that zymosan can tolerize DCs through a TLR2-mediated and dectin-1-mediated pathway involving MAPK/ERK (25). A limited role for TLR1 and especially TLR6, two receptors known for forming heterodimers with TLR2, has been recently reported in case of *C. albicans* recognition (26).
In addition to recognition of *C. albicans*, TLR2 has been suggested to be involved in the recognition of *A. fumigatus* (15). However, TLR2-/-mice do not have an increased susceptibility to aspergillosis (9), and Wang et al. reported no role for TLR2 in the recognition of *Aspergillus* hyphae (14). The role of TLR2 in the recognition of *C. neoformans* is still not clear, because of differences in the results by the various investigators: one study reported that TLR2 can bind cryptococcal glucuronoxylomannan, but this does not lead to translocation of NF-κB (17); an additional study found that TLR2 can mediate cytokine production in response to *C. neoformans* (10); by contrast, Yauch et al. found that TLR2 was not involved in cryptococcal-induced cytokine production (11). In conclusion, TLR2 seems to be involved in the recognition of fungal pathogens, though disagreement persists regarding the precise components recognized, and the amplitude of the effects. Overall, TLR2 ligands seem to induce weaker proinflammatory effects than TLR4 ligands, and TLR2 has also been shown to have immunosuppressive effects, particularly in *C. albicans* host defense, through promoting environments that favor Th2-type or T-reg-type responses (22,24).

TLR9

Unmethylated CpG sequences are the natural ligands for TLR9, and several reports have now suggested that TLR9 can recognize fungal DNA. Blocking of TLR9 in human monocytes and TLR9-deficient mouse macrophages stimulated with *C. albicans* leads to a reduced production of cytokines, mainly IL-10 (27). Another study showed that TLR9 detects *A. fumigatus* DNA, resulting in the secretion of proinflammatory cytokines (28). The same observation has been made for *C. neoformans* DNA, which was able to trigger IL-12p40 and expression of CD40 upon stimulation in murine DCs (29). This study also found that cryptococcal DNA activated NF-kB in TLR9-transfected HEK239 cells. However, Bellocchio et al. have reported that TLR9-/- mice produced less IL-12 and more IL-4 and IL-10, but this had little effects on the overall mortality of the animals (9). In conclusion, most of the data available at this time suggest a role for TLR9 for the recognition of fungal DNA, but the magnitude of this effect for the overall antifungal defense is likely to be overshadowed by redundant signals induced by other PRRs.

CLRs

CLRs comprise a large family of receptors, including dectin-1, the macrophage mannose receptor (MR), the dendritic cell-specific ICAM3-grabbing nonintegrin (DC-SIGN), dectin-2, and the circulating mannose binding lectin (MBL). These receptors share one or more carbohydrate recognition domains that were originally found in the mannose binding lectin and are evolutionary conserved. Importantly, over the recent years these receptors have been shown to be involved in fungal recognition and the modulation of the innate immune response.

Dectin-1

Dectin-1 recognizes β-(1,3)-glucans through which mediates ligand uptake and phagocytosis, and is able to trigger production of both proinflammatory and anti-inflammatory cytokines (30). Dectin-1 signals through the kinase Syk and the adaptor CARD9, and this pathway has been shown to induce IL-2 and IL-10 in DCs (31). It has also been demonstrated that infection with *C. albicans* induces CARD9-dependent Th17 cells (31), and cytokine production induced by *C. albicans* by both human peripheral blood mononuclear cells and murine macrophages is dependent on dectin-1 (32). Although dectin-1 signaling alone is sufficient to induce responses upon fungal recognition, several studies have emphasized that it is also able to cooperate with TLRs leading to synergistic proinflammatory responses. Two independent studies have shown that dectin-1 in collaboration with TLR2 triggers proinflammatory responses upon stimulation with *C. albicans* and zymosan (33,34), and recently dectin-1 has been found to amplify TLR4-dependent pathways in a Syk-dependent manner (35). Furthermore, dectin-1 and TLR2 collaborate for the phagocytosis of *Aspergillus* conidia (36), and *A. fumigatus* can activate the transcription factor AP-1 through a dectin-1/Syk-dependent pathway (37). The first in vivo evidence that dectin-1 plays an important role in innate fungal host defense was reported by a study showing that blocking dectin-1 leads to increased *A. fumigatus* fungal burden in the lung (38). Dectin-1-/-mice are more susceptible to infection with *C. albicans*, resulting in lower survival and increased fungal burdens (39). However, another study using a different strain of dectin-1-knockout mice could not confirm this for *C. albicans* infection, but found an increased susceptibility to *Pneumocystis* infection (40). No role for dectin-1 for the host defense to *C. neoformans* has been observed (41). The adaptor molecule CARD9, involved in the dectin-1-signaling pathway, has also been shown to have a crucial role in the survival during disseminated candidiasis (42). These data suggest an important role for dectin-1 in antifungal immunity, either directly or through collaborative signaling with TLR2 and/or TLR4.

Macrophage mannose receptor (MR)

MR has various domains that can recognize oligosaccharides terminating in GlcNAc (chitin is a polymer of GlcNAc), fucose, and mannose. The MR has been implicated in the recognition of several fungi, including *C. neoformans, C. albicans*, and *Pneumocystis*. Recently, the role of the MR in *C. albicans* has been investigated using mutant *Candida* strains defective in *O*-linked and *N*-linked mannans (20). This study showed that the MR recognizes branched *N*-bound mannans from *C. albicans* and this extended the previous observation that the MR preferentially recognizes branched α-linked oligomannosides (43). In response to

Pneumocystis and *C. neoformans*, the MR activates NF-κB and leads to proinflammatory cytokine production (44,45). However, in the case of *Pneumocystis* the mannose receptor is also capable of inhibiting TNF production, illustrating that the MR can act as a double-edged sword (44).
In vivo data in mice defective for the MR are limited. Although one study suggested only a minor role for MR for the host defense against *Candida* infections (46), this study employed an intraperitoneal model of infection with relatively little relevance to the clinical situation. Another in vivo study on *Pneumocystis* infection in MR-/-mice also showed no difference in survival and only small defects in fungal resistance (47).

Other CLRs

DC-SIGN is primarily expressed on mature DCs and recognizes high-mannose structures in a calcium-dependent way. Recognition has been reported for the pathogenic fungi *C. albicans* (48) and *A. fumigatus* (49) and it mediates uptake and phagocytosis of fungal particles (48). A recent study suggests an immunosuppressive effect through stimulation of IL-10 production. Dectin-2 is also a member of the CLR family and is mainly present on myeloid cells and maturing inflammatory monocytes, which recognizes high-mannose structures (50) and interacts with the FcγR to induce TNF in response to *C. albicans* hyphae (51). Dectin-2 can also recognize *Trichophyton rubrum* and *Microsporum audouinii* with preference to their hyphal components. Therefore, dectin-2 mainly seems to play a role in hyphal recognition, and is the first receptor described to produce proinflammatory cytokines in response to fungal hyphae (51). Galectin-3 is a receptor mainly expressed by macrophages, and it has been shown to be crucial for the recognition of the β-mannosides of *C. albicans*, in close collaboration with TLR2 (52). Mannose binding lectin (MBL) is a soluble CLR that is secreted by the liver, which can bind to *C. albicans* (53) and *A. fumigatus* (54). MBL can also bind to acapsular cryptococcal strains at the level of the budding scar (55). MBL is mainly involved in fungal host defense because of its ability to opsonize fungal yeasts by activating the complement system (56). However, MBL-deficient mice do not show decreased survival to infection with *C. albicans* (57) or *A. fumigatus* (58), though a recent study has demonstrated that MBL administration in a murine model of invasive pulmonary aspergillosis can be protective (59). Recently, a new C-type lectin, Mincle, has been shown to participate in the recognition of *C. albicans* by macrophages. This receptor localizes to the phagocytic cup, but was not essential for phagocytosis. However, knockout mice that lacked this receptor were hypersusceptible to *Candida* infection, and macrophages in which the Mincle receptor was blocked generated significantly reduced levels of TNF when stimulated by *Candida* yeast cells. The nature of the PAMP that binds to this LR is not yet known (60).

Interactions between PRRs

Initial studies already appreciated that fungi are able to recruit different PRRs to activate specific arms of innate host defense (12). For example, recognition of *C. albicans* by monocytes and macrophages has been shown to be mediated by at least four recognition systems that sense fungal PAMPs of the *C. albicans* cell wall: recognition of *N*-linked mannans by MR, recognition of *O*-linked mannans by TLR4, recognition of β-glucans by dectin-1/ TLR2, and recognition of β-mannosides by galectin-3/ TLR2 complexes (20). If the fungal cell wall is able to trigger many different PRRs at the same, it is important to realize

that it is a complex interaction between the various pathways that ultimately leads to the host response.
Several interactions between PRRs are well documented. As mentioned earlier, dectin-1 is able to augment the TLR2-mediated MAPK and NF-kB pathways leading to proinflammatory responses (33,34), and to amplify TLR4 responses through a Syk-dependent pathway (35). Galectin-3, a PRR which recognizes β-(1,2)-mannosides, has recently been shown to associate with TLR2, and this leads to the ability to discriminate between the pathogenic *C. albicans* and the nonpathogenic *S. cerevisiae* (52). In addition, the TLR2 pathway itself is able to inhibit TLR4-mediated production of IL-12 through stabilization of c-Fos (61). Another study demonstrated that when TLRs activate NF-κB, *C. albicans* can induce DC-SIGN-dependent signals which subsequently lead to acetylation of the NF-κB subunit p65 (62). This results in prolonged and increased IL-10 production that shifts the proinflammatory response induced by TLRs to a more anti-inflammatory response (62). All these observations imply that crosstalk between PRRs is essential to the complexity and flexibility of the innate immune response against fungi (Figure 1).

Convergence and specificity shape the fungal innate immune response

Although we are still at the beginning of elucidating the combinatorial use of innate defense mechanisms that define the initial host response, a general concept of the innate antifungal defense can be proposed. In order to recognize and respond to the many different fungi the organism encounters, the host evolved germline PRRs that can identify conserved fungal cell wall components - the fungal PAMPs. In this way, specific recognition of fungal nonself is reduced to a handful of specific pathways that interact with each other: the various mannan structures are recognized by TLR4, MR, DC-SIGN, dectin-2, and galectin-3, while the β-glucans are detected by dectin-1, TLR2, and CR3 (63). These pathways converge into a limited set of shared adaptor molecules and transcription factors (Figure 2). One such example is that of TLRs and CLRs sharing NF-κB during stimulation of proinflammatory cytokines. However, despite converging into certain pathways, the innate immune response still maintains its specificity through the activation of a specific mosaic of PRRs that is determined by the available fungal PAMPs and the innate immune cells involved. In addition, specificity is also preserved by the interactions between the PRR pathways (Figure 1). This response will eventually lead to nuclear translocation of transcription factors that have the competence to activate specific genes. The specificity insured by these mechanisms will determine a divergence of the final type of response. In this way, the innate host response has the capability of transforming converging pathways into tailored responses (Figure 2).

Conclusions

In this review, we have presented a synthesis of the current knowledge on the recognition of fungal pathogens by the innate immune system of the mammalian host. The very active research of the past few years has greatly improved our understanding of how the fungal pathogens are recognized as nonself by the host defense. Our understanding how TLRs and CLRs contribute and collaborate for the recognition of fungi permitted us to propose an integrated model of innate pattern recognition of these important human pathogens. We have also discussed and speculated how the signals induced by these receptors are integrated to bring about efficient activation of the host innate response. This model that is

pertinent conceptually to many host-fungal interactions, may permit in the near future the design of new therapeutic strategies to improve the outcome of patients suffering from these life- threatening infections.

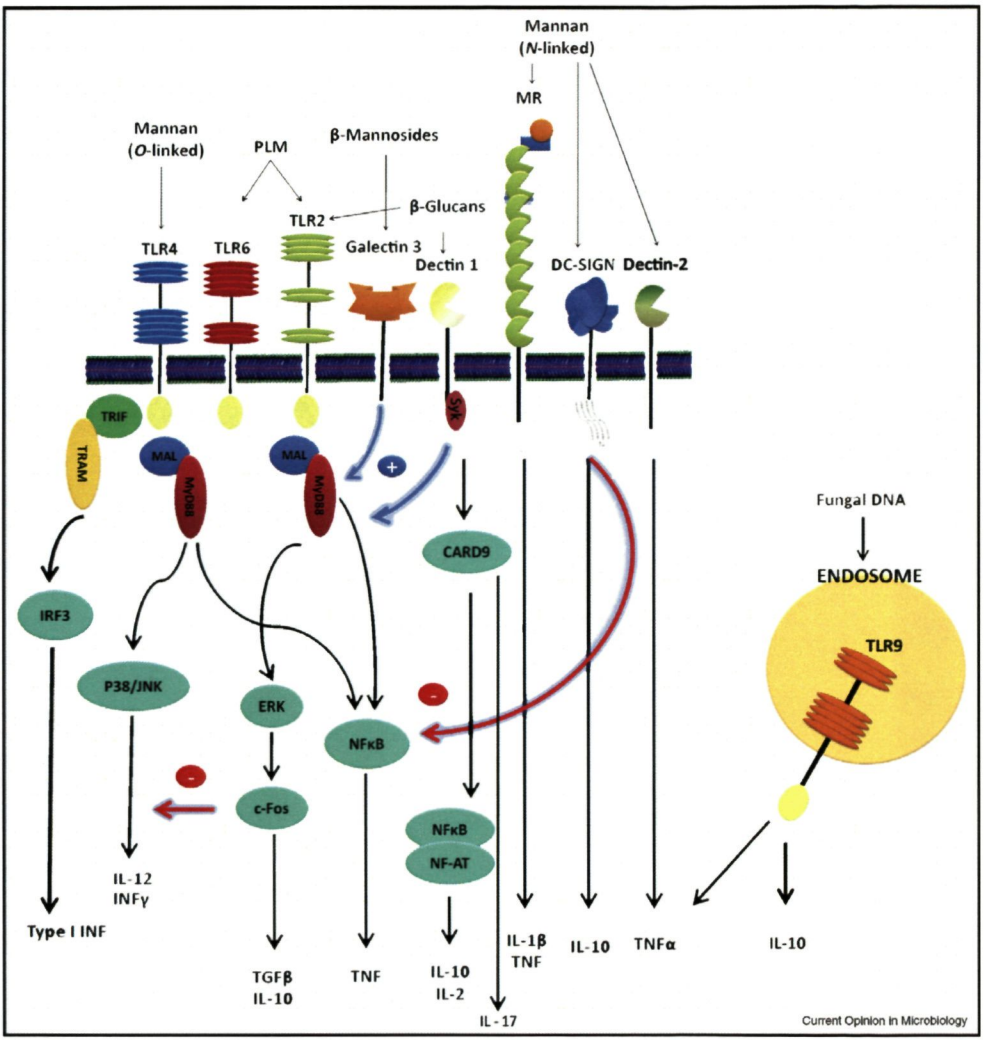

Figure 1. The major pattern recognition pathways of fungal pathogens.
Activation of host response by fungal pathogens at the level of the cell membrane is mediated by Toll-like receptors (TLRs) and C-type lectin receptors (CLRs). TLR4 mainly induces proinflammatory signals through the MyD88-Mal- mediated NF-kB and MAPK pathways, while stimulating type I interferons through IRF3. TLR2 has weaker proinflammatory effects, but induces strong stimulation of the anti-inflammatory cytokines IL-10 and TGFβ and can lead to immunological tolarization in DCs through an ERK/MAPK-dependent mechanism. On the contrary, proinflammatory responses induced by TLR2 can be amplified by dectin-1 and galectin-3. Dectin-1 can also induce cytokine production independently of TLR2, and can lead to Th17 responses through the Syk–CARD9 pathway. The MR induces proinflammatory cytokines such as IL-1β and TNF. DC-SIGN can modulate TLR responses and induces production of IL-10 in DCs. Dectin-2 mainly recognizes mannans from hyphae and leads to the production of TNF.

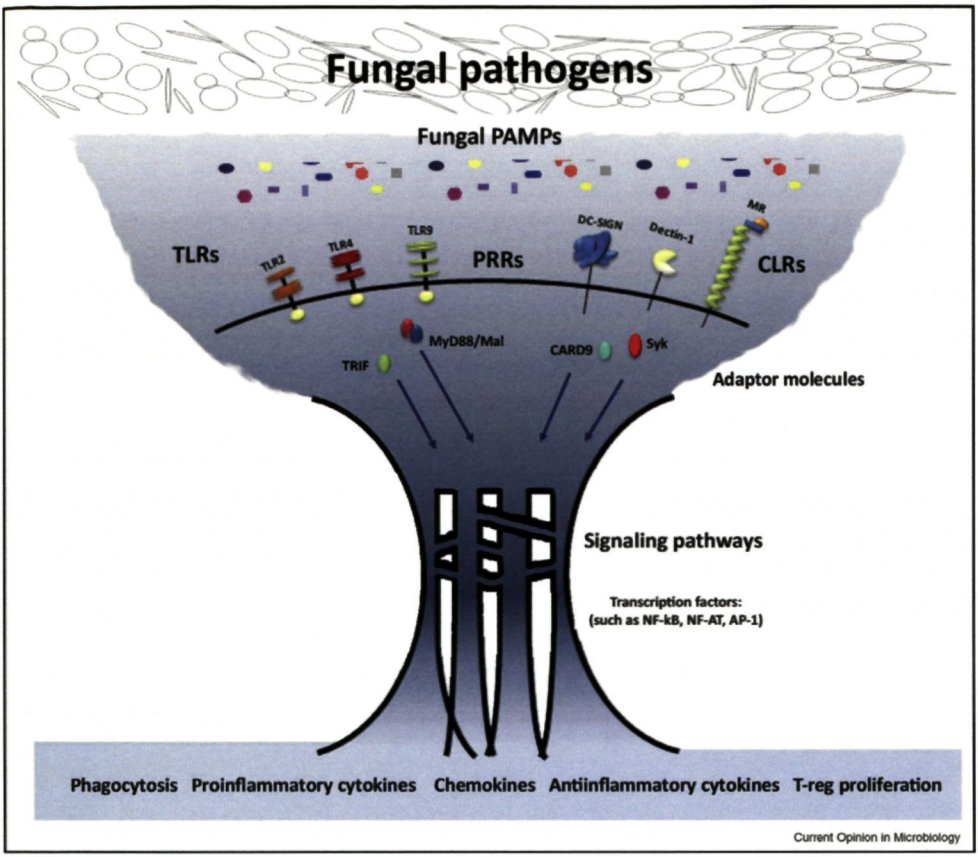

Figure 2. A general model of fungal pattern recognition.
The recognition of the many fungal species is mediated by the interaction between conserved fungal PAMPs and a limited number of PRRs from the TLR and CLR families. These signals further converge because of the use of common adaptor molecules, intracellular pathways, and transcription factors. However, the specificity of the host response is maintained by the different mosaic of receptors stimulated by certain fungi, as well as by the complex interactions between the various pathways. This will determine a divergence of the final type of response elicited by each pathogenic microorganism, and in this way the innate host response has the capability of transforming converging pathways into tailored responses.

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Redundant role of TLR9 for anti-*Candida* host defense

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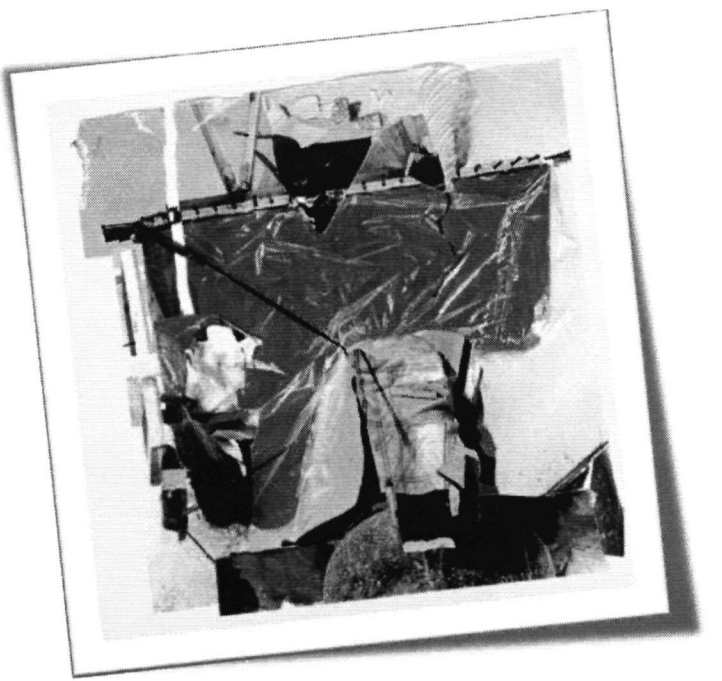

van de Veerdonk FL, Netea MG, Jansen TJ, Jacobs L, Verschueren I, van der Meer JW, Kullberg BJ.

Summary

The role of Toll-like receptor 9 (TLR9) in the recognition of *Candida albicans* and anti-*Candida* host defense was investigated in a murine model of disseminated candidiasis and in human peripheral blood mononuclear cells (PBMC). Blocking TLR9 by a specific inhibitor of human TLR9 or stimulation of cells isolated from TLR9-deficient (TLR9-/-) mice resulted in a 20–30% reduction in cytokine production induced by *C. albicans*. However, this defect was not accompanied by differences in mortality and organ fungal growth between TLR9-/- and TLR9+/+ mice. In conclusion, TLR9 is a pathogen-recognition receptor for *C. albicans*, and TLR9 is involved in the induction of cytokines in response to *C. albicans*. However, the cytokine defect in TLR9-/- mice is compensated by alternative pathways, and the TLR9-dependent pathway seems to be redundant in the disseminated candidiasis model in mice.

Introduction

Invasive infection caused by the fungus *Candida albicans* is a serious threat to hospitalized patients, with a mortality reaching 30–40% (1). New strategies to overcome these infections may include vaccination against *Candida* species. To that purpose, understanding the mechanisms through which the immune response recognizes and responds to this pathogen has become increasingly important. The innate immune response occurs within minutes or hours after infection or vaccination. Cells of the innate immune system sense components of microorganisms through so-called pattern recognition receptors (PRR) of which Toll-like receptors (TLR) are probably the most important (2,3). Earlier studies by our group and others have described a role for TLR4 (2,4,5) and TLR2 (6,7) in the recognition of cell wall components of *C. albicans*. In addition, TLR6 has recently been shown to be involved in the recognition of *C. albicans* (8).

TLR9 recognizes unmethylated CpG dinucleotides, which are common in most bacteria and DNA viruses, but not in vertebrate DNA (9). Its involvement in fungal infections has yet to be determined. The stimulation of TLR9 with CpG DNA leads to the activation of MyD88-dependent cellular signaling pathways, resulting in predominantly Th1-type innate and adaptive immune responses. The strong proinflammatory effects of TLR9 towards activation of cellular responses make CpG oligodeoxynucleotides of considerable interest for use in human medicine, especially as vaccine adjuvants (9-11). Considering the importance of TLR9 for recognition of microbial DNA, we investigated in the present study the role of TLR9 in the recognition of *C. albicans* and the activation of antifungal host defense.

Materials and methods

Animals

TLR9-deficient C57BL/6J (TLR9-/-) mice were kindly provided by Dr. S. Akira (Tokyo University, Tokyo, Japan) (12). TLR9-/- mice and control littermates that weighed 20–25g and were 6–8 weeks old were used for the experiments. The mice were fed sterilized laboratory chow (Hope Farms, Woerden, The Netherlands) and water ad libitum. The experiments were approved by the Ethics Committee on Animal Experiments of Radboud University Nijmegen.

C. albicans and growth conditions

C. albicans ATCC MYA-3573 (UC 820), a strain well described elsewhere (13), was used in all experiments. *Candida* was grown overnight in Sabouraud broth at 37°C, cells were harvested by centrifugation, washed twice, and resuspended in culture medium (RPMI-1640 Dutch modification, ICN Biomedicals, Aurora, OH) (14). To generate pseudohyphae, *C. albicans* blastoconidia were grown at 37°C in culture medium, adjusted to pH 6.4 by using hydrochloric acid. Pseudohyphae were killed for 1 h at 100°C and resuspended in culture medium to a hyphal inoculom size that originated from 106 per ml blastoconidia (referred to as 106 per ml pseudohyphae) (14).

In vitro cytokine production

Separation and stimulation of peripheral blood mononuclear cells (PBMC) was performed as described elsewhere (2). Briefly, venous blood was drawn from six healthy volunteers into 10ml EDTA tubes (Terumo, Leuven, Belgium) and the PBMC fraction was obtained by density centrifugation of diluted blood (1 part blood to 1 part pyrogen-free saline) over Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden). PBMC were washed twice in saline and suspended in culture medium supplemented with gentamicin 1%, L-glutamine 1%, and pyruvate 1%. The cells were counted in a Bürker counting chamber, their number was adjusted to 5x10e6 cells/ml, and cells were incubated with *C. albicans* blastoconidia or pseudohyphae at 1x10e6 microorganisms/ml in the absence or presence of a TLR9 specific antagonist; ODN TTAGGG: 50-ttt agg gtt agg gtt agg gtt agg g-30 (InvivoGen, San Diego, USA), for 24 h at 37 1C.

Recognition of C. albicans by macrophages of TLR9-/- mice

Resident peritoneal macrophages were harvested from groups of five TLR9-/- and their control littermates by injecting 4ml of sterile PBS containing 0.38% sodium citrate (15). After washing, the cells were resuspended in culture medium in 96-well microtiter plates (Greiner, Alphen, The Netherlands) at 10e5cells/well, in a final volume of 200ml. The cells were stimulated with either control medium or heat killed (1h, 100°C) *C. albicans* at 1x10e7 microorganisms/ml. After 24h of incubation at 37°C, the plates were centrifuged (500g, 10min), and the supernatant and cell lysate (three freeze-thaw cycles) were collected and stored at 801C until cytokine assays were performed.

Cytokine assays

TNFα was determined by specific radioimmunoassay (detection limit 20 pg/ml), as previously described (16). IL-6 and IL-10 concentrations were measured by a commercial ELISA (Biosource, Camarillo, CA; detection limit 16pg/ml), according to the instructions of the manufacturer.

C. albicans infection model

TLR9-/- mice and their control littermates were injected intravenously with *C. albicans* blastoconidia (5x10e5 CFU/mouse) in a 100 ml volume of sterile pyrogen-free phosphate-buffered saline (PBS). Survival was assessed daily for 2 weeks. Subgroups of 5 animals were killed on days 3, 7, or 14 of infection. To assess the tissue outgrowth of the microorganisms, the liver and the kidneys of the sacrificed animals were removed aseptically, weighed, and homogenized in sterile saline in a tissue grinder. The number of viable *Candida* cells in the tissues was determined by plating serial dilutions on Sabouraud dextrose agar plates as

previously described (17). The CFU were counted after 24h of incubation at 37°C, and expressed as log CFU/g tissue.

Phagocytosis and killing of C. albicans by macrophages

Phagocytosis and killing of *C. albicans* blastoconidia were assessed according to a method described elsewhere(17,18). Exudate peritoneal phagocytes from groups of 5 TLR9-/- mice and their control littermates were elicited by an i.p. injection of 10% proteose peptone. After 72 h, cells were collected in separate sterile tubes by washing the peritoneal cavity with 4ml of ice-cold PBS that contained 50U/ml heparin. Phagocytes were centrifuged (for 10 min at 2250g), counted in a Bürker counting chamber, and resuspended in culture medium. The processes of phagocytosis and intracellular killing were studied in an adherent monolayer of phagocytes, as described (18). Briefly, 5x10e5 cells were dispensed into the wells of a 96-well flatbottom plate (Costar), allowed to adhere for 2h, and washed to remove nonadherent cells. Subsequently, the cells were incubated with 1x10e4 CFU *C. albicans*, which were opsonized for 45 min at 24°C in modified Eagle's medium (MEM; Gibco Life Technologies) containing 2.5% fresh mouse serum (effector:target ratio, 40:1). After 15 min, supernatants were aspirated, and monolayers were gently washed with MEM to remove noningested microorganisms. The supernatant and well washings that contained the noningested *Candida* blastoconidia were plated in serial dilutions on Sabouraud agar plates. The percentage of phagocytized microorganisms was defined as [1-(number of uningested CFU/CFU at the start of incubation)] x 100. Killing of *C. albicans* by phagocytes was assessed in the same monolayers. After removal of the nonphagocytized *Candida*blastoconidia, 200 ml of culture medium, consisting of Sabouraud in MEM (50% vol/vol), was added to the monolayers. After 3h of incubation at 37°C in air and 5% CO2, the wells were scraped gently with a plastic paddle and washed with 200 ml distilled H2O to achieve lysis of phagocytes in 3 cycles, and 10-fold dilutions of each sample were spread on Sabouraud agar plates and incubated at 37°C for 24 h. The percentage of yeast killed by the phagocytes was determined as follows: [1-(CFU after incubation/ number of phagocytized CFU)] x 100. Phagocyte-free incubations of blastoconidia were included as a control for yeast viability. In earlier experiments we have shown that 90–97% of attached/internalized *C. albicans* are intracellular (19).

Statistical analysis

The differences between groups were analyzed by the Mann–Whitney U test, and where appropriate by the Wilcoxon rank test. The level of significance between groups was set at $p<0.05$. All experiments were performed at least twice, and the data are presented as cumulative results of all experiments performed.

Results

In vitro cytokine production

Blocking TLR9 on human PBMC reduced the TNFα production induced by heat-killed *C. albicans* blastoconidia, and reduced IL-10 production (Fig. 1). IL-6 was marginally increased. Likewise, a similar trend towards decreased TNFα and IL-10 production was observed when cells were stimulated with heat-killed *C. albicans* pseudohyphae, although the difference was not statistically significant. Stimulation with live *C. albicans* resulted in overall lower cytokine production. Blocking TLR9 in experiments with live yeast increased TNFα and IL-6

production, whereas IL-10 production was reduced (Fig. 1).

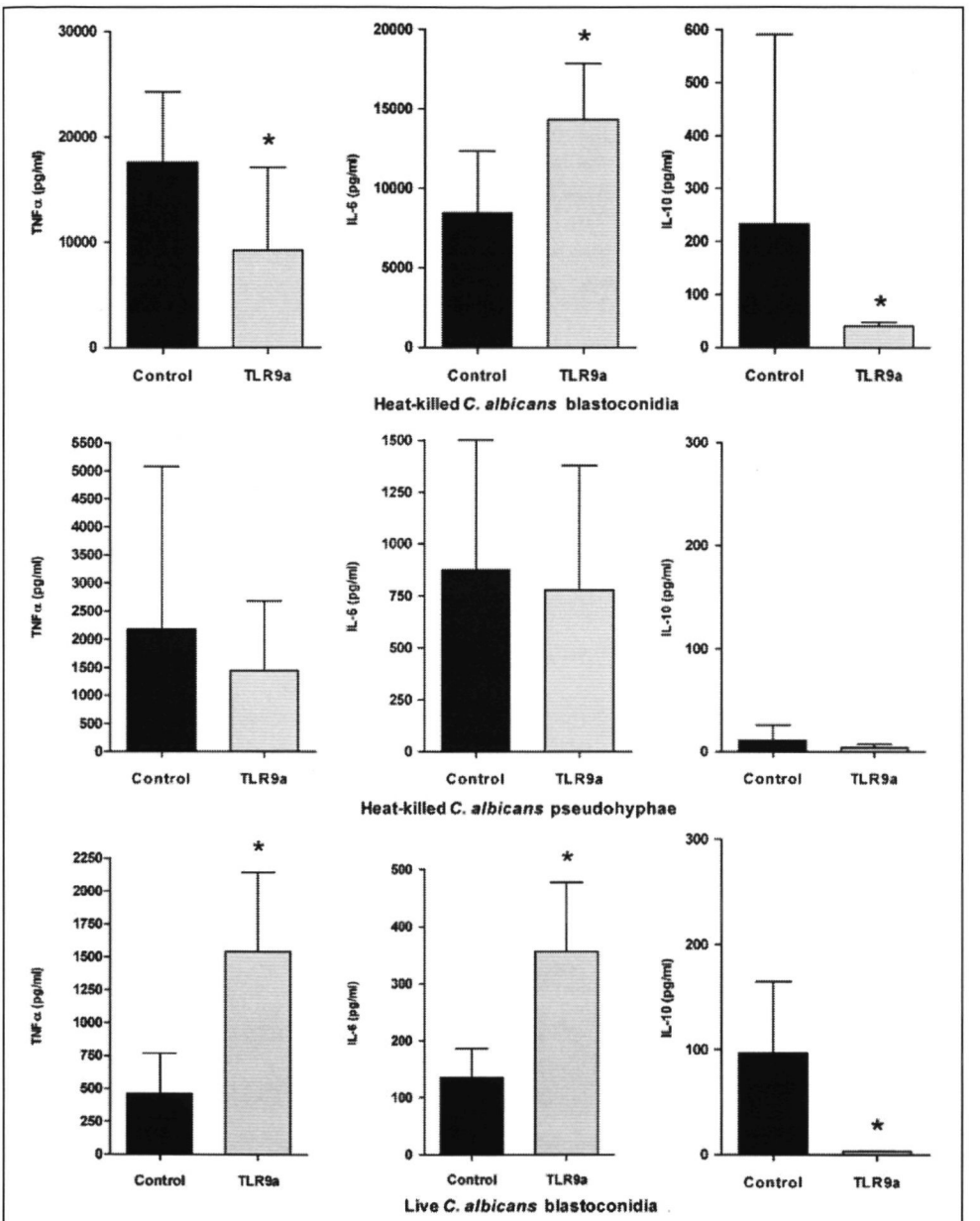

Figure 1. In vitro cytokine production. Human PBMC were stimulated with heat-killed *C. albicans* blastoconidia (1x10e6 microorganisms/ml), heat-killed *C. albicans* pseudohyphae (obtained from 1x10e6 blastoconidia) or live *C. albicans* in the presence or absence of a TLR9 antagonist (TLR9a). Production of TNFα, IL-6 and IL-10 in the supernatants was measured after 24 h of stimulation at 37°C. Data is pooled from 2 separate experiments with a total of 6 healthy volunteers (median ± IQR, *p<0.05, Wilcoxon rank test).

Recognition of C. albicans by TLR9-/- macrophages

To investigate the role of TLR9 for the recognition of *C. albicans*, resident peritoneal macrophages of TLR9-/- and TLR9+/+ control mice were stimulated with heat-killed *C. albicans* blastoconidia and pseudohyphae in vitro. Cytokine production by unstimulated macrophages of all mouse strains was below the detection limit for all cytokines studied (not shown). After stimulation with *Candida* blastoconidia, the production of TNFα, IL-6, and IL-10 did not differ between TLR9-/- and control mice (Fig. 2). However, after stimulation with *Candida* pseudohyphae, the release of IL-6 and IL-10 release was significantly reduced as compared to the TLR9+/+ control mice (Fig. 2).

Disseminated C. albicans infection in TLR9-/- mice

To determine the role of TLR9 in the host defense against invasive *C. albicans* infection, TLR9-/- and TLR9+/+ control mice were infected with *Candida* blastospores and compared for their susceptibility to infection. No significant difference was observed in the survival (80% in both groups) during disseminated candidiasis. The fungal burden in the kidneys, the target organ of disseminated candidiasis in mice, was similar in TLR9-/- and TLR9+/+ control mice on day 3, 7, and 14 of infection (Fig. 3). The fungal burden in the livers was 10–100-fold lower than that in the kidneys, and did not differ between TLR9+/+ and TLR9-/- mice (data not shown).

Phagocytosis and killing of C. albicans by macrophages

Phagocytosis of *C. albicans* by TLR9-/- macrophages was similar to that by macrophages of their control littermates (21% vs 19% phagocytized in 15min; p>0.05). TLR9-/- macrophages killed 70% of phagocytized *Candida* blastoconidia in 3 h, which was not different from the killing activity of TLR9+/+ control macrophages (73%, p>0.05, Fig. 3).

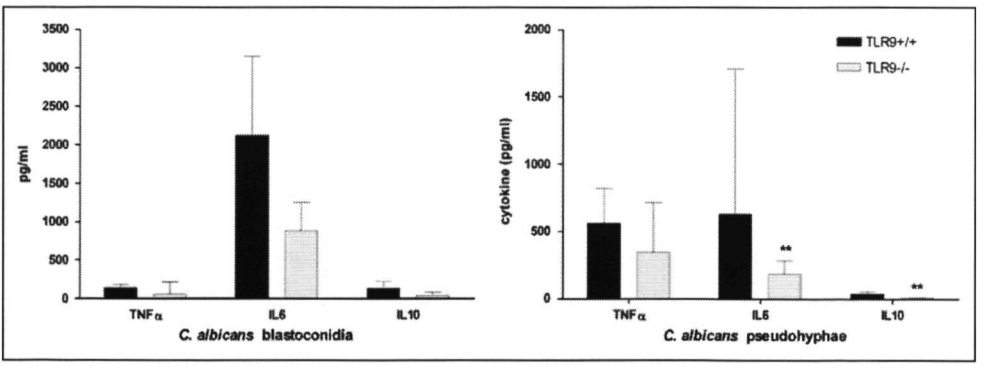

Figure 2. Recognition of *C. albicans* by macrophages of TLR9-/- mice. Resident peritoneal macrophages from TLR9-/- mice and TLR9+/+ mice were stimulated with either 1x10e7 microorganisms/ml *Candida* blastoconidia or pseudohyphae. Production of TNFα, IL-6 and IL-10 in the supernatants was measured after 24 h of stimulation at 37°C. The results are pooled data from two separate experiments with a total of 8 TLR9-/- mice and 7 TLR9+/+ mice (median ± IQR, **p<0.01, Mann–Whitney U test).

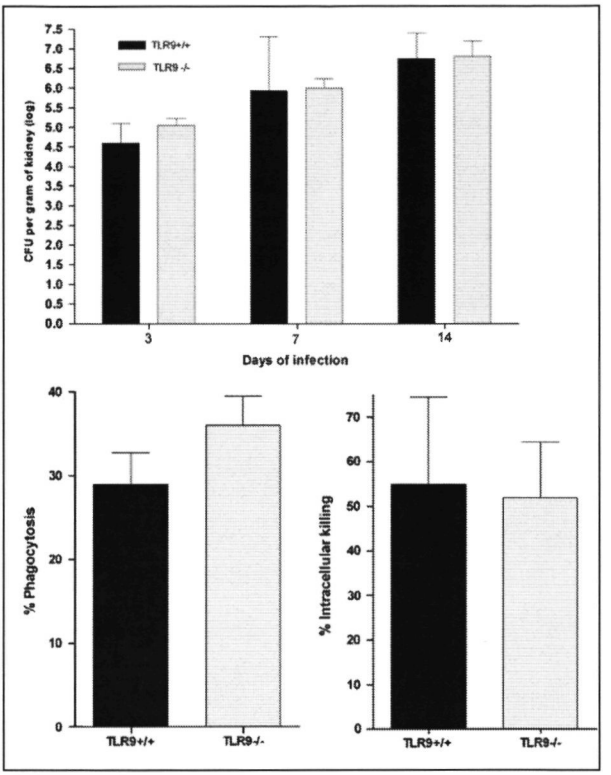

Figure 3. *C. albicans* infection in TLR9-/- mice. (Upper panel) TLR9-/- mice and TLR9+/+ mice (wt) were infected i.v. with *C. albicans* blastoconidia (5x10e5 CFU/mouse). Subgroups of animals were killed on days 3, 7 or 14 of infection, and fungal outgrowth was assessed in the kidneys. The results are pooled data from two separate experiments with at least 5 mice per group (median ± IQR). (Lower panel) Phagocytosis and killing of *C. albicans* by TLR9-/- mice and TLR9+/+ macrophages. Percentage of *Candida* blastoconidia of the initial inoculum that was ingested by macrophages of TLR9-/- and TLR9+/+ after 15 min of phagocytosis and percentage of phagocytized *Candida* blastoconidia that was killed after incubation at 37°C for 3h is shown. The results are pooled data with a total of 3 TLR9-/- mice and 6 TLR9+/+ mice (median ± IQR).

Discussion

In the present study, the role of TLR9 in the recognition of *C. albicans* and the anti-*Candida* host defense was investigated by using a murine model of disseminated candidiasis and ex-vivo stimulation of human PBMC. A moderate decrease in cytokine production was observed when TLR9 was blocked in human PBMC, and when TLR9-/- peritoneal mouse macrophages were stimulated with *C. albicans*. However, this was not accompanied by an increased susceptibility to disseminated candidiasis, arguing for a redundant role for TLR9 in anti-*Candida* host defense.

TLR9 is known to be involved in cytokine stimulation in response to bacterial DNA (20) and has been implicated in the recognition of viral DNA (21). In line with these data, we observed that stimulation with *C. albicans* led to a reduction in cytokine production, most obviously for IL-10 production, in human PBMC and mouse macrophages when the TLR9-pathway was blocked. This is supported by other studies, which have shown that recognition of bacterial

DNA by TLR9 is involved in the stimulation of cytokine production when human PBMC are stimulated with whole bacteria, e.g. *Streptococcus pneumoniae* or *Neisseria meningitidis* (22). Similarly *Brucella abortus* engages TLR9 in murine dendritic cells, resulting in a Th1 cytokine response (23), DNA from *Trypanosoma cruzi* stimulates cytokine production in a TLR9-dependent manner (24), infected TLR9-/- mice display defective *Mycobacteria tuberculosis*-induced cytokine responses in vivo (25), and TLR9 is required for the induction of IFNγ by *Propionibacterium acnes* (26).

Despite a decrease in cytokine production, we did not observe increased susceptibility of TLR9-/-mice to infection with *C. albicans*. Several explanations could account for this. Firstly, the decrease in cytokines observed was moderate, and alternative routes are likely to compensate for the loss of TLR9 signaling. Several other pathways are involved in the recognition of *C. albicans*, leading to cytokine induction: TLR4 stimulates CXC chemokine production and neutrophil recruitment (5), the mannose receptor pathway induces IL-1β, IL-6, and granulocyte-macrophage colony-stimulating factor (27), and TLR2/dectin-1 signaling induces TNFα and IL-12 production (28). Secondly, no significant difference in phagocytosis and killing was observed. This was not unexpected as TLR9 is located intracellularly (30) and probably has no role in phagocytosis. Our findings are also supported by Bellocchio et al. (4). In their model using *Candida* blastoconidia, these authors found very small differences in fungal burden in the kidneys, and there was no significant difference in survival between TLR9-/- mice and TLR9+/+ control mice infected with *Candida* blastoconidia (4). An important additional aspect of our study, compared to the previous data in the literature from experiments performed only in experimental animals, was the assessment of the role of human TLR9 for recognition of *C. albicans*. The data in human cells mostly overlap that in mice. To increase the biological relevance of the data, stimulations were performed with both heat-killed and live *Candida*. Interestingly, TLR9 was mainly involved in the induction of IL-10. The lack of IL-10 when TLR9 was blocked lead to increased IL-6 production, and in the case of stimulation with live *Candida* also increased TNFα release. TNFα production by heat-killed or live *Candida* was differentially modulated by blocking TLR9. This is most likely due to the differences in the PAMP/PRR interaction in live and heat-killed *Candida* as previously shown by our group and others (2,31).

However, the fact that we found no significant difference in survival and fungal load in organs between TLR9-/- mice and TLR9+/+ control mice does not necessarily indicate that TLR9 is not important in anti-*Candida* host defense. The importance of parallel signaling routes has been exemplified during a M. tuberculosis mouse model (25). Whereas TLR2-/- and TLR9-/- single knock-out mice had almost similar susceptibility to *M. tuberculosis* compared to wild-type mice, TLR2-/- TLR9-/- double knock-out mice displayed a significant increased susceptibility (25). This increased susceptibility of TLR2-/- TLR9-/- mice compared to TLR2-/- and TLR9-/- mice has also been observed for infection with *T. cruzi* (24). This may also very well be the case for *C. albicans* infection, in which deficiency of TLR9 alone leads to no significant difference in survival, while TLR9 deficiency in combination with other factors may lead to a significantly increased susceptibility to *Candida* infection. The viability of this hypothesis and the potential role of TLR9 in human disease is currently tested in our laboratory by assessing the role of TLR9 polymorphisms in patients with candidemia.

In conclusion, TLR9 is involved in the production of cytokines in response to *C. albicans*. Although alternative TLR and non-TLR pathways can compensate for an isolated TLR9 deficiency, in the case of a "multiple-hit" condition, TLR9 deficiency could prove significant.

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Chapter 4

Role of TLR1 and TLR6 in the host defense against disseminated candidiasis

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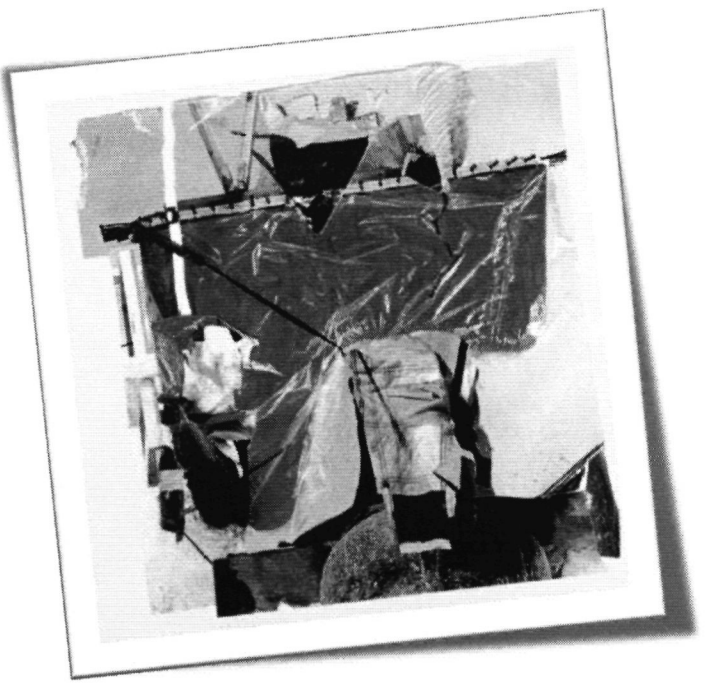

Netea MG, van de Veerdonk FL, Verschueren I, van der Meer JW, Kullberg BJ.

Summary

Toll-like receptor-1 (TLR1) and TLR6 are receptors of the TLR family that form heterodimers with TLR2. The role of TLR1 and TLR6 for the recognition of the fungal pathogen *Candida albicans* was investigated. TLR1 is not involved in the recognition of *C. albicans*, and TLR1 knock-out (TLR1-/-) mice showed a normal susceptibility to disseminated candidiasis. In contrast, recognition of *C. albicans* by TLR6 modulated the balance between Th1 and Th2 cytokines, and TLR6 knockout mice displayed a defective production of IL-10 and an increased IFN-γ release. Production of the monocyte-derived cytokines tumor necrosis factor, IL-1, and IL-6 was normal in TLR6-/- mice, and this was accompanied by a normal susceptibility to disseminated candidiasis. In conclusion, TLR6 is involved in the recognition of *C. albicans* and modulates the Th1/Th2 cytokine balance, but this results in a mild phenotype with a normal susceptibility of TLR6-/-mice to *Candida* infection.

Introduction

Invasive fungal infections, especially those caused by *Candida albicans*, are a serious clinical threat in immunocompromised patients and those who undergo major surgical procedures, with mortality reaching 30–40% despite the availability of new classes of antifungal drugs (1,2). New therapies for the treatment of disseminated fungal infections are therefore needed, and adjunctive immunotherapy is one promising, yet unfulfilled, strategy. Understanding the mechanisms through which the host immune system recognizes and eliminates fungal pathogens is an important step towards achieving this goal.

Much has been done to elucidate the host defense mechanisms against systemic candidiasis. The innate host defense mechanisms leading to elimination of *Candida* during infection have been characterized, and comprise the release of proinflammatory cytokines, leading to activation of phagocytosis and killing of the fungus by neutrophils and macrophages (3-5). Recently, it has been shown that recognition of microbial structures called pathogen-associated molecular patterns (PAMPs) by pattern-recognition receptors (PRRs) is essential for the effective activation of host defense mechanisms in general, and for cytokine production in particular. Earlier studies by the authors' group and others have described the role of several PRRs for the recognition of components of the *C. albicans* cell wall, including recognition of mannans and mannoproteins by mannose receptor and Toll-like receptor-4 (TLR4) (6-11), of phospholipomannan (PLM) by TLR2 (12), and of β-mannosides by galectin-3 (13). In addition, several studies have demonstrated the important role of these receptors for the host defense in experimental models of disseminated candidiasis. The TLR-adapter molecule MyD88 has been demonstrated to be essential for the resistance to disseminated candidiasis (9,14,15), and individual receptors such as TLR4 (7,9), TLR2 (10,14,15) and dectin-1 (16) have been reported to be involved in the defense against *C. albicans* infection.

TLR1 and TLR6 are two receptors of the TLR family that have been shown to form heterodimers with TLR2 (17). Through heterodimerization, receptor complexes are formed that recognize specific microbial structures: TLR2/TLR1 heterodimers recognize bacterial triacyl lipopeptides (18) while TLR2/TLR6 heterodimers recognize bacterial diacyl lipopeptides and lipoteichoic acid (19,20). The specificity in recognition of various microbial components by TLR heterodimers permits an adjusted response to different classes of

microorganisms. Although the role of TLR2 for the recognition of *C. albicans* is well established, nothing is known about the potential involvement of TLR1 and/or TLR6 in the recognition of *Candida* and anti-*Candida* host defense. The possibility that heterodimers between TLR2 and other TLRs may be involved in *Candida* recognition has been suggested by an earlier report showing that TLR2/TLR6 heterodimers recognize zymosan from Saccharomyces cerevisiae (21). In the present study we investigate the role of TLR1 and TLR6 for the recognition of *C. albicans* and the antifungal host defense.

Materials and methods

Animals

TLR1-/- and TLR6-/- mice were kindly provided by Dr. Shizuo Akira (Osaka University, Japan). In all experiments, knock-out mice and their control littermates (20–25g, 6-8-weeks old) were used. The mice were fed sterilized laboratory chow (Hope Farms, Woerden, The Netherlands) and water ad libitum. The experiments were approved by the Ethics Committee on Animal Experiments of Radboud University, Nijmegen.

Recognition of C. albicans and in vitro cytokine production

Groups of five TLR1-/- and TLR6-/-mice and their control littermates were sacrificed, and resident peritoneal macrophages were harvested by injecting 4mL of sterile phosphate-buffered saline (PBS) containing 0.38% sodium citrate (22). After washing, the cells were resuspended in RPMI 1640 containing 1mM pyruvate, 2 mM L-glutamine, 100 μg/mL gentamicin and 2% fresh mouse plasma (culture medium). Cells were cultured in 96-well microtiter plates (Greiner, Alphen, The Netherlands) at 10e5 cells per well, in a final volume of 200 μL. The cells were stimulated with either control medium or heat-killed (1h, 100°C) *C. albicans* at 1x10e7 CFU/mL (ATCC MYA-3573). After 24h of incubation at 37°C, the plates were centrifuged (500g, 10 min), and the supernatant and cell lysate (three freeze–thaw cycles) were collected and stored at -80 1C until cytokine assays were performed.

To assess IFNγ and IL-10 production, primed spleen cells from mice on day 3 of infection with 1x10e5 CFU of *C. albicans* per mouse were stimulated in vitro with heat-killed *Candida* (1x10e7 CFU/mL), zymosan (1 μg/mL), Pam3Cys (10 μg/mL) or MALP (10 μg/mL). Spleen cells were obtained by gently squeezing spleens in a sterile 200 mm filter chamber. Microscopic examination of Giemsa-stained cytospin preparations showed that these cells consisted of 95% lymphocytes, 2% monocytes and 3% granulocytes. The cells were washed and resuspended in RPMI1640, counted in a Bürker counting chamber and the number was adjusted to 5x10e6/mL. One milliliter of the cell suspension was stimulated with 1x10e7 heat-killed *C. albicans* yeasts (E : T ratio 2 : 1). Measurement of IFN and IL-10 concentrations was performed in supernatants collected after 48h of incubation at 37°C in 5% CO2 in 24-well plates.

Cytokine assays

IL-1α, IL-1β and TNFα were determined by specific radio-immunoassays (detection limit 20pg/mL), as described previously (23). IL-6, IFNγ and IL-10 concentrations were measured by a commercial enzyme- linked immunosorbent assay (ELISA) (Biosource, Camarillo, CA; detection limit 16 pg/mL), according to the instructions of the manufacturer.

Candida albicans infection model

Candida albicans ATCC MYA-3573 (UC820), a strain well- described earlier (24), has been used in all experiments. TLR1-/-and TLR6-/- mice and their control littermates were injected intravenously with *C. albicans* (5x10e5 CFU per mouse) in a 100 mL volume of sterile pyrogen-free PBS. Survival was assessed daily for 2 weeks. Subgroups of five animals were sacrificed on days 3, 7 or 14 of infection. To assess the tissue outgrowth of the microorganisms on these days, the liver and the kidneys of the sacrificed animals were removed aseptically, weighed and homogenized in sterile saline in a tissue grinder. The number of viable *Candida* cells in the tissues was determined by plating serial dilutions on Sabouraud dextrose agar plates as described previously (25). The CFU were counted after 24h of incubation at 37°C, and expressed as log CFU/g tissue.

Statistical analysis

The differences between groups were analyzed by the Mann–Whitney U-test and, where appropriate, by Kruskal-Wallis ANOVA. The level of significance between groups was set at $P<0.05$. All experiments were performed at least twice, and the data are presented as cumulative results of all experiments performed.

Results

Recognition of C. albicans by macrophages of TLR1-/- and TLR6-/- mice

To investigate the role of TLR1 and TLR6 for the recognition of *C. albicans*, peritoneal macrophages of TLR1-/-, TLR6-/- and control mice were stimulated with heat-killed *Candida* blastospores in vitro. Cytokine production by unstimulated macrophages of all mouse strains was below the detection limit for all cytokines studied (not shown). *Candida*-stimulated production of TNFα, IL-1β and IL-6, as measured by ELISA in the supernatants of the stimulate cells, did not differ between TLR1-/-, TLR6-/- and control mice (Fig. 1). Similarly, the concentrations of intracellular IL-1α measured in the cell lysates did not differ between the knock-out and control mice (Fig. 1).

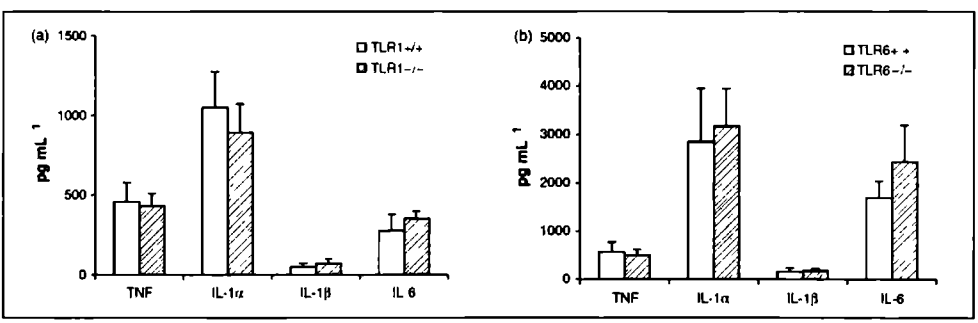

Figure 1. Recognition of *Candida albicans* by macrophages of TLR1-/- and TLR6-/- mice.
Peritoneal macrophages of TLR1+/+ and TLR1-/- mice (a) as well as TLR6+/+ and TLR6-/- mice (b) were stimulated with 1x10e6 yeast cells/mL of heat-killed *C. albicans*. Production of TNF, IL-1β and IL-6 in the supernatants and of IL-1α in cell lysates was measured by specific ELISA after 24 h of stimulation at 37°C. The results are pooled data from two separate experiments with a total of 10 mice per group (means ± SD).

Role of TLR1 and TLR6 for the Th1/Th2 cytokine balance

The balance between Th1-derived proinflammatory cytokines such as IFNγ and Th2-derived

anti-inflammatory cytokines such as IL-10 plays an important role in the host defense against disseminated candidiasis (26). When primed splenocytes of TLR1-/- mice were stimulated with *C. albicans* blastospores, they released similar amounts of IFNγ and IL-10 as splenocytes isolated from control animals did (Fig. 2a). In contrast, primed splenocytes of TLR6-/- mice released greater amounts of IFNγ compared with control mice, while IL-10 production was significantly lower compared with controls (Fig. 2b). Similar results were obtained when IL-10 and IFN production was stimulated with zymosan (not shown). In contrast, none of the two lipopeptides (Pam3Cys and MALP) stimulated IFNγ production either in control or in the knockout mice (concentrations below the 16 pg/mL detection limit of the assay), demonstrating that complex interaction or several types of receptors with fungal PAMPs of the cell wall of fungi are responsible for IFNγ induction, rather than TLR2/TLR1 or TLR2/TLR6 complexes alone. As expected, Pam3Cys did not induce IL-10 in TLR1-/- mice, and MALP did not stimulate IL-10 production in TLR6-/- mice. Pam3Cys induced normal amounts of IL-10 in TLR6-/- mice (322 ± 57 vs. 299 ± 29 pg/mL in control mice, P>0.05), and MALP induced similar amounts of IL-10 in TLR1-/- mice (145 ± 39 vs. 188 ± 51 pg/mL in control mice, P > 0.05).

Disseminated C. albicans infection in TLR1-/-and TLR6-/- mice

To determine the role of TLR1 and TLR6 in the host defense against *C. albicans*, TLR1-/- , TLR6-/- and control mice were infected with *Candida* blastospores and their susceptibility to infection was compared. No difference was observed in either the survival (90% in both groups) or the fungal burden in the kidneys, the target organ of disseminated candidiasis in mice, between TLR1-/- and control mice (Fig. 3a). The fungal burden in the livers was 10–100-fold lower than that in the kidneys, and did not differ between TLR1+/+ and TLR1-/- mice (not shown). The survival of TLR6 +/+ mice at 2 weeks after infection with *C. albicans* was 80%, and that in TLR6-/- mice was 90% (P = NS). The growth of *C. albicans* in the kidneys (Fig. 3b) and liver (not shown) of the TLR6-/- mice was also similar to that in TLR6+/+ mice. Although fewer *Candida* microorganisms tended to be recovered from the kidneys of TLR6-/- mice than from of control animals on Day 14 of infection, the difference did not reach statistical significance (Fig. 3b).

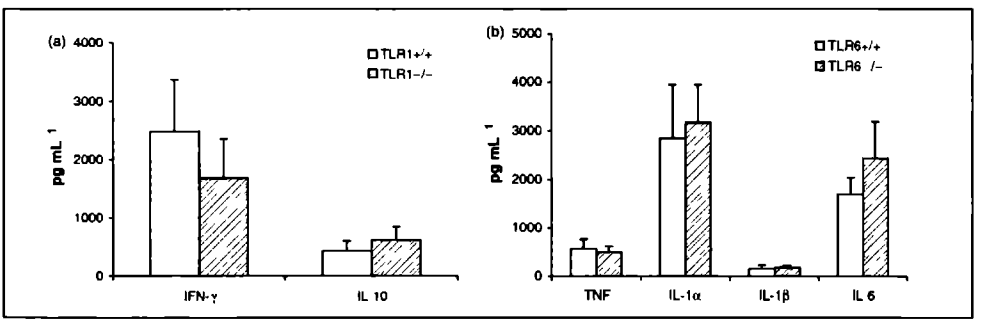

Figure 2. Role of TLR1 and TLR6 in the Th1/Th2 cytokine balance.

Mice (TLR1-/,a; TLR6-/-, b) were infected intravenously with *C. albicans* (1x10e5 CFU/mouse), and after 3 days splenocytes were harvested and stimulated in vitro with 1x10e7 heat-killed *C. albicans* blastospores (E : T ratio 2 : 1). Measurement of IFNγ and IL-10 concentrations was performed in supernatants collected after 48 h of incubation at 37°C. The results are pooled data from two separate experiments with a total of 10 mice per group (means ± SD, P<0.05).

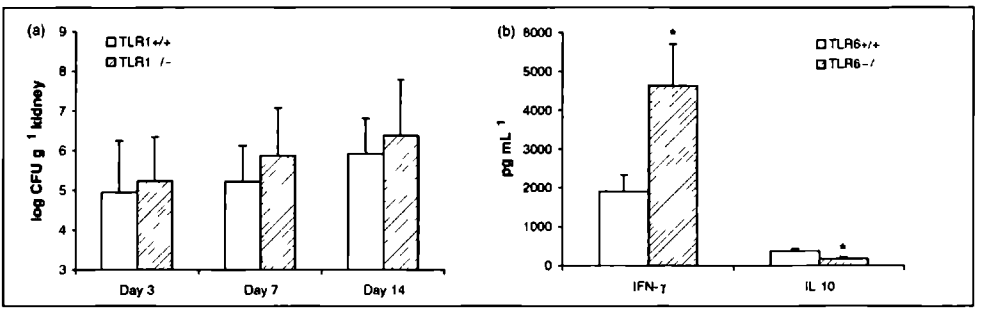

Figure 3. Disseminated *Candida albicans* infection in TLR1-/- and TLR6-/- mice.
TLR1+/+ and TLR1-/- (a) and TLR6+/+ and TLR6-/- mice (b) were infected intravenously with *C. albicans* (5x10e5 CFU per mouse). Subgroups of animals were sacrificed on Days 3, 7 or 14 of infection, and fungal outgrowth was assessed in the kidneys. The results, expressed as log CFU/g tissue, are pooled data from two separate experiments with a total of 10 mice per group (means ± SD).

Discussion

This study investigated the role of TLR1 and TLR6 for the recognition of *C. albicans* and the host defense during disseminated candidiasis. TLR1 is not involved in the recognition of *Candida* by the innate immune system, and did not affect susceptibility to disseminated candidiasis. In contrast, recognition of *C. albicans* by TLR6 modulated the balance between Th1 and Th2 cytokines, with TLR6 knockout mice displaying a more proinflammatory cytokine profile, characterized by increased IFNγ production and reduced IL-10. However, this Th2-biased pattern was not accompanied by a significant modulation of the susceptibility to disseminated candidiasis.

The involvement of TLR6 in the recognition of *C. albicans* is indirectly supported by the established role of TLR2 in the recognition of *C. albicans*, as TLR2 is known to form heterodimers with TLR6 and TLR1 (17). Recognition of *S. cerevisiae*-derived zymosan by TLR2/TLR6 heterodimers has been described (21). In the present study, in vitro stimulation of cells isolated from TLR6-/- mice with *C. albicans* blastospores has demonstrated a role for TLR6 in the recognition of *Candida* and subsequent stimulation of cytokines. However, these effects seem to be cell type-specific, with little effect of TLR6 on the *Candida*-induced production of cytokines by resident murine peritoneal macrophages, while the production of cytokines by splenocytes was strongly influenced by the absence of TLR6.

The defective production of IL-10 by splenocytes of TLR6-/- mice in response to *C. albicans* or zymosan, which was accompanied by an increase in IFNγ, supports the hypothesis that TLR6, most likely in the form of a heterodimer with TLR2, is mainly involved in mediating the release of Th2-cytokines (27). In this respect, the phenotype of TLR6-/- mice resembles that of TLR2-/- mice (9,10), supporting the concept of a functional unity between these receptors. However, it should be acknowledged that the phenotype of TLR6-/- mice is milder than that of TLR2-/- mice, consistent with the notion that TLR2 is the main signaling receptor, while TLR6 has an adjunctive function. No defects in the release of monocyte/macrophage-derived cytokines were observed in the TLR6-/- mice, in contrast to the severely affected cytokine production by TLR2-/- cells, as described earlier (14,15). The milder phenotype of TLR6-/- mice at the level of cytokine production most likely explains the

normal susceptibility to *Candida* infection.

The structural cell wall component of *C. albicans* that is recognized by TLR6 remains to be elucidated. The original identification of zymosan as a ligand for TLR2/TLR6 heterodimers suggests that a polysaccharide component, either β-glucan or a mannan, is the ligand for TLR6. In case of *C. albicans*, the most likely candidate is phospholipomannan, as it has been recently proposed that stimulation of cytokines by phospholipomannan is mediated by both TLR2 and TLR6 (12). As phospholipomannan and other β-mannoside structures are also recognized by galectin-3 on the surface of macrophages (13), it may be speculated whether a TLR2/TLR6/galectin- 3 receptor complex may be involved in the recognition of phospholipomannan. Similar receptor complexes between TLR and non-TLR receptors have been described for the recognition of LPS (TLR4/MD2/CD14/CD11c/18) (28), lipoteichoic acid and diacyl lipoproteins (TLR2/TLR6/CD14/ CD36) (Triantafilou et al., 2006) and β(1,3)-glucan (TLR2/dectin-1) (29,30). β(1,3)-glucan may be considered as an additional candidate ligand, as the β-glucan receptor dectin-1 forms a complex with TLR2, and a recent study has suggested the recognition of β-glucans of *Tinospora cordifolia* by TLR6 (31).

In contrast to TLR6, no role of TLR1 in the recognition of *C. albicans* or the anti-*Candida* host defense has been observed. The absence of the involvement of TLR1 in the recognition of *C. albicans* is not completely unexpected, as no function of TLR1 has been reported in the recognition of zymosan (21). Of note, the antifungal agent amphotericin B, itself a product obtained from the fungus *Streptomyces nodosus*, activates production of cytokines through complexes of TLR2/TLR1 (32). This does not seem to be the case for *C. albicans*.

In conclusion, in this first study on the role of TLR1 and TLR6 in the recognition of *C. albicans*, the absence of a major function of TLR1 in the host defense against disseminated candidiasis is reported. In contrast, TLR6 is involved in the stimulation of the anti-inflammatory Th2-type cytokine IL-10, resulting in a skewed proinflammatory profile in TLR6 knockout mice. However, this phenotype was not associated with a significant difference in susceptibility to disseminated candidiasis. The absence of a role of TLR1 and the moderate effects of TLR6 deficiency on disseminated candidiasis in the mouse model do not preclude a role of either receptor in innate immunity to *Candida* infections in humans, as differences between murine and human TLRs are known to be present (33). Interestingly, a recent study suggested an association between TLR6 and TLR1 polymorphisms and susceptibility to invasive aspergillosis (34), and a potential role of these TLRs in a large cohort of patients with disseminated candidiasis is currently being investigated.

Acknowledgements

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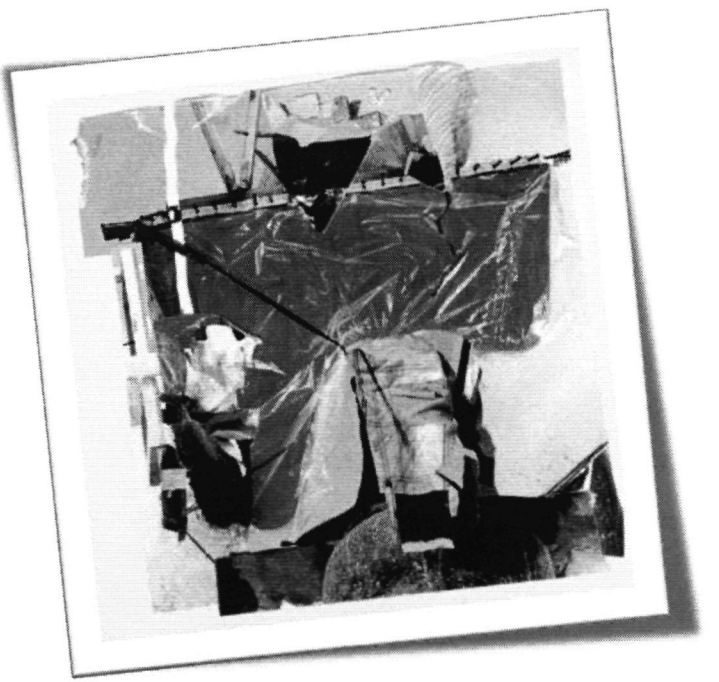
The role of the inflammasome for the host defense against *Candida albicans*

Chapter 5

The role of NLRs and TLRs in the activation of the inflammasome

Expert Opin Biol Ther. 2008 Dec;8(12):1867-72



Netea MG, van de Veerdonk FL, Kullberg BJ, Van der Meer JW, Joosten LA.

Summary

Background: Interleukin-1β is one of the most important pro-inflammatory cytokines. In contrast to other cytokines, activation of IL-1β requires processing from an inactive precursor by the cysteine protease caspase-1. Caspase-1 forms a protein platform called the inflammasome, together with proteins of the nucleotide-binding oligomerization domain-like receptor (NLR) family. Objective/methods: A review of literature investigating the stimulation of IL-1β production by microbial pathogens and their components. Results/conclusions: To produce IL-1β, macrophages need a double stimulation with Toll like receptor (TLR) ligands that induce gene transcription, and NLR agonists (such as ATP or muramyl dipeptide (MDP)) that activate the inflammasome. Monocytes can release active IL-1β upon stimulation with TLR ligands alone. This probably represents an adaptation of each cell type to its environment.

Interleukin-1 (IL-1) in the pathogenesis of human diseases

IL-1α and IL-1β are proinflammatory cytokines exerting similar biological activities after interaction with a receptor complex formed by IL-1 type I receptor (IL-1RI) and the IL-1R accessory protein [1]. Mice deficient in IL-1 have an increased susceptibility to infections, and administration of recombinant human IL-1α or IL-1β have indicated a protective role for IL-1 in infections [2,3]. However, in addition to its beneficial roles in infections, IL-1 also exerts important deleterious effects during the inflammation of autoimmune diseases such as rheumatoid arthritis, Crohn's disease or type 1 diabetes [4].

In addition to its role in infections and autoimmune diseases, an increased interest in the biology of IL-1β has been generated after the discovery that an entire group of autoinflammatory disorders that specifically respond to blockade of IL-1 receptor with the IL-1 receptor antagonist (IL-1Ra), or neutralization of IL-1β by monoclonal anti-IL-1β antibodies. These syndromes include familial Mediteranean fever [5], familial cold auto-inflammatory syndrome [6], Muckle-Wells syndrome [7], neonatal onset multisystem inflammatory disease [8], hyperimmunoglobulin D syndrome [9], and adult-onset Still's disease [10]. Blood monocytes from patients with some of these disorders, especially cryopyrinopathies, readily release more IL-1β than monocytes from unaffected controls, revealing a loss of the tight control regulating processing and release of active IL-1β.

Processing of IL-1b: caspase-1 activation by the NLR inflammasomes

Because IL-1β is one of the most active pro-inflammatory cytokines, which can also exert deleterious effects when overproduced in the circulation, several control mechanisms have evolved to modulate its production and activity (Figure 1): transcription of IL-1β mRNA, translation, processing of the 31-kDa inactive IL-1β precursor form into the bioactive 17-kDa IL-1β [11], and the release from secretory lysosomes through K+-dependent mechanisms [12,13]. In contrast to IL-1β that requires processing before being secreted from the cell, IL-1α is not processed and it mainly remains cell-associated [1]. However, it has been reported that IL1α does partially need caspase-1 and calpain for processing and secretion [14]. In addition to the regulatory mechanisms at the level of processing, the control of IL-1 activity is also exerted by IL-1Ra or the type II decoy receptors [15] that block the interaction of

bioactive IL-1 with the signaling type I IL-1receptor.

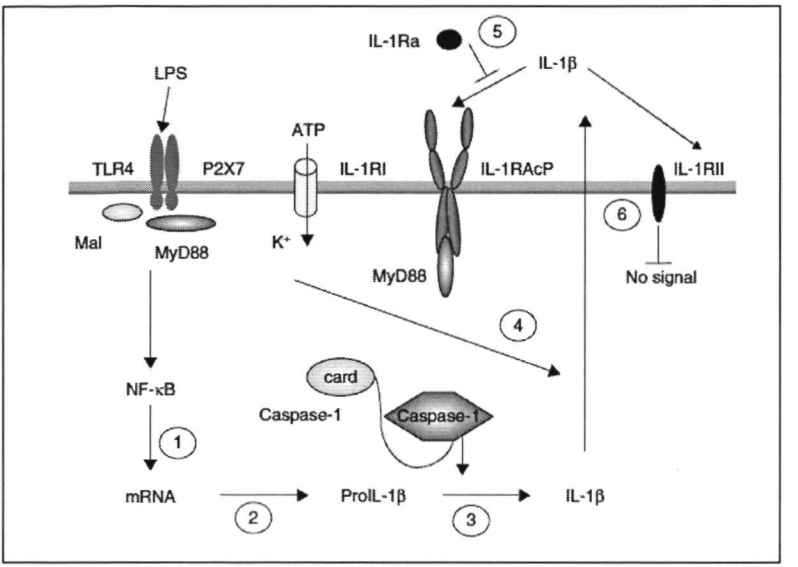


Figure 1. Regulatory steps of IL-1β biological activity. Several control mechanisms have evolved to modulate the production and activity of IL-1β: (1) transcription of IL-1β mRNA; (2) translation; (3) processing of the 31-kDa inactive IL-1β precursor form (ProIL-1β) into the bioactive 17-kDa IL-1β; (4) release through K+-dependent mechanisms; (5) control of IL-1β activity by the IL-1 receptor antagonist (IL-1Ra); (6) the type II decoy receptors (IL-1 RII); (7) blockade of the interaction of bioactive IL-1β with the signaling type I IL-1R-I/interleukin 1 receptor accessory protein (IL-1RAcP) complex. Card: Caspase recruitment domain; LPS: Lipopolysaccharide; mal: Myelin and lymphocyte protein; MyD88: Myeloid differentiation primary response gene 88; P2X7: Purinergic receptor P2X; ligand-gated ion channel 7; TLR Toll-like receptor.

One of the most important steps in the regulation of IL-1β production is the processing of the inactive pro-IL-1β into the bioactive IL-1β. Cleavage of the 31-kDa precursor is mainly exerted by a cysteine protease initially called IL-1 converting enzyme, and later termed caspase-1. In its turn, caspase-1 is also initially produced as inactive pro-caspase-1. Its autocatalytic activation depends on changes in the three-dimensional conformation of a protein complex termed the inflammasome [16]. Several protein platforms/inflammasomes have been described for the activation of caspase-1, and each of them include members of the nucleotide-binding oligomerization domain-like receptor (NLR) family of proteins [17]. The most intensely studied have been the inflammasomes formed by the NLR family members NACHT leucine rich repeat and PYD (pyrin domain)-containing (NALP)3 and NALP1, that, in addition to caspase-1 and NLR proteins, also includes the adapter protein apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) (Figure 2). In addition to the NALPs, another NLR member, IL1-β converting enzyme-protease activating factor (IPAF), forms an inflammasome that activates caspase-1 in response to intracellular flagellin but also in response to flagellin-deficient *Pseudomonas* and non-flagellated *Shigella* [18,19]. While several studies have suggested that the IPAF inflammasome activate caspase-1 in an ASC-independent manner [20,21], other studies suggested a role for ASC in the function of the IPAF inflammasome [18]. Mutations in NALP3 exits in familial cold-induced autoinflammatory syndrome (FCAS) [6], Muckle-Wells Syndrome (MWS) [7], and neonatal

onset multisystem inflammatory disease (NOMID) [8], whereas specific NALP1 polymorphisms have been associated with vitiligo and autoimmune diseases [22].

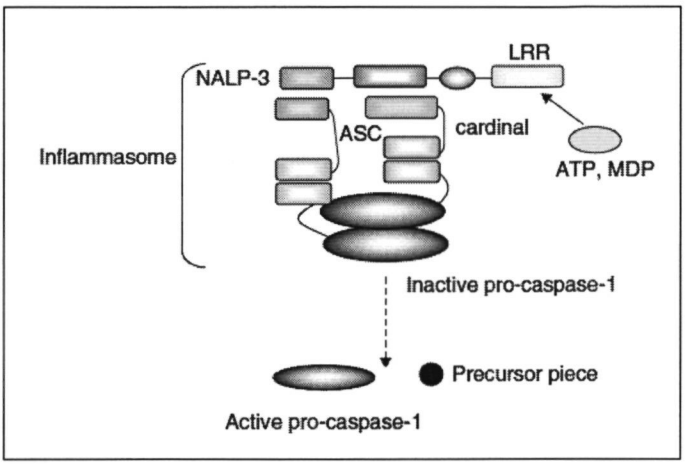


Figure 2. The NALP3 inflammasome. The inactive form of caspase-1 forms a complex with the NLR proteins NALP3, apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) and cardinal. Upon interaction of ATP or muramyl dipeptide (MDP) with the leucine rich repeat (LRR) domain of NALP3, conformational changes in the inflammasome de-repress caspase-1 which is released from the complex in its active form. It should be noted that cardinal is not present in the mouse. NALP3: NLR family apoptosis inhibitory protein, class II transactivator, heterockaryon incompatibility E and transition protein 1 (NACHT)-domain leucine rich repeat and PYD (pyrin domain)-containing 3.

In addition to caspase-1, a cysteine protease mainly present in monocytes and macrophages, a secondary mechanisms for the processing of IL-1β is represented by a group of serine proteases mainly released by the neutrophils: protease-3, cathepsin G and elastase, that have been reported to cleave proIL-1β into a 21-kDa fragment with biological activity [23]. Among these proteases, protease-3 has been recently shown to be the dominant enzyme processing IL-1β in the neutrophil [24]. However, the biological relevance of this secondary pathway of IL-1β processing is less well established.

TLR engagement and the stimulation of IL-1β production

On the basis of results obtained using transfected cell lines and/or NALP3-knockout mice, a broad panel of stimuli have been proposed to activate the NALP3 inflammasome, including bacterial products such as peptidoglycans and muramyl dipeptide (MDP) [25], bacterial toxins [26], silica [27], alum [28], asbestos [27] but also endogenous products such as uric acid [29], ATP [26] or amyloid-β [30]. In addition to NALP3, the peptidoglycan component MDP also induces association of the NLR proteins NOD2 and NALP1, and this complex mediates caspase-1-dependent secretion of IL-1β [31]. A similar process mediates activation of IL-1β by *Bacillus anthracis* infection [31].

Based on responses in the THP-1 cell line, a concept has arisen that IL-1β production induced by the Gram-negative bacteria cell-wall component lipopolysaccharide (LPS) is due to contamination with non-LPS ligands such as peptidoglycans [25], while LPS by itself is ineffective as a stimulator of IL-1β release. According to this model, LPS induces activation of IL-1β gene transcription and during the 15 (up to 30) minute interval when cells are exposed

to ATP is used as a parameter of IL-1β production. In contrast, 'classical' IL-1β stimulation is performed in primary human monocytes, in which the release of the processed cytokine takes place over 24 – 48 h stimulation of the cells.
These methodological differences can explain the apparent discrepancies between these various studies on the one hand but also hint at important biological phenomena. Firstly, there is a very strong body of evidence demonstrating that two signals are needed for the stimulation of IL-1β production in THP-1 cells and primary macrophages. When stimulated with TLR ligands such LPS (TLR4) or tripalmitoyl-S-glyceryl cysteine (Pam3Cys) (TLR2) alone, macrophages or THP-1 cells do not release IL-1β. The lack of IL-1β release was observed not only for cell lines or in vitro-differentiated monocyte-derived macrophages but also for alveolar macrophages isolated from healthy volunteers [42,43]. Only after a TLR ligand is combined with ATP, an inflammasome activator, can the macrophages release active IL-1β. Secondly, these studies suggest that monocytes are functionally different from macrophages in terms of IL-1β release, and they can produce active IL-1β in response to TLR stimulation alone. The precise molecular mechanisms leading to TLR-induction of IL-1β production in monocytes remain however to be elucidated.

The capability of monocytes to release IL-1β secretion after stimulation with TLR ligands alone explains much experimental data. On the one hand, this explains the many clinical studies showing stimulation of IL-1β by TLR ligands in whole-blood assays, or after infusion of clinical-grade LPS in human volunteers [44]. Similarly, mice defective for the inflammasome components ASC [45] or NALP3 [46] show resistance to lethal endotoxemia, an observation that can be explained only if a circulating cell population (e.g., monocytes) releases active IL-1β after the infusion of LPS. One possible explanation that could unify the findings of the 'classical' long-exposure assays, and the short-stimulation assays used for inflammasome activation, is that during the 24h needed for stimulation of IL-1β by TLR ligands, danger signals (e.g., ATP, uric acid) are released from the cells and collaborate with TLR ligands in the release of active IL-1β.

On the other hand, these data explain the profile of IL-1β release from monocytes of patients with autoinflammatory diseases. Although they are highly responsive to TLR stimulation and they release IL-1β, blood monocytes also respond to ATP challenge, leading to a greater release of IL-1β [12]. This is highly relevant to the role of NALP3 in monocytes isolated from patients with autoinflammatory diseases. Monocytes from patients with Muckle-Wells syndrome and NALP3 mutations do not respond to additional ATP stimulation for the release of IL-1β [47]. Thus, the NALP3 mutations result in an inflammasome, which is already maximally stimulated without the need for a second signal from ATP [47]. Having a maximally activated inflammasome that efficiently processes IL-1β even after minimal stimulation may explain the inflammatory attacks in these syndromes, which are induced by most trivial of stimuli [6,48].

Conclusions

This review of published studies investigating the stimulation of IL-1β production by microbial pathogens and their components reveals an important dichotomy in the capacity of myeloid cells to release active IL-1β. While macrophages need a double stimulation with TLR ligands that induce gene transcription, and NLR agonists that activate the inflammasome

for caspase-1 activation and processing of proIL-1β, monocytes can release active IL-1β upon stimulation with TLR ligands alone (Figure 3). The relevance of this difference is apparent when one considers the role of IL-1β in the host response to exogenous challenges. The single (TLR only) stimulation of IL-1β in monocytes compared with the double (TLR/ATP) stimulation in macrophages probably represents an adaptation of each cell type to their respective environments. Circulating monocytes are responsible for the surveillance of an essentially pathogen-free environment, so they must respond promptly to any danger signal (especially of microbial origin). In contrast, macrophages are confined to an environment (e.g., alveolar space, mucosal surfaces) in which they are constantly exposed to microbial stimuli. A sensitive response in macrophages to such stimuli for the release of IL-1β at each encounter with such exogenous stimuli would result in recurring and deleterious inflammatory reactions. Thus, repeated bouts of inflammation are probably reduced by the requirement for a second stimulus for the activation of the inflammasome and release of active IL-1β.

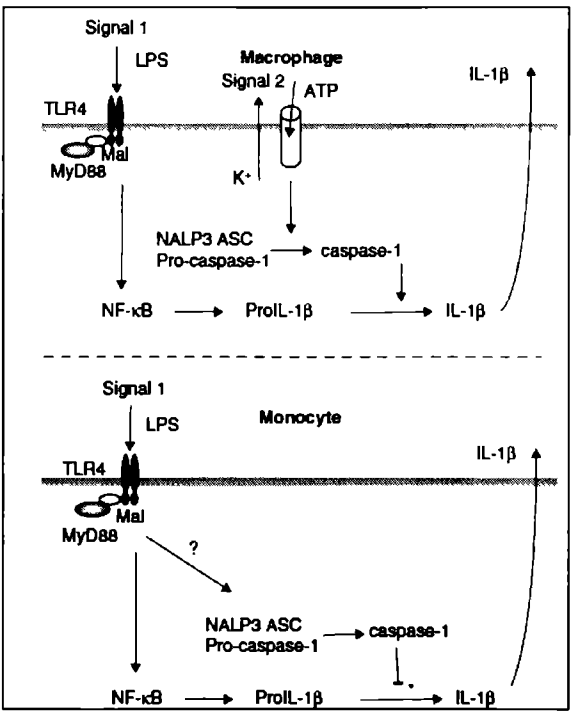


Figure 3. Diagram representing the IL-1β activation pathways in macrophages and monocytes. Macrophages need a double stimulation for IL-1β production: one stimulus (TLR-ligands) induces transcription, and a second stimulus (ATP) induces IL-1β secretion. In contrast, monocytes release mature IL-1β after single stimulation with TLR ligands alone.

Declaration of interest

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Chapter 6

Bypassing pathogen-induced inflammasome activation for the regulation of interleukin-1β production by the fungal pathogen *Candida albicans*

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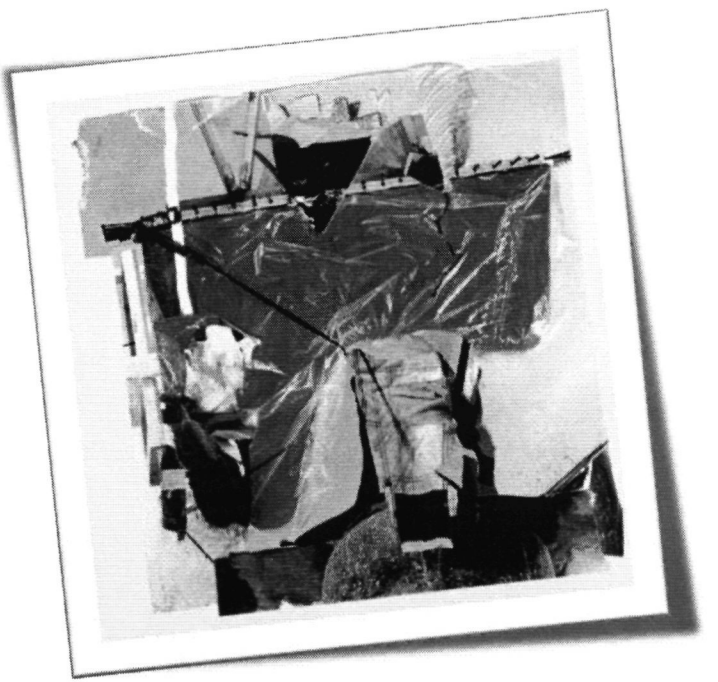

van de Veerdonk FL, Joosten LA, Devesa I, Mora-Montes HM, Kanneganti TD, Dinarello CA, van der Meer JW, Gow NA, Kullberg BJ, Netea MG.

Summary

Background: Interleukin (IL)-1β has an important role in antifungal defense mechanisms. The inflammasome is thought to be required for caspase-1 activation and processing of the inactive precursor pro-IL-1β. The aim of the present study was to investigate the pathways of IL-1β production induced by *Candida albicans* in human monocytes.

Methods: Human mononuclear cells were stimulated with *C. albicans* or mutant strains defective in mannosylation or chitin. Receptors were blocked with specific antagonists, and the IL-1β concentration was measured.

Results: Human primary monocytes produce bioactive IL-1β when stimulated with *C. albicans*. The transcription of IL-1β was induced through mannose receptor (MR), Toll-like receptor (TLR) 2, and dectin-1 but not through TLR4 and TLR9. *N*-mannan-linked residues, chitin, and β-glucan from *C. albicans* are important for IL-1β stimulation. Surprisingly, processing and secretion of IL-1β in monocytes did not require pathogen-mediated inflammasome activation, because of the constitutive activation of caspase-1 and the capability of monocytes to release endogenous adenosine-5'-triphosphate.

Conclusions: This study is the first dissection of the molecular mechanisms of IL-1β production by a fungal pathogen. Transcription through mannan/chitin/MR and β-glucan/dectin-1/TLR2 induces production of IL-1β by *C. albicans* in human monocytes, whereas processing of IL-1β is mediated by constitutively active caspase-1.

Introduction

Candida species are among the most common nosocomial bloodstream pathogens in the United States and Europe (1,2), and candidemia is associated with a high crude mortality rate of 40% (3,4). The main host defense mechanisms in systemic candidiasis are phagocytosis and the killing of *Candida albicans* by neutrophilic granulocytes, monocytes, and macrophages (5,6). The proinflammatory cytokines tumor necrosis factor-α (TNF-α) and interleukin (IL)-1β are essential for anti-*Candida* host defense through neutrophil recruitment and phagocytosis (7), and interferon-γ (IFN-γ) has been shown to be important for nitric oxide (NO) production by macrophages (8). IL-1-α and IL-1β-deficient mice have increased mortality rates, with endogenous IL-1α and IL-1β required for the induction of protective inflammatory responses in disseminated candidiasis (9).

In contrast to other proinflammatory cytokines, IL-1β and IL-18 lack a signal peptide (10). After transcription and translation, pro–IL-1β is processed by the caspase-1 protease; secretion that is dependent on the interaction of adenosine-5'-triphosphate (ATP) with the P2X7 receptor then follows (11). It has been recently shown that activation of caspase-1 in human monocytic leukemia (THP-1) cells and mouse macrophages requires a protein complex known as the "inflammasome" (12,13). Detection of specific pathogen-associated molecular patterns (PAMPs) or danger signals by the inflammasome is achieved by proteins of the nucleotide-binding oligomerization domain–like receptor (NLR) family, such as cryopirin (NALP3) or IL-1-converting enzyme (ICE) protease-activating factor (Ipaf), leading to

a conformational change in caspase-1 and caspase-1 activation (14). Caspase-1 activity is essential for host defense against infection with *Francisella tularensis* (15), *Legionella pneumophila* (16), *Shigella* species (17), and *Pseudomonas aeruginosa* (18). In addition to IL-1β and IL-18, IFNγ is also indirectly dependent on caspase-1 activity, because of its induction by IL-18 (19).

No component of *C. albicans* has yet been reported to be able to interact with the NLR receptors that form the protein platform of the inflammasome. Therefore, the formal possibility exists that *C. albicans* differs significantly from other pathogens in the way that IL-1β is induced. In the present study, we investigated how IL-1β is induced, processed, secreted, and regulated by *C. albicans* in human monocytes, as well as the extent to which caspase-1 plays a role in this pathway. We concluded that the conventional pathway of caspase-1 activation by the inflammasome complex is bypassed during host-*Candida* interaction.

Methods

Volunteers

Blood samples were collected from 14 healthy, nonsmoking volunteers who were free of obvious diseases. After written informed consent was obtained, venipuncture was performed to collect blood into 10-mL ethylenediaminetetraacetic acid (EDTA) syringes (Monoject).

Mice

Ipaf-deficient, NALP3-deficient, and caspase-1-deficient mice on a C57BL/6 background were previously described elsewhere (20-22). Age-matched C57BL/6 mice were used as control mice. Animal experiments were approved by the Institutional Animal Care and Use Committee at St. Jude Children's Research Hospital (Memphis, Tennessee).

Reagents

The irreversible caspase-1 inhibitor Ac-Tyr-Val- Ala-Asp-2,6 dimethylbenzoyl-oxymethylketone (YVAD) was purchased from Alexis Biochemicals, reconstituted in 10 mmol/L dimethyl sulfoxide (DMSO), and subsequently diluted to the desired concentration in medium (RPMI 1640). The proteinase 3 inhibitor and lipopolysaccharide (LPS) (*Escherichia coli* serotype 055:B5) were purchased from Sigma. LPS was repurified as described elsewhere (23). Mouse anti-human monoclonal anti-Toll-like receptor (TLR) 4 HTA125 antibody (aTLR4) was provided by Kensuke Miyake (Saga Medical School, Saga, Japan). Mouse anti-human monoclonal anti-TLR2 antibody (aTLR2) was provided by Douglas Golenbock (University of Massachusetts, Boston). Mouse anti-human monoclonal anti-CD14 WT14 antibody (aCD14) was a gift of Wil Tax (Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands). Anti-dectin-1 antibody (aDectin-1) was a gift of Gordon Brown (University of Capetown, Capetown, South Africa). Mouse control antibody was purchased from Sigma. Chitin was prepared according to protocols described elsewhere (24). *C. albicans* mannan (mannan) was provided by David L. Williams (East Tennessee State University, Johnson City, Tennessee). TLR9 was inhibited with oligodeoxynucleotide TTAGGG: 5'- TTTAGGGTTAGGGTTAGGGTTAGGG-3' (aTLR9) (InvivoGen). Syk inhibitor (Syk-i) was purchased from Calbiochem. P2X7 receptor was inhibited with oxATP (Sigma). In

experiments using pharmacological inhibitors, control cells were treated with an equivalent concentration of vehicle (0.01%-0.1% DMSO).

C. albicans strains and mutants

C. albicans ATCC MYA- 3573 (UC 820) (25) was used, unless otherwise indicated. *Candida* organisms were grown overnight in Sabouraud broth at 37°C, and cells were then harvested by centrifugation, washed twice, and resuspended in culture medium (RPMI 1640; ICN Biomedicals) (26). To generate pseudohyphae, *C. albicans* blas- toconidia were grown at 37°C in culture medium, which was adjusted to a pH of 6.4 by use of hydrochloric acid. Pseudohyphae were killed for 1 h at 100°C and were resuspended in culture medium to a hyphal inoculum size that originated from 1x10e6 microorganisms/mL blastoconidia (referred to as "1x10e6 microorganisms/mL pseudohyphae") (26). The *C. albicans* CAI4 strain (i.e., the wild-type strain), *och1* null mutant (defective in outer, branched *N*-linked glycans) (27), *mnt1/mnt2* double null mutant (lacking the 4 terminal *O*-linked 1,2-mannosyl residues) (28), *mnn4* null mutant (lacking phosphomannan) (29), CAF2 wild-type strain, and *chs1* null mutant (defective in chitin) were used and have been well described elsewhere (30).

Isolation of peripheral blood mononuclear cells (PBMCs) and in-vitro stimulations

Separation and stimulation of PBMCs were performed as described elsewhere (31). Cells were adjusted to 5x10e6 cells/mL. They then were incubated at 37°C in round-bottom 96-well plates (5x10e5 cells in total volume 200 L/well) with either heat-killed *C. albicans* (10e6 microorganisms/mL) or culture medium, with or without caspase-1 inhibitor at different concentrations. In some experiments, PBMCs were preincubated for 1h with antibodies (anti-TLR4, 20 μg/mL; anti-TLR2, 20 μg/mL; anti-CD14, 20 μg/mL; and anti–Dectin-1, 10 μg/mL) or inhibitors (Syk-i, 50 nmol/L; chitin, 20 μg/mL; and mannan, 200 μg/mL) before stimulation with *C. albicans*. After 24h, supernatants were collected and stored at 20°C until assayed. To investigate the role of endogenous ATP in the secretion of IL-1β, stimulations with RPMI and *C. albicans* were performed with or without oxATP for 24 h. For ATP measurements, PBMCs and macrophages were stimulated with RPMI or *C. albicans*. After 24h, the supernatants were collected and directly measured.

Production of cytokines by mouse macrophages

Resident peritoneal mouse macrophages were obtained aseptically with ice-cold PBS. Cells were resuspended in RPMI in a round-bottom 96-well plate (10e5 cells/well). For cytokine production, mouse macrophages were stimulated with culture medium as a negative control or with heat-killed blastoconidia (ATCC MYA- 3573; 107 microorganisms/mL).

Cytokine assays

IL-1β concentrations were measured using a commercial ELISA kit (DY201; R&D Systems; Pelikine-Compact (Sanguin)), according to the manufacturers instructions. The presence of TNFα was determined by specific radioimmunoassay (detection limit, 20 pg/mL), as described elsewhere (32). Murine IL-1α, IL-1β, and IL-6 concentrations were determined using specific radioimmunoassay, as described elsewhere (32). ATP concentrations in the supernatants were assessed using a firefly luciferase assay (ATP determination kit; Invitrogen).

Western blot assay
PBMCs (10e7 cells/well in a total volume of 1 mL) were incubated for 2h (to evaluate caspase-1 activation) and 24h (to assess pro-IL-1β processing) with either heat-killed *C. albicans* (10e6 microorganisms/mL) or culture medium. THP-1 cells were cultured in RPMI medium and 10% fetal calf serum and were used for Western blot analysis of caspase-1. Cells were lysed in 100 μL of lysis buffer and were centrifuged (at 10,000 g for 5 min), and the protein content was determined by use of a bicinchonic acid protein assay (Pierce). Equal amounts of protein were loaded on 12% SDS-PAGE and transferred onto nitrocellulose membranes. For supernatants, 30 μL aliquots were loaded. Membranes were blocked in Tris-buffered saline-Tween (TBS-T) containing 3% w/v skim milk. For measurement of (pro-)IL-1β, membranes were incubated with anti-(pro-)IL-1β polyclonal antibody (1/1000 dilution) (Cell Signaling). For caspase-1 quantitation, membranes were incubated with specific caspase-1 p10 polyclonal antiserum (1/500 dilution) (Santa Cruz). β-actin was quantified as an internal control by use of specific anti-β-actin polyclonal antiserum (1/ 1000 dilution) (Santa Cruz). Blots were washed and incubated with peroxidase-conjugated goat anti-rabbit IgG (1/1000 dilution). After the blots were washed 3 times with TBS-T, they were developed with Hyper ECL (GE Healthcare), according to the manufacturer's instructions.

Quantitative polymerase chain reaction (qPCR)
PBMCs were stimulated as described above. After 4h, the supernatant was removed, and the cells were resuspended in 200μL of RNAzolB RNA isolation solvent (Campro Scientific) and stored at 80°C. mRNA was isolated according to the manufacturer's protocol. cDNA was synthesized from 1 μg of total RNA by use of SuperScript reverse transcriptase (Invitrogen). Relative mRNA levels were determined using the Bio-Rad i-Cycler and the SYBR Green method (Invitrogen). The following primers were used: IL-1β forward primer 5'-TGGCCCAGGCAGTCAGA-3' and reverse primer 5'- GGTTTGCTACAACATGGGCTACA-3' and β2M forward primer 5'-ATGAGTATGCCTGCCGTGTG-3' and reverse primer 5'-CCAAATGCGGCATCTTCAAAC-3' (Biolegio). Values are expressed as fold increases in mRNA levels, relative to those in unstimulated cells.

Statistical analyses
Differences were analyzed by the Wilcoxon rank test or the Mann-Whitney U test. $P<0.05$ was considered to denote significance. All experiments were performed at least twice, and the data presented are the cumulative result of all experiments performed. Data are expressed as the median ± interquartile range (IQR).

Results

Induction of IL-1β production in PBMCs by Candida albicans
Heat-killed *C. albicans* yeast cells, heat-killed *C. albicans* pseudohyphae, and live *C. albicans* are all capable of inducing secretion of IL-1β in the supernatant of PBMCs (Figure 1A). Mature IL-1β, but not pro-IL-1β, was found to be present in the supernatant by use of Western blot analysis (Figure 1B). TaqMan reverse-transcriptase PCR analysis showed that heat- killed *C. albicans* yeast cells or pseudohyphae induced expression of IL-1β mRNA in human PBMCs 4h after stimulation. In line with the findings for IL-1β secretion, stimulation with heat-killed pseudohyphae was lower than stimulation with heat-killed yeast cells (Figure 1C).

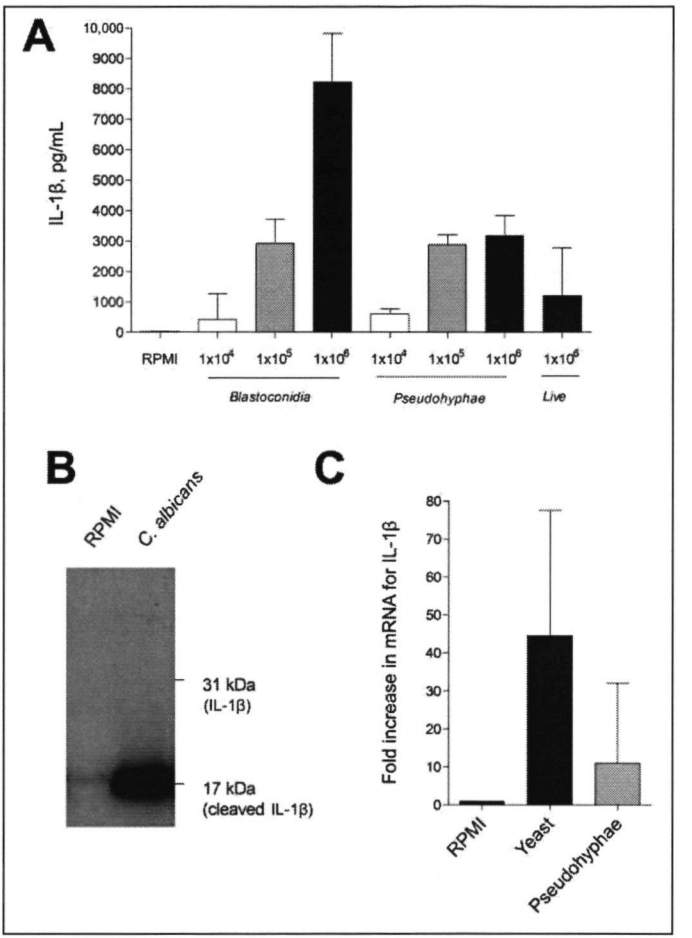

Figure 1. *Candida albicans*–induced production of interleukin (IL)–1β in peripheral blood mononuclear cells (PBMCs).

A, Human PBMCs were stimulated with heat-killed *C. albicans* yeast cells, heat-killed *C. albicans* pseudohyphae, or live *C. albicans* yeast cells (expressed as the no. of microorganisms per milliliter). ELISA was used to measure the production of IL-1β in the supernatants of PBMCs incubated at 37°C for 24h. B, Western blot analysis for IL-1β in the supernatants of PBMCs stimulated with either control RPMI 1640 medium or heat-killed *C. albicans* yeast cells (10e6 microorganisms/mL). C, Reverse-transcriptase quantitative polymerase chain reaction analysis of mRNA for IL-1β production in PBMCs stimulated for 4h with heat-killed *C. albicans* yeast cells or pseudohyphae (10e6 microorganisms/mL) or with RPMI 1640 medium. In panel A, the data are pooled from 3 separate experiments involving a total of 8 healthy volunteers (median ± interquartile range; *P<0.05, by Wilcoxon rank test). Panel B presents findings from 2 separate experiments (n=4), and panel C presents the average value of data from 2 separate experiments (n=4).

Caspase-1-dependent IL-1β production induced by C. albicans

IL-1β, but not TNFα, production induced by *C. albicans* was reduced in the presence of the caspase-1 inhibitor (YVAD), in a dose-dependent manner (Figure 2A). LPS-induced IL-1β production was also inhibited by YVAD, with a reduction of 50%-60% observed (Figure 2A). Interestingly, the proteinase 3 inhibitor alone did not result in a reduction in amounts of IL-1β production; however, when used in combination with caspase-1 inhibitor, there was a

slight yet statistically significant inhibitory effect on IL-1β production (Figure 2A). Neither the PR3 inhibitor nor the caspase-1 inhibitor alone induced IL-1β production (data not shown). Western blot analysis confirmed that the lower IL-1β level denoted a reduction in mature IL-1β (Figure 2B). Endogenous IL-1β can induce its own transcription (33). Indeed, transcription of IL-1β mRNA was reduced by the caspase-1 inhibitor (Figure 2C).

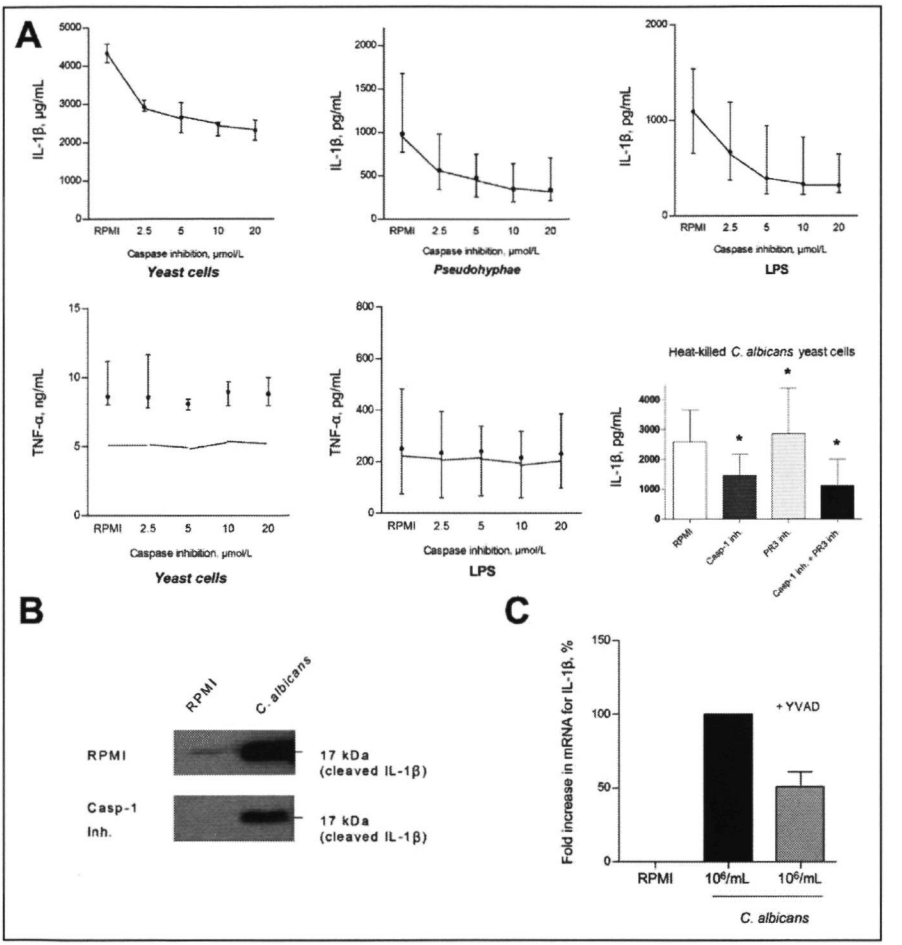

Figure 2. Caspase-1–dependent *Candida albicans*–induced interleukin (IL)–1β production.
A, Human peripheral blood mononuclear cells (PBMCs) were stimulated for 24h with heat-killed *C. albicans* yeast cells or pseudohyphae (106 microorganisms/mL) or with lipopolysaccharide (LPS; 1 ng/mL), with or without various concentrations of the caspase-1 inhibitor (casp-1 inh.; YVAD) and with or without a PR3 inhibitor (PR3 inh.) or a combination of PR3 inh. and YVAD. Production of IL-1β and tumor necrosis factor (TNF)α in the supernatants was measured by ELISA after incubation at 37°C for 24 h. B, Western blot analysis was performed for the detection of IL-1β in supernatants from PBMCs stimulated for 24h with heat-killed *C. albicans* yeast cells (10e6 microorganisms/mL) with or without a casp-1 inhibitor (20 µmol/L). C, Reverse-transcriptase quantitative polymerase chain reaction analysis of mRNA for IL-1β in PBMCs stimulated for 4h with *C. albicans* yeast cells (1x10e6 microorganisms/mL) with or without a caspase-1 inhibitor. In panel A, data are pooled from 3 separate experiments involving a total of 7 healthy volunteers (median ± interquartile range; *P<0.05, by Wilcoxon rank test). The data in panels B and C are the average values from 2 separate experiments (n=4).

Constitutively active caspase-1 in human monocytes and endogenous ATP– dependent secretion of IL-1β

The possibility that *C albicans* stimulation activates caspase-1 was investigated by Western blot analysis Surprisingly, unstimulated PBMCs were found to have activated/processed caspase-1 (p35), and this was not upregulated by heat-killed *C albicans* yeast cells (Figure 3A) To rule out the possibility that activation of caspase-1 occurred during the period of preparation of PBMCs, control experiments were performed using freshly isolated unstimulated PBMCs that were immediately lysed Once again, caspase-1 was found to be present in its processed/active form (Figure 3B) The THP-1 cells commonly used for studies of inflammasome activation showed no constitutively active caspase-1 (data not shown)

We investigated the role of ATP in IL-1β secretion by stimulating monocytes with heat-killed *C albicans* in the presence or absence of a P2X7 receptor inhibitor (oxATP) oxATP strongly reduced IL-1β stimulation by *C albicans*, whereas the intracellular concentration was elevated (Figure 3C) This finding suggested that endogenous ATP was present in the supernatants, indeed, endogenous ATP was released in the supernatants by monocytes but not by macrophages (data not shown) To assess whether induction of IL-1β by *C albicans* was dependent on the inflammasome, peritoneal macrophages from mice deficient in NALP3, Ipaf, or caspase-1 were stimulated with heat-killed *C albicans* The production of IL-1β was dependent on caspase-1 (Figure 3D) However, the absence of NALP3 or Ipaf did not alter IL-1β production (Figure 3D) In contrast to IL-1β, the production of IL-1β and IL-6 was not reduced in macrophages from mice deficient in either caspase-1 or NALP3/Ipaf (data not shown)

Mediation of IL-1β production through TLR2/dectin-1, mannose receptor (MR), and chitin receptor pathways

The *C albicans och1* null mutant is defective in outer, branched *N*-linked glycan formations, the *mnt1/mnt2* double null mutant lacks the 4 terminal *O*-linked 1,2-mannosyl residues but has normal *N*-mannan, the *mnn4* null mutant lacks phosphomannan, and the *chs1* null mutant has a 70% reduction in the chitin content of the wall PBMCs stimulated with the *och1* null mutant or the *chs1* null mutant released significantly lower amounts of IL-1β than did the wild-type strains (Figure 4A) In contrast, were no differences in IL-1β production between the *mnt1/mnt2* double null mutant and *mnn4* null mutants The *och1* and *chs1* null mutants expressed lower levels of mRNA for IL-1β than did their wild-type strains (Figure 4B), whereas transcription of the IL-1β gene was not affected when PBMCs were stimulated with the *mnt1/mnt2* and *mnn4* mutants (data not shown).

To investigate which receptors were involved in IL-1β production, PBMCs were stimulated with heat-killed *C albicans* yeast cells in the presence of anti-TLR4 antibodies (aTLR4), aCD14, aTLR9, aTLR2, aDectin-1, and mannan Chitin was used to block chitin-binding sites Inhibition of the mannan and chitin-signaling pathway resulted in reduced IL-1β production, whereas TLR4, CD14, and TLR9 inhibition did not modify IL-1β production (Figure 4C) A second mechanism of IL-1β stimulation was represented by the TLR2/dectin-1 pathway, because blocking of TLR2 or dectin-1 inhibited the production of IL-1β In addition, pharmacologic Syk inhibition also resulted in reduced IL-1β production (Figure 4C) *C albicans* hyphae shield their β-glucans from recognition (34) PBMCs stimulated with heat-

killed *Candida* pseudohyphae showed no reduction in IL-1β production when the dectin-1 receptor was blocked (Figure 4D).

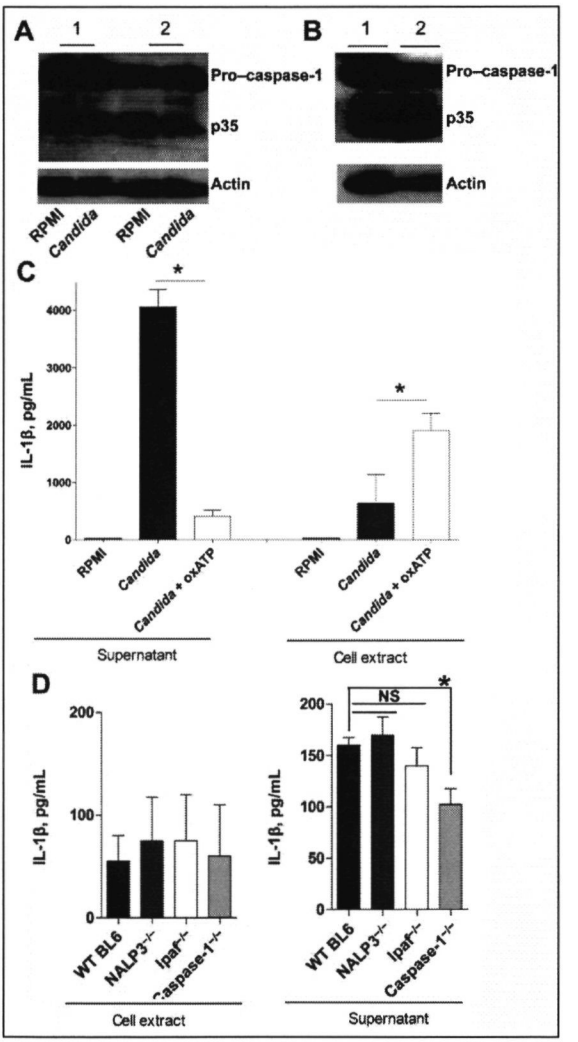

Figure 3. Constitutively active caspase-1 in human monocytes and adenosine-5'-triphosphate (ATP)-dependent interleukin (IL)-1β secretion in human peripheral blood mononuclear cells (PBMCs).
A, Western blot analysis for the detection of caspase-1 and β-actin in cellular lysates from PBMCs incubated with RPMI 1640 or *Candida albicans* at 37°C for 2h. B, Western blot analysis for the detection of caspase-1 and β-actin in cellular lysates from unstimulated PBMCs directly lysed after isolation. C, Human PBMCs were stimulated with heat-killed *C. albicans* yeast cells (10e6 microorganisms/mL) in the presence or absence of the P2X7 receptor inhibitor oxATP. Production of IL-1β in the supernatants was measured by ELISA after stimulation at 37°C for 24h. Data are pooled from 2 separate experiments involving a total of 5 healthy volunteers (median ± interquartile range (IQR); *P<0.05, by Wilcoxon rank test). D, In vitro IL-1β production by resident peritoneal macrophages (10e5 microorganisms/mL) stimulated with heat-killed *C. albicans* (10e7 microorganisms/mL). The data in panel D are pooled from 2 separate experiments involving a total of 8 mice (median ± IQR; *P<0.05, by Mann-Whitney U test). Ipaf, IL-1 converting enzyme protease-activating factor; NALP3, cryopirin; NS, nonsignificant; WT BL6, wild-type C57BL/6.

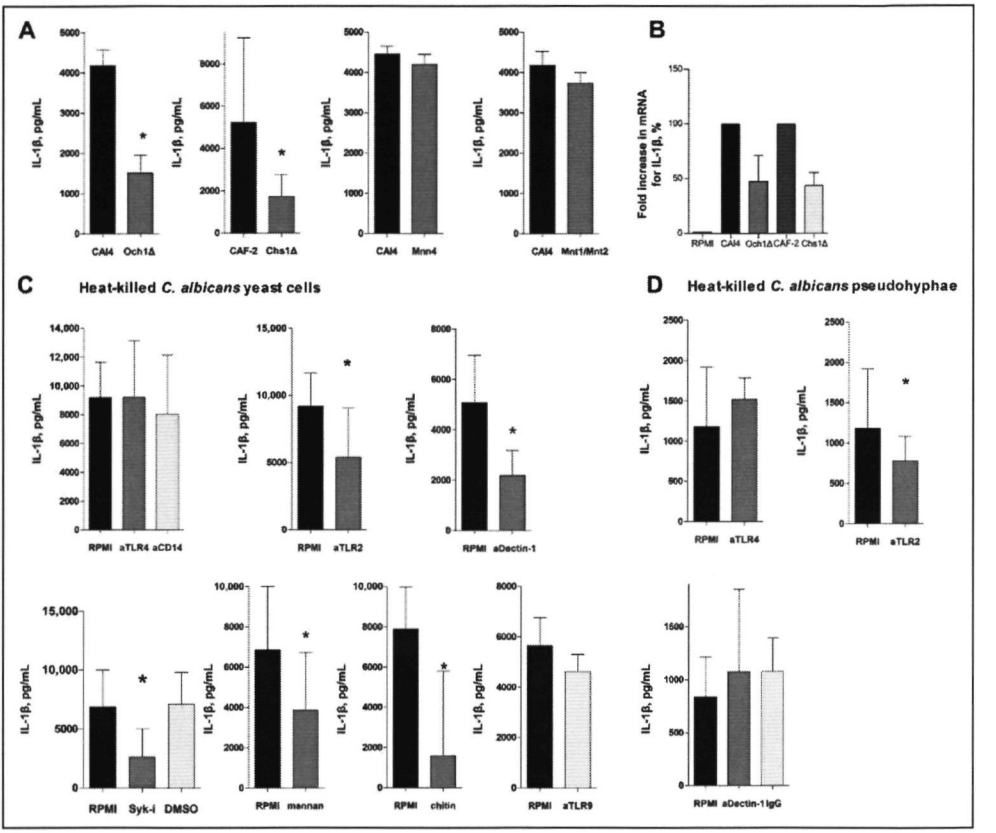

Figure 4. Dependence of interleukin (IL)-1β production on Toll-like receptor (TLR) 2, dectin-1, mannose receptor, and chitin pathways.

A, Human peripheral blood mononuclear cells (PBMCs) were stimulated with heat-killed wild-type *Candida albicans* CAI4 or CAF2 and null mutants affected in cell wall glycosylation at a concentration of 10e6 microorganisms/mL for 24h. IL-1β concentrations were measured by ELISA. B, Reverse-transcriptase quantitative polymerase chain reaction analysis of mRNA for IL-1β in PBMCs stimulated with *C. albicans chs1* and *och1* null mutants and their wild types. Human PBMCs were stimulated with heat-killed *C. albicans* yeast cells (C) or heat-killed pseudohyphae (10e6 microorganisms/mL) (D) in the presence or absence of specific receptor inhibitors (anti-TLR4 antibody (aTLR4), 20 μg/mL; anti-TLR2 antibody (aTLR2), 20 μg/mL; anti-CD14 WT14 antibody (aCD14), 20 μg/mL; and antibody to the dectin-1 receptor (aDectin-1), 10 μg/mL), mannan (200 μg/mL), or chitin (20 μg/mL) or Syk inhibitor (50 nmol/L). Production of IL-1β in the supernatants was measured by ELISA after stimulation at 37°C for 24h. Data are pooled from at least 2 separate experiments involving a total of at least 5 healthy volunteers (median ± interquartile range; *P<0.05, by Wilcoxon rank test). DMSO, dimethylsulfoxide; Syk-i, Syk inhibitor.

Discussion

In the present study, we demonstrated that *C. albicans*-induced production of IL-1β in human PBMCs is dependent on the recognition of *N*-mannan-linked residues, chitin, and β-glucan components of the *C. albicans* cell wall. This recognition is mediated through the MR and dectin-1/TLR2. Caspase-1 is the main enzyme responsible for IL-1β processing, whereas the serine protease PR3 plays a secondary role. However, although IL-1β production is

caspase-1 dependent, the regulation of its production mainly takes place at the level of transcription. This is because of the constitutive activation of caspase-1 in human PBMCs, as well as the capability of PBMCs to release endogenous ATP, leading to the secretion of the processed IL-1β. The implication of these findings is that *Candida*-induced activation of the inflammasome complex is not required for the induction of IL-1β by this fungal pathogen.

The importance of understanding the mechanisms responsible for the production of IL-1β by *C. albicans* is underlined by the important role that IL-1β plays in anti-*Candida* host defense (6). It is known that IL-1β is produced during infection with *C. albicans* (35). Accordingly, we show that, after contact with *C. albicans*, human PBMCs up-regulate mRNA for IL-1β and secrete mature IL-1β. To determine which receptors of human PBMCs and components of *C. albicans* are responsible for these developments, receptor inhibitors and *C. albicans* cell wall mutants were used. The outer layer of the *C. albicans* cell wall is enriched with mannoproteins, whereas the inner layer is composed of chitin and β1,3- and β1,6-glucan. Recently, we and other investigators have shown that mannoproteins, phospholipomannan, and β-glucans are involved in cytokine stimulation induced by *C. albicans* (36-39). Recognition of mannosyl residues is mediated by MR binding to *N*-linked mannosyl residues and by TLR4 binding to *O*-linked mannosyl residues (40).
A second pathway of cytokine production is mediated by the recognition of β-glucan through the dectin-1/TLR2 receptor complex. This has been demonstrated for TNFα and IL-6, in which transcriptional regulation of cytokine production is very important. We demonstrated that stimulation of monocytes with *C. albicans* lacking *N*-linked mannosyl residues or blockade of the MR leads to a reduction in IL-1β production. In addition, chitin has also been reported to be recognized by MR (41). When chitin-binding sites were blocked, IL-1β production by *C. albicans* was significantly impaired.

The lectin receptor dectin-1 recognizes β-glucan and interacts with TLR2 (42, 43). TLR2 and dectin-1 are also involved in IL-1β production after activation by *C. albicans* yeast cells in human monocytes. Interestingly, TLR2, but not dectin-1, is involved in the induction of IL-1β by pseudohyphae, which is in line with the observation that *C. albicans* is able to completely mask its β-glucans after transition from the yeast to the hyphal form (34). It is tempting to speculate that the loss of the additional effect of dectin-1 on TLR2 explains the lower amount of pseudohyphae-induced IL-1β production. Syk tyrosine kinase, which can be phosphorylated by the immunoreceptor tyrosine-based activation motif of dectin-1 (44), is also involved in the induction of IL-1β.

In contrast, TLR4 is not involved in IL-1β production induced by *C. albicans*, because neither blocking of TLR4 or CD14 nor stimulation with the *mnt1/mnt2*double null mutant lacking the terminal 4 *O*-linked 1,2-mannosyl residues that bind to TLR4 (29) altered IL-1β production. This observation is surprising, considering that (1) *C. albicans* possesses known TLR4 ligands, such as *O*-linked mannosyl residues, that induce production of such proinflammatory cytokines as TNFα, and (2) LPS leads to potent IL-1 production after contact with human monocytes, suggesting that TLR4 is involved in induction of IL-1β (45). These findings show that redundant mechanisms are responsible for IL-1β stimulation by *C. albicans*, and they un- derline the principle that specific receptors can induce transcription of different subsets of genes in response to the same ligand. This results in the ability of immune cells to induce

many different cytokine responses from a limited repertoire of receptors, according to the specific PAMPs that are present, and it helps to explain how the immune system induces tailored responses to specific pathogens. Similarly, phosphomannan is not involved.

One has to acknowledge differences in the stimulation of cytokines - in this case IL-1β- by live or dead *Candida* organisms. Both of these forms of *Candida* organisms stimulated IL-1β. However, exposure of β-glucans at the cell wall surface is more prominent when yeasts are heat killed (34). It is likely that the role of dectin-1 in IL-1β induction is more prominent when heat-killed *Candida* organisms are used instead of live yeasts.

It has been suggested that processing of IL-1β is mediated by caspase-1 activation by the "inflammasome," a protein complex formed mainly by receptors of the NLR family (12). Several inflammasomes activate IL-1β during bacterial infections, including NALP3 (20) and Ipaf inflammasomes (46). Of note, no component of *C. albicans* has yet been reported to be able to interact with NLR receptors. We demonstrated that caspase-1 is the main enzyme that processes IL-1β produced by human monocytes stimulated with *C. albicans*. However, the inflammasome components NALP3 and Ipaf were not needed for *Candida*-induced production of IL-1β. The fact that we observed a reduction in mRNA transcription for IL-1β in PBMCs treated with a caspase-1 inhibitor can be explained by the known feedback loop of IL-1β transcriptional induction and confirms that the endogenous IL-1β production is bioactive (33). Furthermore, we observed that caspase-1 was already present in its active form in freshly isolated human PBMCs, and p35 was not up-regulated by *C. albicans*. This observation suggests that inflammasome activation in human monocytes is not an important regulatory step in IL-1β synthesis and release after stimulation with *C. albicans*, and it shows that, similar to IL-6 and TNFα production, IL-1β production by monocytes encountering *C. albicans* is mainly regulated at the level of transcription. In addition to caspase-1, the serine protease PR3 has also been proposed to be able to process pro-IL-1β (47). Our data show that PR3 has an additional effect, albeit a relatively limited one, in the processing of IL-1β. Other serine proteases, such as cathepsin G and elastase, can play similar additional roles. This likely explains the residual IL-1β production observed after maximal caspase-1 inhibition.

Although secretion of IL-1β is incompletely understood, evidence points to the P2X7 receptor, a membrane receptor for extracellular ATP, as a key player in this process (48). In line with this evidence, we found that blocking the P2X7 receptor leads to a reduction in IL-1β secretion by stimulated monocytes. In addition, it has been reported that monocytes can release endogenous ATP (49). We also observed that monocytes could release endogenous ATP and thus were able to provide their own signal for IL-1β secretion.

In conclusion, we dissected the molecular mechanisms responsible for production of IL-1β by a fungal pathogen. Several important pathways are involved in these processes: *C. albicans* activates IL-1β through *N*-mannan and chitin recognition by MR, and β-glucan recognition by dectin-1/TLR2. It has also been suggested by Reese et al. (50) that chitin plays an important role as a PAMP in the innate immune system; however, whether receptors other than MR can recognize chitin remains to be elucidated. The tyrosine kinase Syk plays a role in the intracellular pathway inducing IL-1β gene activation (see the diagram presented

in Figure 5). We demonstrated that caspase-1 activation is constitutive and does not need recognition of *C. albicans* by the inflammasome in human primary mononuclear cells. These data question the role of the inflammasome in the induction of IL-1β by fungal pathogens. A deeper knowledge of the mechanisms of cell activation by *Candida* organisms may represent the basis for the future design of immunotherapeutic strategies in fungal infections.

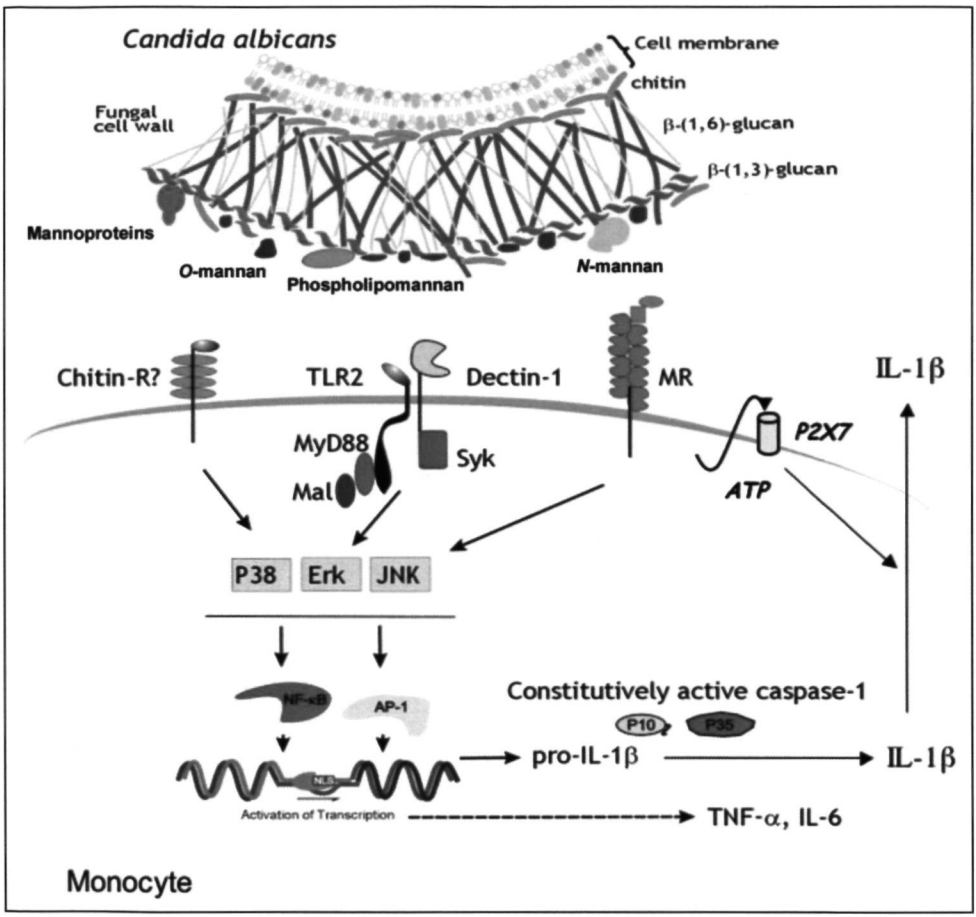

Figure 5. Pathway of activation of interleukin (IL)-1β by *Candida albicans* in human monocytes.
Schematic representation of the induction and signaling pathway of IL-1β induced by the fungal pathogen *C. albicans* in the human primary monocyte. Induction involves a dectin-1/Toll-like receptor (TLR) 2 complex that recognizes β-glucan, as well as mannose receptor (MR) recognizing *N*-linked mannan and chitin, leading to transcription of mRNA for IL-1β. There is a possibility that chitin induces cytokines through other receptors, but this has not yet been confirmed. The dectin-1 signaling pathway is dependent on Syk and also induces transcription of mRNA for pro-IL-1β. Pro-IL-1β, in its turn, is cleaved by active caspase-1, which is constitutively activated in human monocytes, resulting in IL-1β processing and secretion. The end result of this cascade is production of bioactive endogenous IL-1β. AP-1, activator protein-1; JNK, Jun N-terminal kinase; TNF, tumor necrosis factor.

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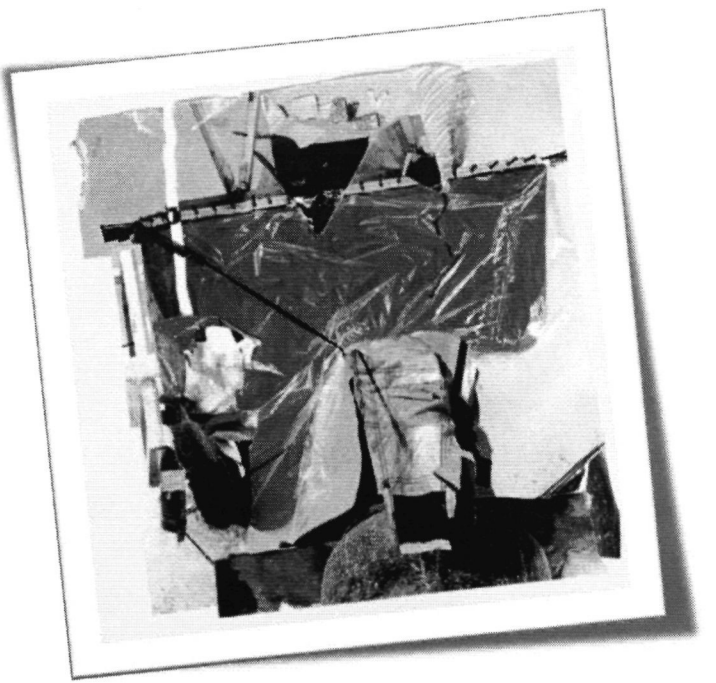
The inflammasome component Nlrp3 is redundant in host defense against disseminated candidiasis

Submitted



van de Veerdonk FL, Joosten LA, Shaw PJ, Malireddi S, Smeekens SP, van der Meer JW, Kullberg BJ, Netea MG, Kanneganti TD.

Summary

Introduction IL-1β is crucial for antifungal host defense and its processing is mediated by caspase-1, a protease that is activated by the Nlrp3 inflammasome Several studies have suggested that the inflammasome component Nlrp3 is involved in caspase-1 activation by *Candida* components In the present study we investigated the role of Nlrp3 in an in-vivo model model of murine disseminated candidiasis

Materials and Method Bone marrow derived dendritic cells (BMDCs) from wild type (WT) and Nlrp3-/- mice were cultured for four hours in the presence or absence of live *Candida albicans* and western blots for capase-1 were performed on cell lysates WT and Nlrp3-/- mice were intravenously infected with live *Candida albicans* and survival, fungal loads in the kidney, splenocyte restimulation with *C albicans,* and histology of the kidneys was assessed

Results Western blots demonstrated that Nlrp3 was essential for caspase-1 activation by *C albicans* in BMDCs in vitro However, no differences were observed on survival or fungal outgrowth in the kidneys between wild type mice and Nlrp3/- mice IL-1β production by Nlrp3-/- splenocytes that were restimulated at day 3 and day 7 of infection with *C albicans* was not different from WT splenocytes In addition, concentrations of bioactive IL-1 in kidney homogenates was not different between Nlrp3-/- mice and WT mice Histological images of the kidneys from Nlrp3-/- mice were indistinguishable from the kidneys isolated from WT mice

Conclusions The in-vitro induction of caspase-1 activation by *C albicans* involves engagement of Nlrp3 However, Nlrp3 is redundant for Il-1β processing in vivo and for the host defense against disseminated candidiasis in mice

Introduction

IL-1β plays an important role in anti-*Candida* host defense Mice deficient in IL-1β are more susceptible to disseminated *Candida* infection, and generation of reactive oxygen species is diminished in IL-1β-/- granulocytes (3) Furthermore, IL-1β-/- mice show decreased recruitment of granulocytes in response to intraperitoneal *C albicans* infection (3) In contrast to other proinflammatory cytokines, IL-1β lacks a signal peptide After transcription and translation, the inactive pro-IL-1β precursor is processed by caspase-1 into the active cytokine The activation of caspase-1 in macrophages and the subsequent processing of IL-1β is dependent on the inflammasome, a multimeric protein complex composed of ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain), caspase-1 and one or more nucleotide-binding domain leucine-rich repeat receptors (NLRs) (4) The NLRs link pattern recognition to caspase-1 activation and subsequently IL-1β production (5).

Recent studies suggest that the inflammasome component Nlrp3 transduces *Candida* recognition into caspase-1 activation, and that this process is important for antifungal host defense (6, 7) However, a previous in-vitro investigation in human primary cells suggested IL-1β induction by *C albicans* in a Nlrp3-independent pathway (8) To address the role of the Nlrp3 inflammasome in invasive *Candida* infection, we studied the susceptibility to

disseminated candidiasis of mice deficient in Nlrp3. We report that Nlrp3 has a redundant role for host defense in disseminated *Candida* infection.

Results

In vitro caspase-1 activation is dependent on Nlrp3

Upon recognition of pathogens the intracellular microbial sensors (such as Nlrp3) will be activated. Nlrp3 will then interact and recruit the adaptor molecule ASC. Subsequently, ASC will bind and recruit caspase-1 to the inflammasome (4). The key function of this process is to convert inactive pro-caspase-1 (p45) into its active form caspase-1 (p10). We examined whether Nlrp3 was essential for the *C. albicans*-induced caspase-1 activation. When BMDCs were stimulated with *Candida,* caspase-1 activation was dependent on Nlrp3 (Figure 1). These data are in line with previous studies reporting that Nlrp3 is crucial for caspase-1 activation in-vitro.

Nlrp3$^{-/-}$ mice are equally susceptible to disseminated candidiasis

Twenty two days after intravenous injection of $2x10^5$ colony forming units (CFU) of *C. albicans*, 83% of the WT mice survived the infection (Figure 2A). In contrast to the recently reported data, Nlrp3 -/- mice did not show an increased susceptibility (Figure 2A). In addition, no differences were observed in the fungal loads in the kidneys of Nlrp3-/- mice on day 3 and day 7 of infection compared to the WT mice (Figure 2B). We repeated this experiment on day 7 of infection and again found no significant difference: control mice 4.2 ± 0.6 (mean ± SD) versus Nlrp3-/- mice 3.4 ± 2.3 (mean ± SD) Log/CFU per gram kidney (P=0.52).

Cytokine production in Nlrp3 knockout mice

Because it has been suggested that Nlrp3 controls IL-1β production in response to *C. albicans* (6, 7, 9, 10), we tested the importance of Nlrp3 in the production of IL-1β by splenocytes. Splenocytes from WT mice and Nlrp3 deficient mice were isolated on day 3 and day 7 of infection and re-stimulated with heat killed *C. albicans*. The ability of *C. albicans* to transform into hyphae is crucial for providing the second signal for Nlrp3 inflammasome activation and subsequently IL-1β production (9), therefore we stimulated with *C. albicans* yeast cells and hyphae. We observed no difference in the amount of IL-1β produced by either wild-type splenocytes or Nlrp3-/- splenocytes (Figure 3A,B). Furthermore, IFNγ and the inflammasome independent cytokine TNFα were also not impaired in the Nlrp3-/- and mice (Figure 3A,B).

Equal inflammatory response in the kidneys of Nlrp3-/- mice compared to WT mice

Although Nlrp3 deficiency did not result in increased susceptibility or a different cytokine profile, it did play a crucial role in the induction of caspase-1 in-vitro. Therefore the kidneys were examined by histology for abnormalities. Nlrp3 deficient mice did not display any difference in inflammatory characteristics on day 3 or day 7 when compared to WT mice (Figure 4A). When cytokines were measured in the kidney homogenates, there was no difference in the amount of bioactive IL-1 or IFNγ between control and Nlrp3-/- mice (Figure 4B).

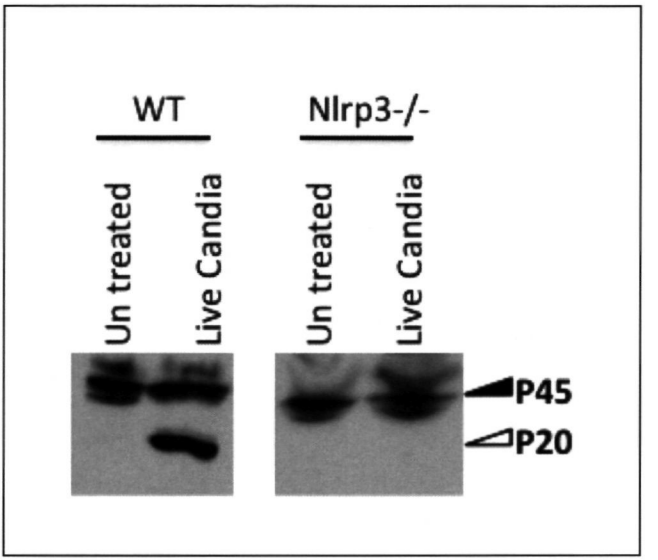


Figure 1. In vitro caspase-1 activation is dependent on Nlrp3.
Lysates from bone marrow-derived dendritic cells (BMDCs) from WT and Nlrp3-/- mice were collected 4 hours after exposure to $1x10^4$ CFU live *C. albicans*/ml, and immunoblotted with anti-caspase-1 antibody. p45 indicates procaspase-1 and p20 processed caspase-1.

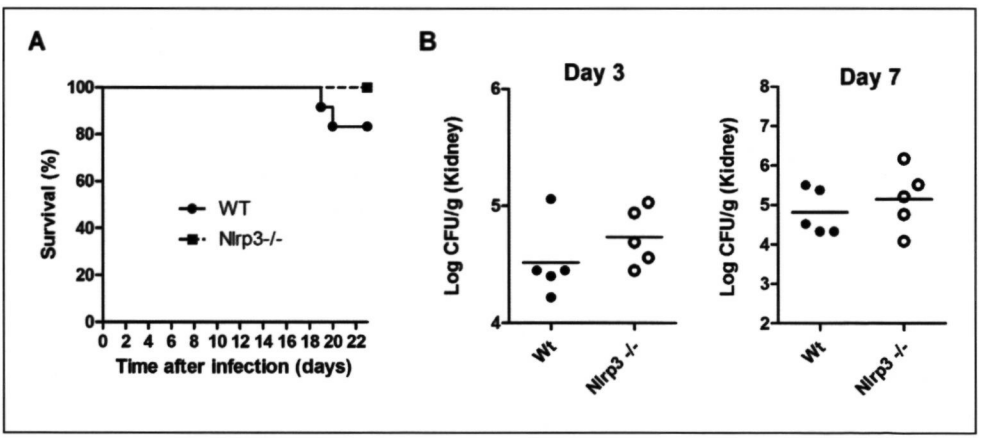


Figure 2. Nlrp3$^{-/-}$ mice are equally susceptible to disseminated candidiasis.
(A) Kaplan-Meier survival plots of WT and Nlrp3-/-mice after intravenous infection with $2x10^5$ CFU *C. albicans*/mouse. n=10 mice per group. (B) Fungal burden of kidneys of WT and Nlrp3-/- mice after intravenous infection with $2x10^5$ CFU *C. albicans*/mouse. n=5 mice per group.

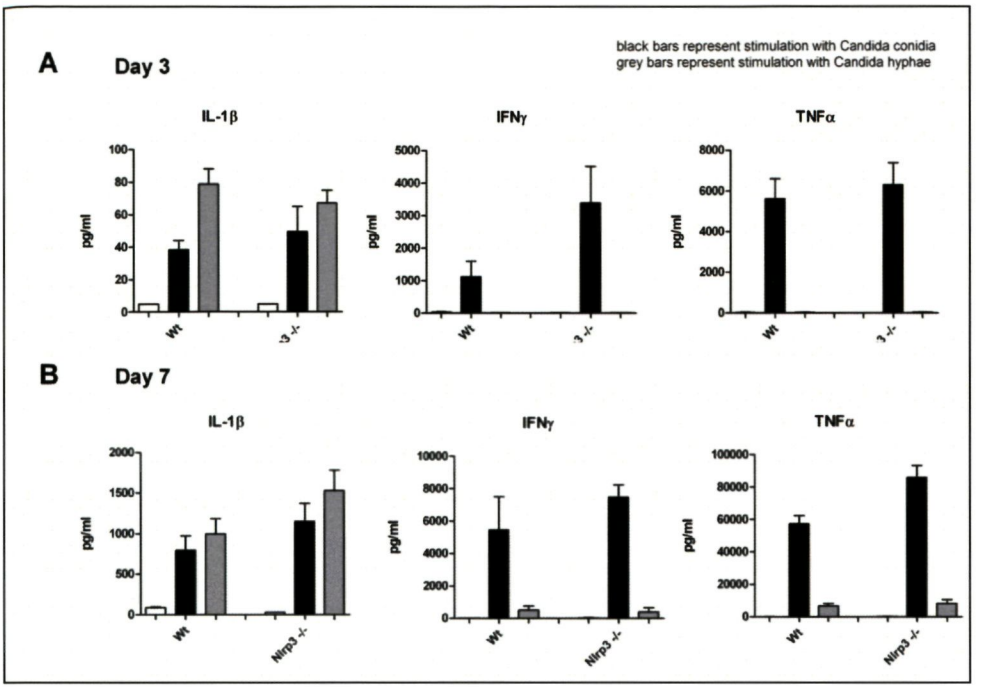


Figure 3. Cytokine production in Nlrp3 knockout mice.
(A) Splenocytes from WT and Nlrp3-/- mice were restimulated with $1x10^6$ *C. albicans* yeast cells/ml (black bars) or $1x10^6$ *C. albicans* hyphae/ml (grey bars) at day 3 (A) and day 7 (B) after intravenous infection with *C. albicans*. Cytokines were measured 48 hours after stimulation with ELISA. *p<0.05. n=5 mice per group.

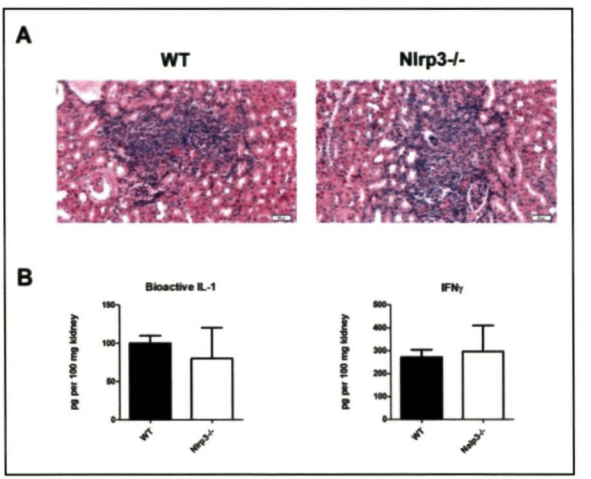


Figure 4. Equal inflammatory response in the kidneys of Nlrp3-/- mice compared to WT mice.
(A) Histopathologic assessment of the kidneys of WT and Nlrp3-/- mice after intravenous injection with $2x10^5$ CFU *C. albicans*. No differences were seen in the inflammatory reaction on day 3 and 7 after infection between Nlrp3-/- mice and WT mice. (A) Neutrophilic infiltrates in the kidneys of WT and Nlrp3-/- mice with disseminated candidiasis at day 3 of infection; images represent the observed equal inflammation in WT and Nlrp3-/- mice at day 3 and day 7 of infection. (B) IFNγ and bioactive IL-1β measured in the kidney homogenates from WT and Nlrp3-/- mice, 7 days after intravenous infection with *C. albicans*. n=5 mice per group.

Discussion

Despite the fact that several studies have attempted to investigate the activation of the inflammasome by *C. albicans*, the role of the inflammasome in vivo remains controversial (11). On the one hand, IL-1β is important for mounting an efficient anti-*Candida* host defense and its tight regulation is of critical importance during infection (3). Recently, it has been proposed that the production of IL-1β in response to *C. albicans* was dependent on the Nlrp3 inflammasome (6, 7, 9). Nlrp3 links pattern recognition of *Candida* to caspase-1 activation and subsequently IL-1β production. On the other hand, these novel studies are in contradiction with the study of Mencacci et al., who suggested that caspase-1 is not essential during primary disseminated infection with *C. albicans* in a murine model (12), and the report that IL-1β induction in human primary cells circumvents activation of the inflammasome (8). Another controversial observation was made by Gross et al. who reported that all Nlrp3 deficient mice with disseminated candidiasis died within 7 days and fungal loads were 10.000 times higher in the kidneys (6), while previous reports have shown that IL-1β deficient mice during disseminated candidiasis have a moderate mortality and much less increase in fungal loads (3).

Because of all these inconsistencies in the literature concerning this potentially important anti-candidal host defense mechanism, we aimed to assess the role of Nlrp3 during disseminated candidiasis. Although we were able to demonstrate that the in-vitro induction of caspase-1 by live *Candida* was Nlrp3-dependent, Nlrp3 deficient mice were not more susceptible to disseminated candidiasis. It is of course possible that the use of Nlrp3 deficient mice generated in different institutes, and infection with different *Candida* strains could explain the contradictory observations found in our study and that of Gross et al. It must however be mentioned that the differences found are remarkably striking. Even when we used a double dose of intravenously administered live *Candida* compared to that used by Gross et al., we observed that 100% of the Nlrp3 deficient mice survived 22 days after the initiation of infection and they had no significant differences in fungal loads, while in the study of Gross et al. there was 100% mortality after 7 days and a 10.000 higher fungal burden in the kidneys (6). While we can confirm the in-vitro role of Nlrp3 for caspase-1 activation, as reported by Joly et al., their in-vivo experiments involved only four mice, precluding any strong conclusions (9). In contrast, the study of Hise et al. investigated a model of oral candidiasis (7) that is known to have a different pathophysiology. Additional studies are needed to address these contradictory findings.

Notably, our observation that Nlrp3 is not essential for bioactive IL-1 at the site of infection underscores the argument that IL-1β processing during disseminated *Candida* infection does not require pathogen-mediated Nlrp3 activation (8). This observation suggests that during the early stages of infection proIL-1β can be processed without the need of inflammasome induction and subsequently caspase-1 activation, most likely through the activity of neutrophil-derived serine proteases (13, 14). In line with this, histology of the kidneys at day 3 and day 7 of infection predominantly shows neutrophil infiltrates in both WT and Nlrp3 deficient mice, and neutrophils are the main source of proteinase-3 that is also able to process pro-IL-1β into mature bioactive IL-1β.

In conclusion, in the present study we have dissected the role of Nlrp3 for IL-1β production and antifungal host defense in vitro and in vivo. These experiments clearly demonstrate a crucial role for Nlrp3 in the activation of caspase-1 by live *Candida* in-vitro. However, in in-vivo disseminated candidiasis, Nlrp3-mediated antifungal host defense was redundant. The Nlrp3-induced activation of the inflammasome by *Candida* most likely represents one of the mechanisms by which the host can regulate IL-1β in vivo, but in the absence of Nlrp3 other mechanisms such as neutrophil derived proteinase-3 can compensate for Nlrp3 deficiency during infectious states.

Materials and Methods

Mice and bone marrow derived dendritic cells (BMDCs)
Nlrp3-/- mice backcrossed to C57BL/6 background for at least 10 generations have been described before (15, 16). Bone marrow was prepared from the leg bones of 8–20-week-old mice. The legs were dissected, and the bone marrow flushed out. DCs were differentiated from bone marrow cells cultured with RPMI-1640 supplemented with 20ng/ml GM-CSF along with 10% heat inactivated fetal bovine serum (Invitrogen), 100 U/ml penicillin and 100 mg/ml streptomycin at 37 °C in 5% CO2 for 7 days. Mice were housed in a pathogen-free facility.

C. albicans growth conditions
C. albicans ATCC MYA-3573 (UC 820), a strain well described elsewhere (17), was used in all experiments. *Candida* was grown overnight in Sabouraud broth at 37°C, cells were harvested by centrifugation, washed twice, and resuspended in culture medium in culture medium (RPMI-1640 Dutch modification, ICN Biomedicals, Aurora, OH) (18). For in-vitro experiments, *C. albicans* was heat-killed for 1h at 100°C. To generate pseudohyphae, *C. albicans* blastoconidia were grown at 37°C in culture medium, adjusted to pH 6.4 by using hydrochloric acid. Pseudohyphae were killed for 1h at 100°C and resuspended in culture medium to a hyphal inoculom size that originated from 10^6/ml blastoconidia (referred to as 10^6/ml pseudohyphae) (18).

C. albicans infection model
Knock-out mice and WT mice were injected intravenously with *C. albicans* blastoconidia (2 x 10^5 CFU/mouse) in a 100 µl volume of sterile pyrogen-free phosphate-buffered saline (PBS). Survival was assessed daily for 22 days. Subgroups of 5 animals were killed on days 3 or 7 of infection. To assess the tissue outgrowth of the microorganisms, the kidneys of the sacrificed animals were removed aseptically, weighed, and homogenized in sterile saline in a tissue grinder. The number of viable *Candida* cells in the tissues was determined by plating serial dilutions on Sabouraud dextrose agar plates as previously described (19). The CFU were counted after 24h of incubation at 37°C, and expressed as log CFU/g tissue. For histologic analysis, kidneys of subgroups of mice (5 mice/group) were fixed in buffered formaldehyde (4%). Paraffin-embedded sections were stained with hematoxylin-eosin.

In vitro cytokine production by primed splenocytes
To assess cytokine production, primed spleen cells from mice on day 3 and day 7 of infection with $2x10^5$ CFU of *C. albicans* per mouse were stimulated in vitro with heat-killed *Candida* conidia or hyphae ($1x10^6$ microorganisms/ml). Spleen cells were obtained by gently

squeezing spleens in a sterile 200 mm filter chamber. The cells were washed and resuspended in RPMI1640, counted in a Bürker counting chamber and the number was adjusted to $5x10^6$/ml. 500 µL of the cell suspension was stimulated with $1x10^6$ heat killed *C. albicans*/ml. Measurement of IL-1β, IFNγ and TNFα concentrations was performed in supernatants collected after 48 h of incubation at 37 1C in 5% CO2 in 48-well plate.

Cytokine quantification
A Bioplex mouse X-plex assay (BioRad) was used according to the manufacturer's instructions in order to evaluate the quantity of the following cytokines: TNFα, IL-1β and IFN-γ. Bioactive IL-1 secretion was determined in a bioassay using the murine thymoma cell line EL4/NOB 1 as an IL-1-specific cell-producing IL-2 response. IL-2 was subsequently determined by ELISA (R&D).

Immunoblotting for caspase-1
BMDCs were first stimulated for 4 hours with live *C. albicans* in a concentration of $1x10^4$ CFU/ml and subsequenlty washed twice with phosphate-buffered saline and scraped in lysis buffer solution (150 mm NaCl, 10 mm Tris, pH 7.4, 5 mm EDTA, 1 mm EGTA, 0.1% Nonidet P-40) supplemented with a protease inhibitor mixture tablet (Roche Applied Science). Samples were clarified, denatured with SDS buffer, and boiled for 5 min. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were immunoblotted with primary antibodies and proteins detected with appropriate secondary anti-rabbit antibody conjugated to horseradish peroxidase followed by enhanced chemiluminescence. Rabbit anti-mouse caspase-1 was a generous gift from Dr. P. Vandenabeele (Gent University, Belgium).

Statistical analysis
Data were analyzed using GraphPad software. The differences between groups were analyzed by the Mann-Whitney U test or Student's t-test where appropriate. Comparison of two survival curves was done using the Logrank test. The level of significance between groups was set at $p<0.05$. Reproducibility of data was tested in two separate experiments.

Acknowledgements

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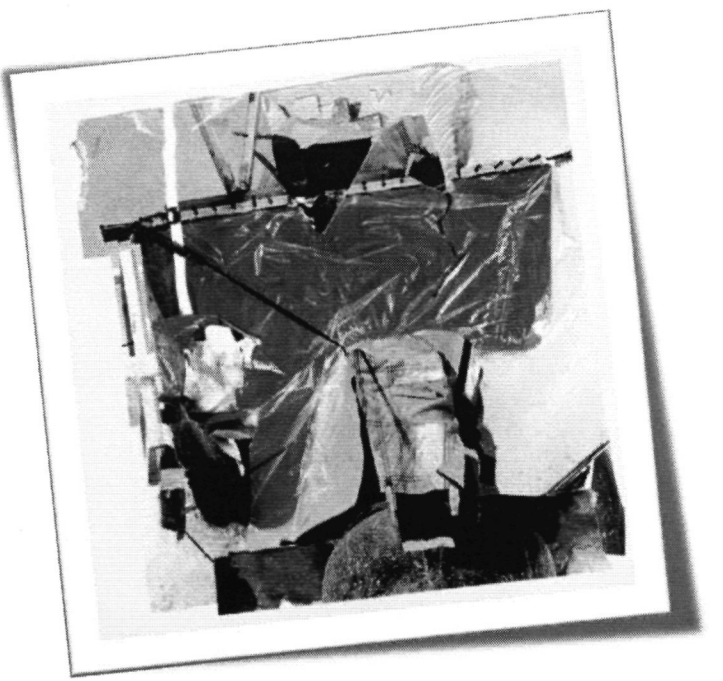
Th1 and Th17 responses in disseminated candidiasis are critically dependent on caspase-1 and ASC

Submitted



van de Veerdonk FL, Joosten LA, Shaw PJ, Malireddi S, Smeekens SP, van der Meer JW, Kullberg BJ, Netea MG, Kanneganti TD.

Summary

The Nlrp3 inflammasome has been proposed to play an important role in antifungal host defense. However, the in-vivo role of the inflammasome component ASC in disseminated candidiasis has not been studied, and previous studies examining the role of caspase-1 in anti-*Candida* host defense are contradictory. Furthermore, the main mechanisms controlled by the inflammasome that are responsible for antifungal host defense have not yet been elucidated. In the present study we demonstrate an essential role for caspase-1 and ASC in disseminated candidiasis through regulating antifungal Th1 and Th17 responses. These observations identify a critical role for the inflammasome in controlling protective adaptive immune responses during invasive fungal infection.

Introduction

The available treatment options have not reduced the mortality and morbidity associated with invasive *Candida* infections over the recent years, and a better understanding of the host defense against *Candida* is crucial to develop new strategies that will improve clinical outcome in patients with disseminated candidiasis. IL-1β and IL-18 play an important role in anti-*Candida* host defense and these cytokines are specifically controlled by the inflammasome, a multimeric protein complex composed of ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain), caspase-1 and one or more nucleotide-binding domain leucine-rich repeat receptors (NLRs) (1). The NLRs serve as danger sensors which connect upstream signals to caspase-1 activation (2).

Recently, it has been reported that Nlrp3 is the crucial NLR family member that connects *Candida* recognition to caspase-1 activation. Mice deficient in Nlrp3 were highly susceptible to disseminated candidiasis (3) and mucosal candidiasis (4). It has therefore been suggested that the inflammasome is crucial for antifungal host defense. However, the in-vivo role of the inflammasome component ASC has not been studied in disseminated candidiasis, and previous studies examining the role of caspase-1 in anti-*Candida* host defense are contradictory (5). In the present study we have addressed the role of the inflammasome in invasive candidiasis by studying the susceptibility of caspase-1, ASC, or P2X7 deficient mice to disseminated candidiasis, and observed that caspase-1 and ASC, but not P2X7, have strong protective antifungal capacities by controlling Th1 and Th17 responses during disseminated candidiasis.

Results and discussion

Caspase-1$^{-/-}$ and ASC$^{-/-}$, but not P2X7$^{-/-}$ mice, are more susceptible to disseminated candidiasis

Recent studies suggest that the production of IL-1β in response to *C. albicans* is critically dependent on the Nlrp3 inflammasome (3). However, the essential role of Nlrp3 and caspase-1 in anti-*Candida* host defense is controversial (5, 6). Because of all these inconsistencies in the literature concerning this potentially important anti-*Candida* host defense mechanism, we aimed to assess the role of the inflammasome during disseminated candidiasis using knock-out animals deficient in the crucial components of the inflammasome; caspase-1 and ASC. Twenty two days after intravenous injection of $2x10^5$

colony forming units (CFU) of *C. albicans*, 83% of the WT mice survived the infection (Figure 1A). When caspase-$1^{-/-}$ mice were infected, they showed a higher susceptibility to disseminated candidiasis, with 50% overall survival after 22 days of infection (Figure 1A). $ASC^{-/-}$ mice also showed a higher mortality than WT mice with 54% survival of $ASC^{-/-}$ mice 22 days after infection (Figure 1B).

Mice with hematogenously disseminated candidiasis die because of progressive sepsis (7). Notably, kidney fungal burden is correlated with severity of renal failure and systemic acidosis, which are hallmarks of severe sepsis (7). We therefore investigated the fungal loads in the kidneys of the WT mice and knockout mice. In line with the higher susceptibility to disseminated candidiasis demonstrated by the survival experiments, caspase-$1^{-/-}$ mice had a 62-fold increase, and ASC-/- mice had a 13-fold increase in fungal load on day 7 compared to wild-type mice (Figure 1C). Histology showed that large amounts of *C. albicans* had accumulated on day 7 of infection in the collecting ducts of caspase-$1^{-/-}$ mice that directly invaded the tissue (Figure 1D), which was not observed in the WT mice. These findings are different from a previous study which reported that caspase-1 deficiency had no critical effect on primary disseminated candidiasis (5). In the latter study however, infection with a virulent *C. albicans* strain resulted in 100% mortality in both the control group and caspase-1 deficient mice, while infection with an avirulent *C. albicans* strain resulted in almost 100% survival in both groups. This makes it difficult to draw conclusions about the susceptibility of the caspase-1 deficient mice during these infections, as no intermediary (LD50) mortality rates were performed in any of the experiments. Overall our data clearly demonstrate an important role for the inflammasome components ASC and caspase-1 in antifungal host defense against disseminated candidiasis.

Processing of IL-1β and IL-18 by the inflammasome in macrophages and DCs requires two signals. One signal such as LPS is needed to provide the substrate, pro-IL-1β or pro-IL-18, and a second signal is required to activate the caspase-1. A second signal that is able to induce caspase-1 activation is ATP, and this effect is dependent on the ATP membrane receptor P2X7. Although P2X7 could therefore be crucial in the induction of the inflammasome in vivo it did not play a major role in disseminated candidiasis, since there was no significant difference in survival or fungal outgrowth compared to WT mice (Figure 1E). Furthermore, *C. albicans* was able to induce ASC-dependent caspase-1 activation in BMDC without the need of ATP (Figure 1F). Therefore, the P2X7 receptor is redundant for *Candida*-induced activation of the inflammasome in vitro and in vivo.

Caspase-1 and ASC deficiency results in impaired Th1 and Th17 responses

Caspase-1, the essential enzyme of the inflammasome, is able to cleave the precursors pro-IL-1β an pro-IL-18 into their mature active forms (8, 9). IL-1β plays an important role in the induction of the Th17 response, which is characterized by the production of IL-17, a cytokine that is important for recruiting neutrophils and maintaining optimal neutrophil responses at the site of infection (10). Th17 responses are crucial for protective anti-*Candida* host defense, since mice that are deficient in the IL-17 signaling pathway are highly susceptible to disseminated candidiasis (11), and patients that are deficient in Th17 responses are highly susceptible to mucosal candidiasis (12).

We therefore tested the hypothesis that the inflammasome could play an important role in the induction of a protective antifungal Th17 response. We observed that splenocytes from mice deficient in caspase-1 or ASC that were infected with *Candida*, had an impaired IL-1β response and were unable to mount normal production of IL-17 after restimulation with *Candida* (Figure 2A). Histology supported these findings since we observed remarkable little influx of neutrophils in the caspase-1 deficient mice (Figure 1D). IL-17 concentrations and levels of bioactive IL-1 were also significantly lower in vivo at the site of infection (Figure 2B). Our data are in contrast with a recent report suggesting that the Nlrp3 inflammasome has no effect on Th17 responses. However, this study only investigated the impact of Nlrp3 deficiency on Th17 responses, but not the impact of caspase-1, ASC, or P2X7 deficiency.

IL-18 production is controlled by the inflammasome and is necessary for an optimal Th1 response. Th1 responses are characterized by IFNγ production, and IFNγ plays an important role in disseminated candidiasis (13, 14). In line with these reports, we observed an impaired Th1 response in response to *C. albicans* on day 7 of infection by splenocytes isolated from caspase-1-/- and ASC-/- mice (Figure 2A). IFNγ levels in the kidney homogenates were also significantly lower in the caspase-1-/- mice (Figure 2B). Although IL-1β, IFNγ and IL-17 were reduced in the caspase-1 and ASC deficient mice, the production of the inflammasome-independent cytokine TNFα was not reduced in the caspase-1 deficient mice and was even significantly elevated in the ASC-/- mice (Figure 2A). It is unknown why the TNF responses differ between ASC-/- and caspase-1-/- splenocytes and this is currently under investigation.

Interestingly, we and others have observed that during early infection (day 3), the caspase-1 deficient splenocytes did not have an impaired IL-1β or IFNγ production when re-stimulated with *C. albicans* (data not shown, (5)). These observations suggest that during the early stages of infection proIL-1β and pro-IL-18 can be processed without the need of inflammasome induction and subsequently caspase-1 activation. Most likely the activity of neutrophil-derived serine proteases, which can also cleave pro-IL-1β and pro-IL-18, is responsible for these effects (15, 16).

We observed that the inflammasome specifically controls optimal IL-1β and IL-18 concentrations during the later stages of infection, a time point where Th1 and Th17 responses are critical for protective immunity. This would indicate that the inflammasome is a critical link between the innate and adaptive immune response during disseminated candidiasis.

A recent study defined the protective mechanisms of immunity during *Candida albicans* bloodstream infections in mice immunized with the recombinant N-terminus of Als3p (rAls3p-N) vaccine plus aluminum hydroxide (alum) (17). Interestingly, vaccination resulted in protective Th1 and Th17 responses that enhanced phagocytic killing of *C. albicans*, increased neutrophil influx, and decreased fungal burden in tissues. Since alum activates caspase-1 (18), it is tempting to speculate that adjuvant alum modulated the immune response towards protective Th1 and Th17 responses by activating the inflammasome, which would be in line with our findings.

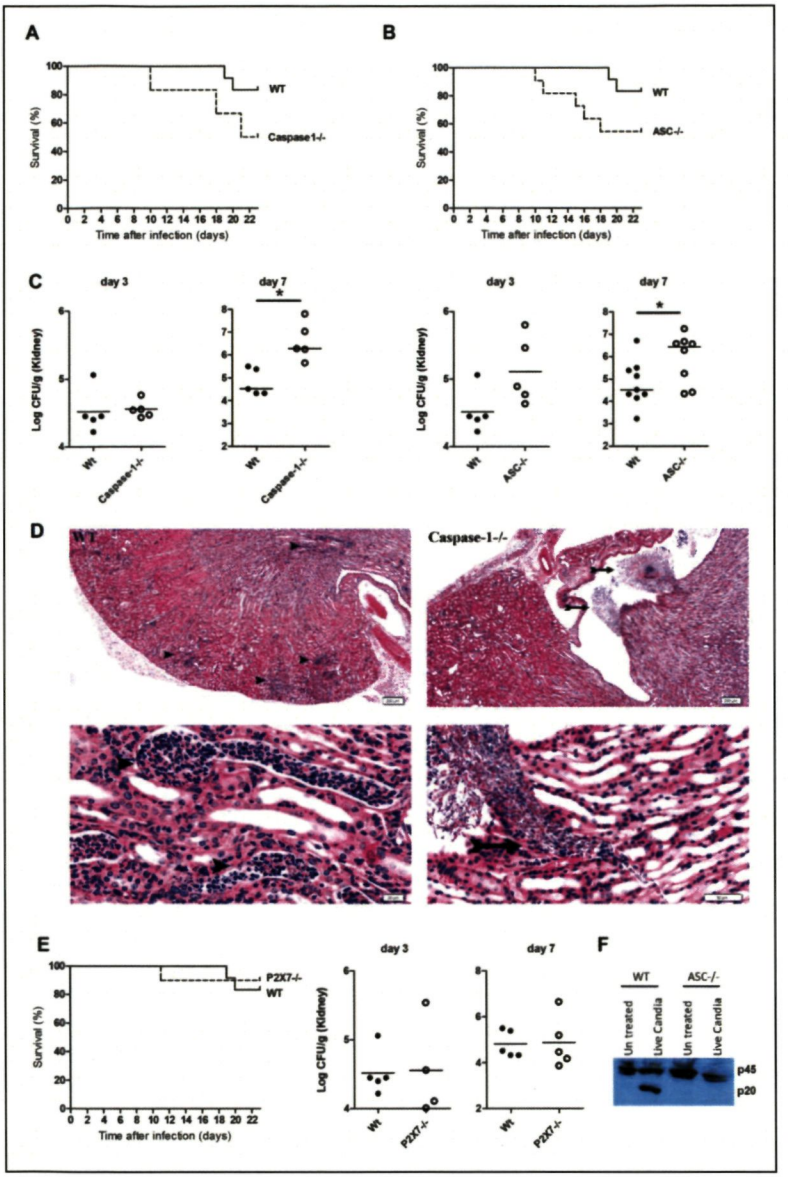


Figure 1. Caspase-1$^{-/-}$ and ASC$^{-/-}$, but not P2X7$^{-/-}$ mice, are more susceptible to disseminated candidiasis. (A) Kaplan-Meier survival plots of WT (n=10), caspase-1-/- (n=6) (B) Kaplan-Meier survival plots of WT, ASC-/-, n=10 mice per group. (C) Fungal burden of kidneys of WT, Caspase-1-/-, and ASC-/- mice at day 3 and 7 after infection. n≥ 5 mice per group. (D) Histopathologic assessment of the kidneys of WT and caspase-1 -/- mice 7 days after intravenous injection with 2x10^5 CFU *C. albicans*. Kidneys from caspase-1-/- mice show *C. albicans* hyphae invading the tissue in the presence of little inflammation. Arrowheads mark neutrophil infiltrates and full arrows mark *Candida* hyphae. (E) Kaplan-Meier survival plots of WT and P2X7-/- mice, n=10 mice per group. (F) Fungal burden of kidneys of WT and P2X7-/- mice at day 3 and 7 after infection. n=5 mice per group. (G) Lysates from bone marrow-derived dendritic cells (BMDCs) from WT and ASC-/- mice were collected 4 hours after exposure to 1x10^4 CFU *C. albicans/*ml, and immunoblotted with anti-caspase-1 antibody. p45 indicates procaspase-1 and p20 processed caspase-1.

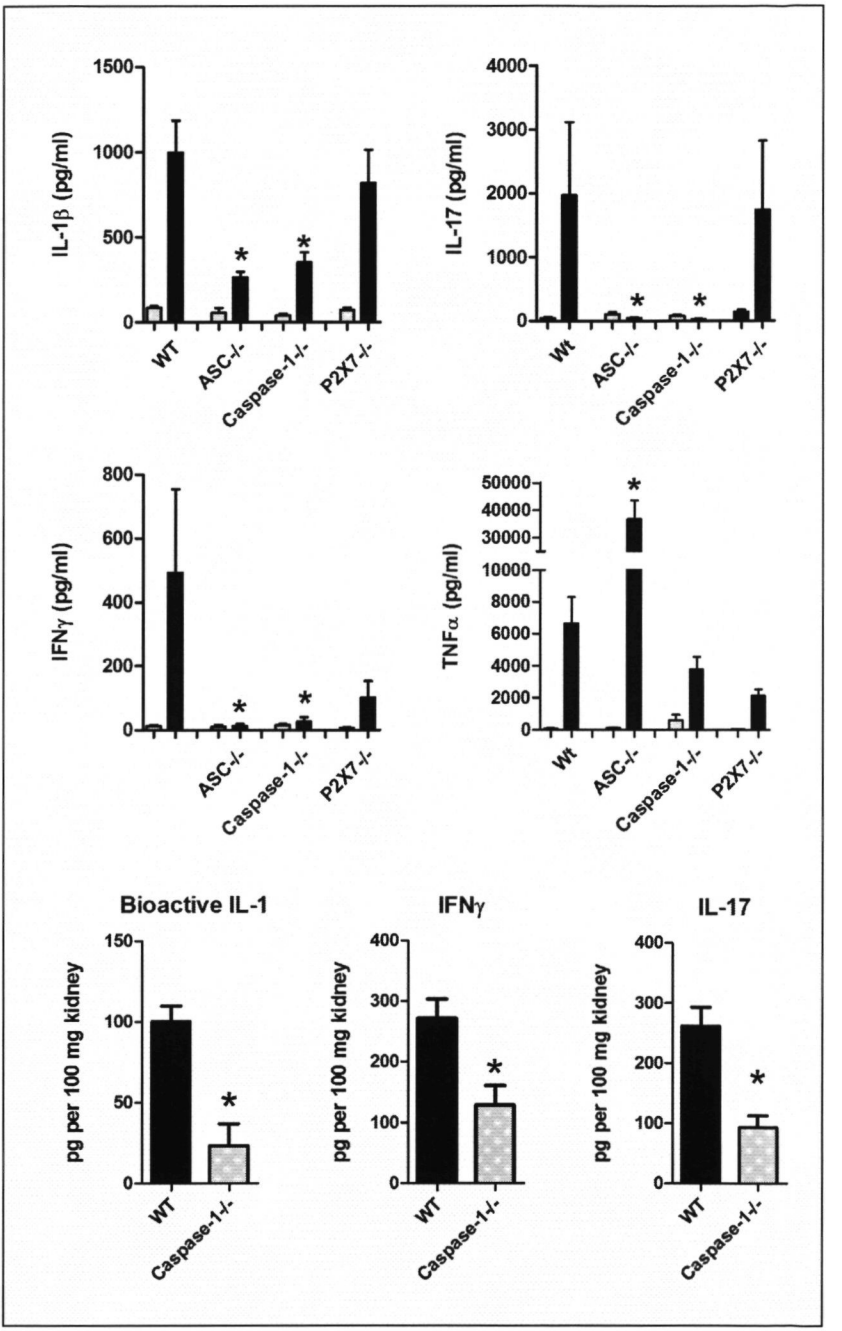


Figure 2. Cytokines in ASC, caspase-1 or P2X7 deficient mice.

(A) Splenocytes from WT, ASC-/-, caspase-1-/-, and P2X7-/- mice were re-stimulated with RPMI (grey bars) or heat-killed $1x10^6$ *C. albicans* hyphae cells/ml (black bars) 7 days after intravenous infection with *C. albicans*. Cytokines were measured 48 hours after stimulation with ELISA. *p<0.05. n=5 mice per group. (B) IFNγ, IL-17 and bioactive IL-1β were measured in the kidney homogenates from WT and caspase-1-/- mice 7 days after intravenous infection with *C. albicans*. n=5 mice per group.

Conclusions

An increasing interest has been elicited in the role of the inflammasome for the host defense against *Candida* infections. In the present study we have dissected the contribution of the inflammasome components caspase-1, ASC and P2X7 to the host defense against disseminated candidiasis. The data reported demonstrate that caspase-1 and ASC are crucial for anti-*Candida* host defense during disseminated candidiasis by promoting optimal antifungal protective Th1/Th17 responses.

Materials and Methods

Mice and bone marrow derived dendritic cells (BMDCs)
ASC-/-, caspase-1-/-, and P2X7-/- mice backcrossed to C57BL/6 background for at least 10 generations have been described before (19, 20). Bone marrow was prepared from the leg bones of 8–20-week-old mice. The legs were dissected, and the bone marrow flushed out. DCs were differentiated from bone marrow cells cultured with RPMI-1640 supplemented with 20ng/ml GM-CSF along with 10% heat inactivated fetal bovine serum (Invitrogen), 100 U/ml penicillin and 100 mg/ml streptomycin at 37 °C in 5% CO2 for 7 days. Mice were housed in a pathogen-free facility.

C. albicans growth conditions
C. albicans ATCC MYA-3573 (UC 820), a strain well described elsewhere (21), was used in all experiments. *Candida* was grown overnight in Sabouraud broth at 37°C, cells were harvested by centrifugation, washed twice, and resuspended in culture medium in culture medium (RPMI-1640 Dutch modification, ICN Biomedicals, Aurora, OH) (22). For in-vitro experiments, *C. albicans* was heat-killed for 1h at 100°C. To generate pseudohyphae, *C. albicans* blastoconidia were grown at 37°C in culture medium, adjusted to pH 6.4 by using hydrochloric acid. Pseudohyphae were killed for 1h at 100°C and resuspended in culture medium to a hyphal inoculom size that originated from 10^6/ml blastoconidia (referred to as 10^6/ml pseudohyphae) (22).

C. albicans infection model
Knock-out mice and WT mice were injected intravenously with *C. albicans* blastoconidia (2 x 10^5 CFU/mouse) in a 100 µl volume of sterile pyrogen-free phosphate-buffered saline (PBS). Survival was assessed daily for 22 days. Subgroups of 5 animals were killed on days 3 or 7 of infection. To assess the tissue outgrowth of the microorganisms, the kidneys of the sacrificed animals were removed aseptically, weighed, and homogenized in sterile saline in a tissue grinder. The number of viable *Candida* cells in the tissues was determined by plating serial dilutions on Sabouraud dextrose agar plates as previously described (23). The CFU were counted after 24h of incubation at 37°C, and expressed as log CFU/g tissue. For histologic analysis, kidneys of subgroups of mice (5 mice/group) were fixed in buffered formaldehyde (4%). Paraffin-embedded sections were stained with hematoxylin-eosin.

In vitro cytokine production by primed splenocytes
To assess cytokine production, primed spleen cells from mice on day 7 of infection with $2x10^5$ CFU of *C. albicans* per mouse were stimulated in vitro with heat-killed Candida conidia or pseudohyphae ($1x10^6$ microorganisms/ml). Spleen cells were obtained by gently

squeezing spleens in a sterile 200 mm filter chamber. The cells were washed and resuspended in RPMI1640, counted in a Bürker counting chamber and the number was adjusted to $5x10^6$/ml. 500 µL of the cell suspension was stimulated with $1x10^6$ heat killed *C. albicans*/ml. Measurement of IFNγ, IL-1β, IL-17, and TNFα concentrations was performed in supernatants collected after 48 h of incubation at 37 1C in 5% CO2 in 48-well plate.

Cytokine quantification
A Bioplex mouse X-plex assay (BioRad) was used according to the manufacturer's instructions in order to evaluate the quantity of the following cytokines: IL-1β, IFN-γ, and IL-17. Bioactive IL-1 secretion was determined in a bioassay using the murine thymoma cell line EL4/NOB1 as an IL-1-specific cell-producing IL-2 response. IL-2 was subsequently determined by ELISA (R&D).

Immunoblotting for caspase-1
BMDCs were first stimulated for 4 hours with live *C. albicans* in a concentration of $1x10^4$ CFU/ml, washed twice with phosphate-buffered saline and scraped in lysis buffer solution (150 mm NaCl, 10 mm Tris, pH 7.4, 5 mm EDTA, 1 mm EGTA, 0.1% Nonidet P-40) supplemented with a protease inhibitor mixture tablet (Roche Applied Science). Samples were clarified, denatured with SDS buffer, and boiled for 5 min. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were immunoblotted with primary antibodies and proteins detected with appropriate secondary anti-rabbit antibody conjugated to horseradish peroxidase followed by enhanced chemiluminescence. Rabbit anti-mouse caspase-1 was a generous gift from Dr. P. Vandenabeele (Gent University, Belgium).

Statistical analysis
Data were analyzed using GraphPad software. The differences between groups were analyzed by the Mann-Whitney U test or Student's t-test where appropriate. Comparison of two survival curves was done using the Logrank test. The level of significance between groups was set at $p<0.05$.

Acknowledgements

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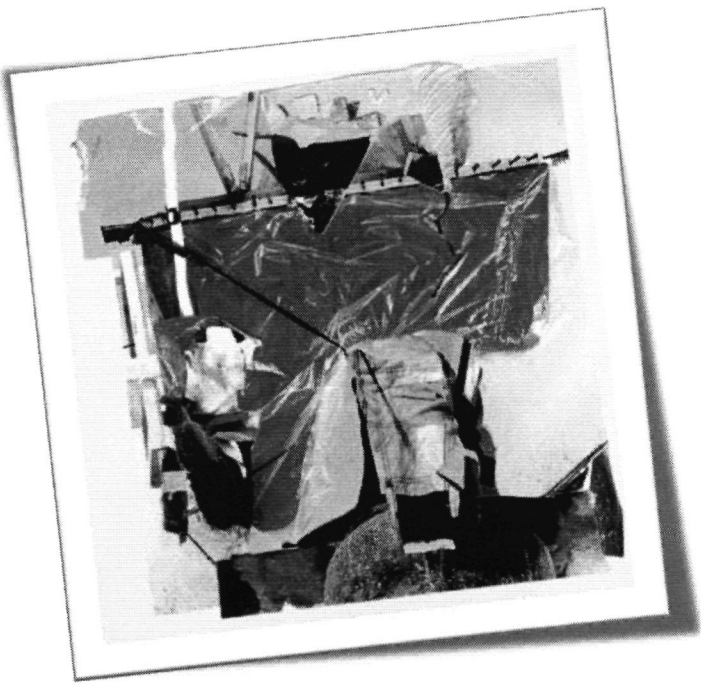
Th17 and antifungal host defense



Chapter 9

Th17 responses and host defense against microorganisms: an overview

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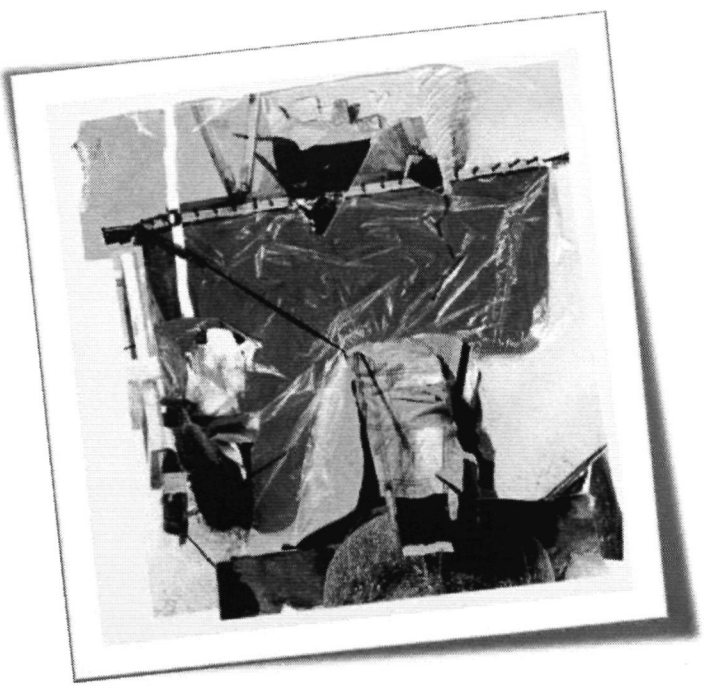

van de Veerdonk FL, Gresnigt MS, Kullberg BJ, van der Meer JW, Joosten LA, Netea MG.

Summary

T helper (Th) 17 cells have recently been described as a third subset of T helper cells, and have provided new insights into the mechanisms that are important in the development of autoimmune diseases and the immune responses that are essential for effective antimicrobial host defense Both protective and harmful effects of Th17 responses during infection have been described In general, Th17 responses are critical for mucosal and epithelial host defense against extracellular bacteria and fungi However, recent studies have reported that Th17 responses can also contribute to viral persistence and chronic inflammation associated with parasitic infection It has become evident that the type of microorganisms and the setting in which they triggers the Th17 response determines the outcome of the delicate balance that exists between Th17 induced protection and immunopathogenesis

Introduction

Mosmann and Coffman have introduced the concept of different sets of T helper (Th) cells, namely Th1 cells and Th2 cells (1) Th1 cells are characterized by the production of interferon-γ, which is essential for the defense against intracellular pathogens Th2 cells are characterized by the production of interleukin (IL)-4 and are important in the host defense against parasitic infections Recently, a new subset of T helper cells called Th17 cells has been described and these cells are characterized by the production of IL-17 (2), which is important for neutrophil recruitment and host defense against extracellular bacteria and fungi Th17 cells produce a distinct cytokine profile, namely IL-17A (IL-17), IL-17F, IL 21 and IL-22 Like Th1 and Th2 cells, the development of Th17 cells from naive T cells is dependent on antigen presentation by professional antigen presenting cells, co-stimulatory stimulation, and a specific cytokine milieu In summary, the cytokines IL-1β, IL-6, TGFβ have been reported to induce the development of Th17 cells and IL-23 has been reported to be important for the maintenance of Th17 cells, whereas IL-12 is important for Th1 differentiation and IL-4 drives activated naive T cells towards Th2 cells (3) (Figure 1) It is important to mention that the cytokine IL-12 is composed of the subunits IL-12p40 and IL-12-p35, and the cytokine IL-23 is composed of IL-12-p40 and IL-23p19 Therefore IL-23p19 deficient mice are generally used as a model to study the role of IL-23, and IL-12p35 deficient mice are used to investigate IL-12 dependent mechanisms of disease The functions of Th17 cells, called Th17 responses, in infectious diseases and autoimmune diseases have only recently started to be elucidated It has become apparent that Th17 responses are associated with chronic inflammation and autoimmune diseases such as multiple sclerosis and rheumatoid arthritis (4) Furthermore, Th17 responses have been shown to be important for the host defense against many microorganisms, although they can also contribute to immunopathology during infection In this review we will provide an overview of the rapidly extending literature that has investigated the role of Th17 responses in relation to viral, bacterial, fungal and parasitic infections

Th17 responses and infections in STAT3 deficient patients

STAT3 deficiency in humans, demonstrated to be the cause hyperIgE syndrome, has provided crucial insights in the role of Th17 responses in the setting of antimicrobial host

defense. STAT3 deficient patients suffer from *S. aureus* skin and pulmonary infections and mucocuteneous candidiasis (5). The cause of these complications has been linked to a defective Th17 response against *C. albicans* and gram positive bacteria, such as *S. aureus* and *S. pyogenes* (6). Intriguingly, defective Th17 responses were also seen with mitogenic stimulation of CD4 T helper cells from these patients, suggesting a severe defect in mounting an optimal Th17 response against many stimuli. These observations provide strong evidence that Th17 responses are needed to control fungal colonization at the mucosal level, and play an important role in host defense against extracellular bacteria, especially *S. aureus,* in the lung and skin.

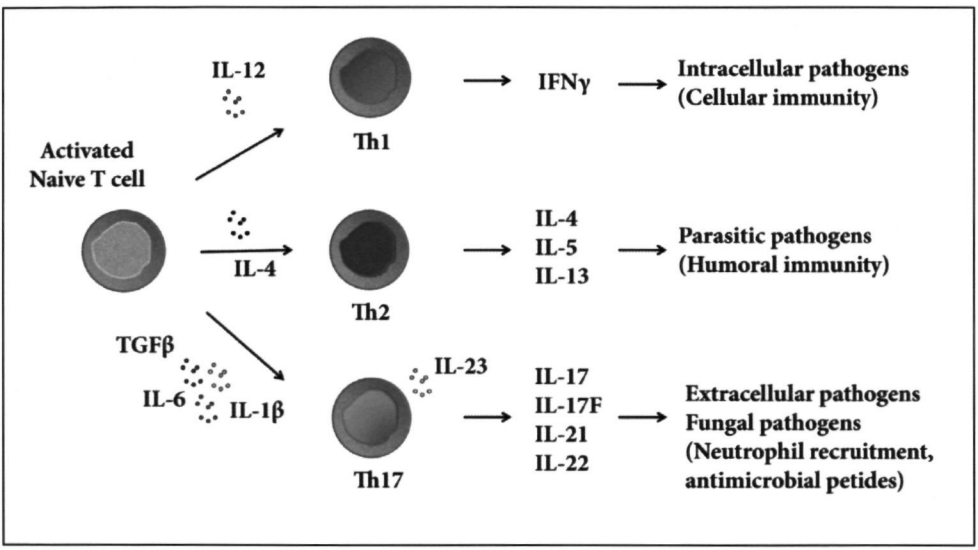

Figure 1. Different types of T helper (Th) subsets.
The cytokine profile which is responsible for the differentiation from activated naïve T cells into the three distinct Th subsets is shown. The main effector cytokines of and functions for each Th subset is shown.

Fungal infections

Candida

Involvement of Th17 responses in protective antifungal host defense was first demonstrated in IL-17RA deficient mice that showed increased susceptibility to disseminated *C. albicans* infection (7). In addition, a model for oropharyngeal candidiasis in mice showed that IL-23p19 or IL-17 deficiency resulted in severe oropharyngeal candidiasis, while mice deficient for IL-12p35 did not show this (8). However, negative effects of Th17 mediated inflammatory responses to intragastric *C. albicans* and intranasal *Aspergillus fumingatus* infection in mice have also been reported. IL-23p19 deficiency was shown to reduce fungal burden and IL-17 inhibited Th1 responses to *C. albicans* (9). Furthermore, mice with defects in Toll IL1R8 (TIR8), a negative regulator of Th17 responses, showed higher susceptibility to *Candida* and *Aspergillus* and more immunopathology (10). Differences in the animal model, *Candida* strains and mouse strains could account for these contradictory observations. However, patients with impaired *Candida* specific Th17 responses such as patients with hyper IgE

syndrome or chronic mucocutaneous candidiasis (CMC), are highly susceptibility to mucosal *C. albicans* infections (5, 11). These observations strongly indicate that Th17 responses are important for human anti-*Candida* mucosal host defense. Th17 induction in response to *Candida* infection is strongly dependent on C type lectin receptors (CLRs), since it has been shown that the CLRs: mannose receptor, dectin-1 and dectin-2 are important in the IL-17 production in response to *Candida* (12-14). Furthermore, dectin-1 and dectin-2 signal through the Syk-CARD9 pathway, and CARD9 was shown to be involved in *Candida*-induced Th17 responses (13). Recently, it has been reported that patients with genetic defects in dectin-1 or CARD9 suffer from chronic oychomycosis and mucosal fungal infection (15, 16). Both dectin-1 and CARD9 deficiency was shown to result in deficient fungal induced IL-17 responses. These data further strengthen the crucial role of Th17 cells in human antifungal mucosal defense.

Other fungi

Patients with chronic granulomatous disease (CGD) lack NADPH oxidase activity and do not generate reactive oxygen species, which results in recurrent bacterial and fungal infections especially fungal infections with *Aspergillus*. It has been reported that in the setting of deficient reactive oxygen species generation in a mouse model of CGD, the tryptophan metabolism in mice is deficient, which eventually resulted in increased IL-17 responses (17). These increased IL-17 responses were suggested to be detrimental to the host when CGD mice were infected with *Aspergillus*. It has been suggested that the IL-17 pathway also plays a role in *Pneumocystis carinii* infections. Stimulation of alveolar macrophages with *Pneumocystis carinii* induces IL-23 mRNA. Furthermore, neutralization of IL-23 or IL-17 increased disease severity during *P.carinii* infection in WT mice (18). Another important fungal pathogen is *Cryptococcus neoformans,* which is associated with HIV infection and causes lethal fungal meningoencephalitis in patients suffering from AIDS. Mice deficient of IL-12p40 show much higher mortality and had impaired IL-17 expression when compared to IL-12p35 deficient mice (19). This indicates a role for IL-23 and thus Th17 responses in protection against *C. neoformans* infection. Another study that investigated the role of the Th17 response in antifungal host defense reported that in a mice model of *Paracoccidioides braziliensis* infection, TLR2 deficiency resulted in increased Th17 responses which were associated with protection (20). Overall, these data indicate that Th17 responses can be induced by fungi and that the IL-17 pathway plays an important role in protective antifungal host defense.

Th17 responses in bacterial infections

Borrelia

One of the first studies that reported pathogen specific induced IL-17 production showed that *Borrelia burgdorferi* and *Mycobacterium bovis* BCG lipopetides were able to induce IL-17 in addition to IFNγ in CD4 positive T helper cells (21). Furthermore, it was shown that these responses were dependent on antigen presentation (21). Another study reported that *B.burgdorferi* triggers bone marrow derived DC's to produce IL-23, causing stimulation of IL-17 producing T-cells (22). Codolo et al. presented that *B.burgdorferi* neutrophil-activating protein A (NapA) is capable of generating increased IL-6, IL-1β, IL-23, and TGF-β expression by cells of the innate immune system, thereby inducing Th17 responses in human synovial fluid (23). Notably, in an animal model of *Borrelia*-induced destructive arthritis, anti-IL-17

and anti-IL-17R treatment resulted in prevention of severe destructive arthritis (24) In addition, it was shown that IL-23, which is a survival factor for Th17 cells, was associated with Lyme arthritis, and depletion of IL-23p19 or blockade of IL-23 resulted in the absence of *Borrelia* induced arthritis in mice (25) These studies provide evidence that while Th17 effector functions are likely to be involved in the host defense against *Borrelia spp* , they also play an important role in mediating the strong immunopathology associated with chronic *Borrelia* infection

Helicobacter pylori

IL-17 has been shown to be increased in *H pylori*-infected gastric mucosa and stimulated the synthesis of IL-8 which is a strong chemoattractant for neutrophils (26) Another study provided evidence that STAT3 activation in lamina propria mononuclear cells could lead to sustained IL-17 production, resulting in persistent inflammation (27) In an experimental mouse model, IL-17 neutralization resulted in an increased Th1 response with elevated IL-12, TNF-α and IFN-γ mRNAs levels, while replenishing IL-17 showed reduction of the Th1 response (28) This underscores that counter-regulation between Th1 and Th17 responses is present during infection As with many other infections, it remains to be determined which response is protective for host defense against *H pylori* and which response will lead to persistent infection that could be detrimental to the host Notably, it has been shown in a recent publication that IL-17RA signaling regulates gastric B cell recruitment (29) This indicates that IL-17 has an important role in the orchestration of immune cells such as neutrophils and B cells at the site of infection during *H pylori* infection

Klebsiella pneumonia

IL-17 deficient mice suffer from lethal *K pneumoniae* infection in contrast to control mice (30) In addition, overexpression of IL-17 leads to increased IL-1β, TNF-α and MIP-2 and G-CSF, which results in higher leukocyte numbers and increased clearance of infection (30) Experimental *K pneumoniae* infection in mice was reported to induce IL-23p19 and subsequently IL-17 production (31) Furthermore, the importance of the Th17 cytokines for protection against *K pneumonia* infection was shown in mice deficient in IL-12p40, IL-23p19 or IL-17R, which suffer from higher susceptibility and mortality (32) Furthermore, IL-17 administration was able to restore the normal host defense against *K pneumoniae* in IL-23 deficient mice (32) Both IL-17 and IL-22 were shown to be crucial for protective local host defense against *K pneumonia* However, only IL-22 was crucial for defense against transepithelial damage, since in contrast to IL-17, IL-22 was able to enhance repair of transepithelial resistance Interestingly, it was shown that blocking IL-22 in mice infected with *K pneumonia* resulted in 100% mortality within one day, which was significantly earlier than control mice or IL-17 deficient mice Furthermore, IL-22 blockade resulted in increased dissemination of bacteria in both control mice and IL-17 deficient mice These data argue that IL-22 plays a more important role than IL-17 in mucosal host defense against *K pneumonia* A recent study has reported that lipocalin-2 is important for mucosal host defense against *K pneumonia* Although IL-17 can induce Lcn2 in vitro, it was not necessary for in vivo induction (33, 34) IL-22 is also able to induce Lcn2 in vitro (33) However, to what extend IL-22 plays a role in the induction of Lcn2 in vivo remains to be determined In conclusion, Th17 responses provide protective host defense against *K pneumonia*

Citrobacter rodentium

Infection of IL-23p19 deficient mice with *Citrobacter rodentium* showed reduced survival rates due to increased bacterial dissemination as a result of impaired Th17 responses (35). Also, a role for IL-17A and IL-17F induced β-defensin expression in the defense against *C. rodentium* has been described (36). In addition, by using the *C. rodentium* as a mouse model for human intestinal infection, Zheng et al. hypothesized that the influence of the Th17 cytokine IL-22 also plays an important role in human intestinal infection (37). Interestingly, it has recently been shown that Th17 differentiation in the small intestine is dependent on specific commensal flora (38). A single microbe, namely a segmented filamentous bacterium, was responsible for inducing Th17 responses in the lamina propria of the small intestine (39). Colonization with this specific commensal resulted in increased Th17 responses, which were protective during experimental infection with *C. rodentium* (39).

Salmonella spp.

Salmonella enterica serotype Typhimurium can induce Th17 responses in the intestinal mucosa in mice (40). Another study showed that *Salmonella enterica* serotype Typhimurium infection resulted in increased levels of IL-17, IL-22 and IL-23, and induction of MIP-2 and Lcn2 genes in the intestine (41). These responses were mainly driven by IL-23(41). In the setting of IL-12 deficiency, IL-23 dependent IL-22 was shown to be crucial in protection against disseminated infection with *Salmonella enterica* serotype Enteritidis, while IL-17 was redundant in this model (42). However, it has also been shown that IL-17 deficient mice had slightly higher bacterial load in liver and spleen when compared to control mice in this model of disseminated *Salmonella* infection (42). During *Salmonella enterica* serotype Typhimurium infection, IL-17 deficiency in mice resulted in impaired neutrophil recruitment to the intestinal mucosa (43). It has been reported that HIV-infected patients are more susceptible to non-typhoid *Salmonella* bacteremia (44). IL-17 deficiency caused by Simian immunodeficiency virus (SIV) in macaques, which is the primate variant of HIV, resulted in increased translocation of *Salmonella enterica* serotype Typhimurium (43). Interestingly, *S. typhimurium* infection in SIV positive macaques caused significant less IL-17 and IL-22, whereas IFNγ production was normal. These data indicate that Th17 responses play an important role in controlling mucosal host defense against non-thyphoid *Salmonella* and protection against disseminated salmonellosis. In addition, *S. enterica* serotype Typhi can inhibit Th17 responses, which probably contributes to the higher virulence associated with this *Salmonella* spp. (40).

Mycobacteria

M. bovis was one of the first microorganisms that was able to induce IL-17 production in CD4 positive T cells (21). In addition, dendritic cells that are stimulated with *M. tuberculosis* produce IL-12 and IL-23 (45). IL-23 was shown to be crucial for the induction of Th17 responses against *M. tuberculosis* and *M. Bovis* (46). IL-23 has also been shown to be important for *M. tuberculosis*-induced Th1 responses (45). Interestingly, it has been reported that when the IL-17A receptor is absent in mice, no difference in clearance of *M. tuberculosis* infection was observed compared to control mice (47). Th1 cells seem to be more important in the protection against primary *M. tuberculosis* infection, while the absence of Th17 cells does not alter protection against primary infection (48). These data are supported by the absence of mycobacterial infections in patients with hyperIgE syndrome. Although IL-17 does not appear to play a role in primary TB infection, it may play a role in the maintenance

of the inflammatory response. In line with this, granuloma formation in the lungs of IL-17 deficient mice infected with BCG was reported to be impaired and IL-17 was shown to play a role in the trafficing of Th1 cells to the site of infection (49). These data suggest that Th17 responses play an important role in providing long lasting immunity against *M. tuberculosis*, and could therefore be crucial for vaccine development against *M. tuberculosis*.

Bordetella spp.
Although earlier studies have reported that *B. pertussis* infection promotes the Th1 response (50), more recent studies have shown that *B. pertussis* is also able to skew the host response towards a Th17 profile (51). Blocking of IL-17 during *B. pertussis* infection in mice resulted in reduced neutrophil recruitment and modestly increased bacterial burden (51). In addition, *B. pertussis* toxin is able to induce IL-17 responses (52). *B.bronchiseptica* is also able to skew the immune response towards a Th17 response (53). Interestingly, lung tissue from mice infected with *B.bronchiseptica* expressed a strong Th17 response. It remains however to be established if Th17 responses contribute to host defense against *Bordetella spp*.

Other bacteria
Porphyromonas givinalis is an anaerobic bacterium which causes periodontal disease (PD). This is associated with periodontal bone destruction. It has been reported that patients with severe PD have elevated IL-17 responses (54). Despite this observation and the observations that Th17 responses have been associated with bone destruction and the induction of Lyme arthritis, it has been reported that the Th17 response induced by *P. gingivalis* infection prevented bone destruction in mice (55). In case of *Mycoplasma pneumonia* it has been reported that this pathogen triggered alveolar macrophages to produce IL-23, which subsequently contributed to an increase of IL-17 production (56). Depletion of IL-23 resulted in less IL-17 production and reduced lung neutrophil recruitment (56).

However, Th17 responses during bacterial infection are not always beneficial for the host. Patients with cystic fibrosis (CF), which have defects in a chlorine channel which contributes to thick mucus production in the lungs (57), are more susceptible to *Pseudomonas aeruginosa* infection. CF patients with *P. aeruginosa* infection had higher levels of the Th17 cytokines IL-23 and IL-17 (58). An important observation made by others was that mice deficient in IL-23 showed decreased inflammation compared to wild type mice, although they had the same amount of *P. aeruginosa* dissemination (59). These data suggest that the Th17 response against *P. aeruginosa* does not play a crucial role in the host defense against this pathogen, but that it contributes to the pathology of the airways which leads to bronchiectasis seen in CF patients. It must however also be mentioned that IL-17 was recently shown to be a critical factor in a vaccine that induced protection to *P.aeruginosa* (60). Another study has shown that mice injected intraperitoneal with *B. fragilis* formed abscesses in an IL-17 dependant way, and that co-localization of IL-17 producing CD4 positive cells within the abscess wall was shown (61). Furthermore, when these mice were treated with an IL-17 neutralizing antibody, the formation of these abcesses was blocked. These studies suggest that Th17 responses during certain types of bacterial infections can result in deleterious host effects.

Patients with hyperIgE syndrome are especially susceptible to *S. aureus* (6, 62-64). It has recently been reported that human keratinocytes and bronchial epithelial cells were

especially dependent on Th17 cytokines for their anti-staphylococcal host defense, such as secretion of chemokines that recruit neutrophils and the production of antimicrobial peptides (64). In addition, an association between the severity of the defective Th17 response against *S. aureus* and the susceptibility to *S. aureus* pneumonia has been reported (van de Veerdonk et al. in press). Three patients with a STAT3 mutation and hyperIgE syndrome that had a partial *S. aureus*-induced IL-17 deficiency, never developed *S. aureus* pneumonia in contrast to patients with a complete deficiency in *S. aureus* induced IL-17.

Finally, studies that investigated the role of IL-17 in antibacterial host defense have also provided insights in the role of IL-17 production by innate immune cells such as γδ T-cells. γδ T-cells rather than CD4 T helper cells were found to be the main source of IL-17 during mycobacteria infection in mice (65). This was further supported by the observation that patients with TB had higher proportions of γδ T-cells that were able to produce IL-17 in their peripheral blood compared to healthy controls (66). In addition, it has been shown that IL-17 mediates protection against *Listeria monocytogenis* in the liver (67, 68) and this IL-17 production was mainly derived from γδ T-cells (67, 68). Similar findings were reported for *Escherichia coli* in a mouse model of intraperitoneal infection (69), where neutrophils were shown to infiltrate in an IL-17 dependant manner and interestingly and γδ T-cells were the major source for IL-17 (69). These observations suggest that innate immune cells are also an important source of IL-17 during bacterial infections and supports the hypothesis that IL-17 producing γδ T-cells are able to provide an efficient first line of defense against bacterial invasion (70).

Th17 responses in viral infections

Vaccinia virus and Theilers murine encephalomyelitis virus

It is generally accepted that antiviral host defense is mainly mediated through the production of type 1 IFN and IFNγ. Th1 responses have clearly been associated with protective host defense against viruses. Since there were no suggestions that STAT3 deficient patients were more susceptible to viral infections, it may be hypothesized that Th17 responses played a minor role in antiviral host defense and that Th1 and type I interferon responses are the main protective adaptive immune response against viral infection. However, recent evidence suggests that viruses can also induce Th17 cells, although their role in antiviral host defense still remains to be elucidated.

Smallpox, which represents a serious threat as a possible agent for bioterrorism, has been eradicated by the smallpox vaccine that consists of live vaccinia virus (VV). One of the first reports that studied the role of IL-17 in viral infection was from Patera et al. They inserted murine IL-17 into vaccinia virus (VV-IL-17) and showed that VV-IL-17 was much more virulent than wild type VV (71). Mice infected with VV-IL-17 had higher viral burdens and had impaired NK cell cytotoxicity. In contrast, another study reported that VV-IL-17 was less virulent than VV-WT in mice, and IL-17-deficient mice were more sensitive to VV-WT than control mice (72). In addition, VV expressing IL-23 were shown to be less virulent than wild type VV (72). This controversy still has to be addressed in additional studies.

In patients with atopic dermatitis, VV vaccination can result in eczema vaccinatum, which is a disseminated form of vaccinia infection and can be lethal. Recently, two reports showed an immunopathological role for IL-17 in eczema vaccinatum (73, 74). IL-17 was shown to reduce

NK activity in mice with atopic dermatitis, resulting in higher susceptibility to eczema vaccinatum (73). Similar results were obtained by eliciting Th2 responses using ovalbumin, thereby simulating allergic skin inflammation (74). After VV inoculation, mice with sensitized skin showed localized IL-17 expression, increased IL-23, IL-6 levels and neutrophil influx, which resulted in increased viral loads in the skin and organs when compared with mice that had unsensitized skin (74). Moreover, IL-17 neutralization using anti-IL-17 showed decrease of lesions and viral load while IL-17 administration promoted viral replication. In conclusion, these data suggest that IL-17 contributes to viral replication in a model of disseminated vaccinia infection rather than providing host defense against viral infection, and suggests that IL-17 is a potential target during disseminated vaccinia virus infection.

Theilers murine encephalomyelitis virus (TMEV) infects microglial cells, oligodendrocytes, astrocytes and macrophages in the central nervous system of mice, thereby inducing demyelating disease. In response to TMEV, macrophages are able to produce IL-23p19 and IL-12p40 (75). Recently, it has been shown that Th17 cells promote chronic viral infection in an experimental TMEV infection model, and play an important role in demyelinating disease. IL-17 was responsible for the induction of anti-apoptotic mechanisms that resulted in the survival of cells infected with TMEV (76). Blocking IL-17 resulted in more efficient clearance of TMEV and cytotoxic T cell responses and could prevent disease development. It has been proposed that IL-17 could be a potential therapeutic target in chronic diseases associated with viral infections.

Herpes Simplex Virus

An important complication of Herpes simplex virus infection (HSV) is damage to the cornea, also called herpetic stromal keratitis. It has been shown that patients with corneal HSV infection have increased levels of local IL-17, which can bind to IL-17R on corneal fibroblasts (77). This triggers expression of the neutrophil attracting chemokines IL-8 (CXCl-8) and MMP-1, which can subsequently result in neutrophil influx and inflammation that causes damage to the cornea and eventually blindness. In addition, mice lacking the IL-17R have decreased neutrophilic migration in the cornea and corneal pathology, although this effect was transient and control of viral growth in the cornea was not affected in IL-17R-/- mice (78). However, it has also been proposed that the immunopathology in herpetic stromal keratitis is mainly driven by Th1 responses and not Th17 responses (79).

Human Immunodeficiency Virus

Interestingly, early HIV infection is associated with an increase of IL-17 production by CD4 positive and CD4 negative cells in peripheral blood (80). Misse et al. have proposed that IL-22 contributed to antiviral defense by activating acute-phase proteins and induction of IL-22 might be a protective mechanism during HIV infection (81). Although Th17 responses could have a protective role against HIV infection, HIV infection has mainly provided supporting evidence of the role of CD4 positive T cells during specific infections. This is due to the fact that HIV specifically infects CD4 positive T helper cells, which results in a depletion of CD4 positive IL-17 producing cells in humans in vivo. The observation that HIV patients are especially susceptible to oropharyngeal *C. albicans* infection supports the important role for CD4 positive cells that secrete IL-17 in anti-*Candida* host defense. Furthermore, the increased susceptibility to non-typhiod salmonella infections underscores the important role for Th17 cells in the protection against non-typhoid *Salmonella* spp.

Hepatitis viruses

It has been shown that during *hepatitis C* infection virus specific Th17 cells are induced (82). However, *hepatitis C* also induces immunosuppressive cytokines IL-10 and TGF-β, which are able to inhibit Th17 and Th1. Neutralization of TGF-β has been shown to increase IL-17 production in response to *hepatitis C* nonstructural protein 4 (82). In patients with chronic *hepatitis B*, Th1 cells were decreased and Th17 cells were increased (83). The Th17 cytokine IL-22 was shown to be protective, however a protective function for IL-17 was not observed (84). The balance between Th1 and Th17 seems to play an important role in viral hepatitis, although it remains to be elucidated if Th17 responses favor protective host defense.

Other viruses

Several other studies have shown that IL-17 is elevated during viral infection: when mice that are deficient in Th1 responses are infected with lymphocytic choriomeningitis viral infection, they display elevated IL-17 producing CD8 T cells and develop progressive inflammation (85). Human T-cell leukemia virus (HTLV) was shown to up-regulate IL-17 expression in CD4 T-cells (86). In studies where epithelial cells were exposed to human rhinovirus, increased induction of IL-17 was shown, together with specific infiltration of neutrophils in the lungs (87). It was however not clear in these studies if IL-17 provides a protective antiviral role or that it contributes to inflammation that is detrimental for the host. Notably, Th17 responses were shown to be protective in IL-10 deficient mice infected with *Influenza* virus (88).

Th17 responses in parasite infections

Protozoa

Evidence regarding the role of Th17 responses in the host defense against protozoa is relatively limited. IL-4 which is the prototypical effector cytokine of Th2 cells can negative regulate Th17 responses (2), and therefore it may be hypothesized that Th17 responses would not be strongly involved in host defense against parasites. However, new studies have suggested a more subtle view. Oral *Toxoplasma gondii* infection in IL-17 deficient mice leads to higher mortality than in control mice (89). These mice were shown to have less parasite burden and normal neutrophil infiltration during *T.gondii* infection (89). Despite the beneficial effect of IL-17 mediated inflammation which effectively cleared *T.gondii* infection, increased liver and intestinal pathology was observed. Stumhofer et al, have shown that IL-27 deficient mice show a profound Th17 response and severe neuroinflammation (90). These data indicate that Th17 responses can contribute to persistent inflammation in the organs affected by *Toxoplasma*. Furthermore, IL-27 which suppresses Th17 responses was found to be beneficial in infections with the protozoan *Trypanosoma cruzi* (91). In an experimental model of *Leishmania* infection, IL-17 and IL-22 were shown to be important in the protection against *L.donovani* (92), but in *L.major* infection IL-27 was shown to inhibit Th17 responses and this prevented immunopathology (93). These data suggest that the inhibiting effect of IL-27 during infection with protozoa is of utmost importance to control the Th17 responses that contribute to persistent inflammation. Mice deficient in bradykinin receptor 2 were shown to have higher Th1 responses at the cost of lower Th17 responses, and were better protected against intraperitoneal *T. cruzi* infection than control mice, which provides further evidence that Th17 responses can be detrimental for the host during protozoal infection

(94). However, it must be mentioned that a study on *Leishmania braziliensis* showed protection that was mediated by IFN-γ and IL-17 producing T-cells (95).

Helminths

Infection with *Schistosoma mansoni* in mice causes development of hepatic granuloma around the *S.mansoni* eggs (96). Antigens from these parasite eggs have been shown to cause CD4 positive T-cell mediated immunopathology. IL-12p40 deficient mice not able to make IL-12 and IL-23 were completely resistant to immunopathology (96), while mice with IL-12p35 deficiency that are not capable of making IL-12 still showed severe immunopathology. In addition, IL-17 neutralization significantly reduced immunopathology observed in *S. mansoni* infection (96, 97). Despite this immunopathological role for Th17 responses in *S. mansoni* infection, it has also been shown that TGF-β can up-regulated Th17 responses which result in increased resistance against lung-stage schistosomula (98). Evidence for a negative role for Th17 was also described during infection with *Trichuris muris*, which is the murine variant of *Trichuris trichuria*. Down-regulation of the Th2 promoting cytokine IL-25 caused severe inflammation associated with high levels of IFN-γ and IL-17, although it still remains to be elucidated which cytokine contributed most substantial (99). In general, these observations suggest that Th17 responses in the setting of parasitic infections can contribute to host defense, but they have to be controlled, for example by cytokines such as IL-27, in order to prevent immunopathology.

Th17 responses in infection: the balance between protection and immunopathogenesis

Both pathological and protective roles have been described with respect to the Th17 lineage in infectious diseases (Table 1). In infections caused by bacteria and fungi, the pathogen induced Th17 response has been reported as an important mediator of protective mucosal host defense. Th17 cells improve mucosal barrier function during infection by secretion of antimicrobial peptides and additional chemokine signaling for neutrophil reinforcements. The importance of the Th17 subset in mucosal host defense is underscored by the observation that patients with hyperIgE syndrome and chronic mucocutaneous candidiasis suffer from a severe form of mucosal candidiasis and they display specific defects in their Th17 response against *C. albicans*.

However, in viral infections and parasitic infections the role of Th17 responses is less clear and IL-17 has even been reported to be detrimental for the host. Interestingly, Th17 responses inhibit apoptosis of virus infected cells and contribute to persistence of the virus. In parasitic infections IL-17 was shown to contribute to persistent inflammation. Since Th17 cells are associated with autoimmune diseases and hence chronic inflammation, it seems logical that persistent en chronic induction of Th17 responses, triggered by pathogens that are not sufficiently cleared, can results in immunopathology that is detrimental for the host. Th17 responses can effectively recruit and orchestrate neutrophils, and therefore are probably very efficient in inducing the killing of extracellular invading pathogens, while the killing and clearance of intracellular pathogens such as viruses may be more efficient in the setting of a strong Th1 response and host defense against parasitic infection requires an optimal Th2 response. Since skewing towards Th17 responses can result in downregulation of Th1 and possibly Th2 responses, Th17 cells might in this way contribute to a suboptimal host defense against viruses and parasites (Figure 2). Although Th17 cells have only recently

been discovered, they have already provided crucial insights into the host defense against microorganisms. Understanding the Th17 responses and their interactions with the immune repertoire will likely provide crucial insights in the host defense and immune responses, and this will provide new tools for the development of effective immunomodulatory treatment strategies in the setting of infectious diseases.

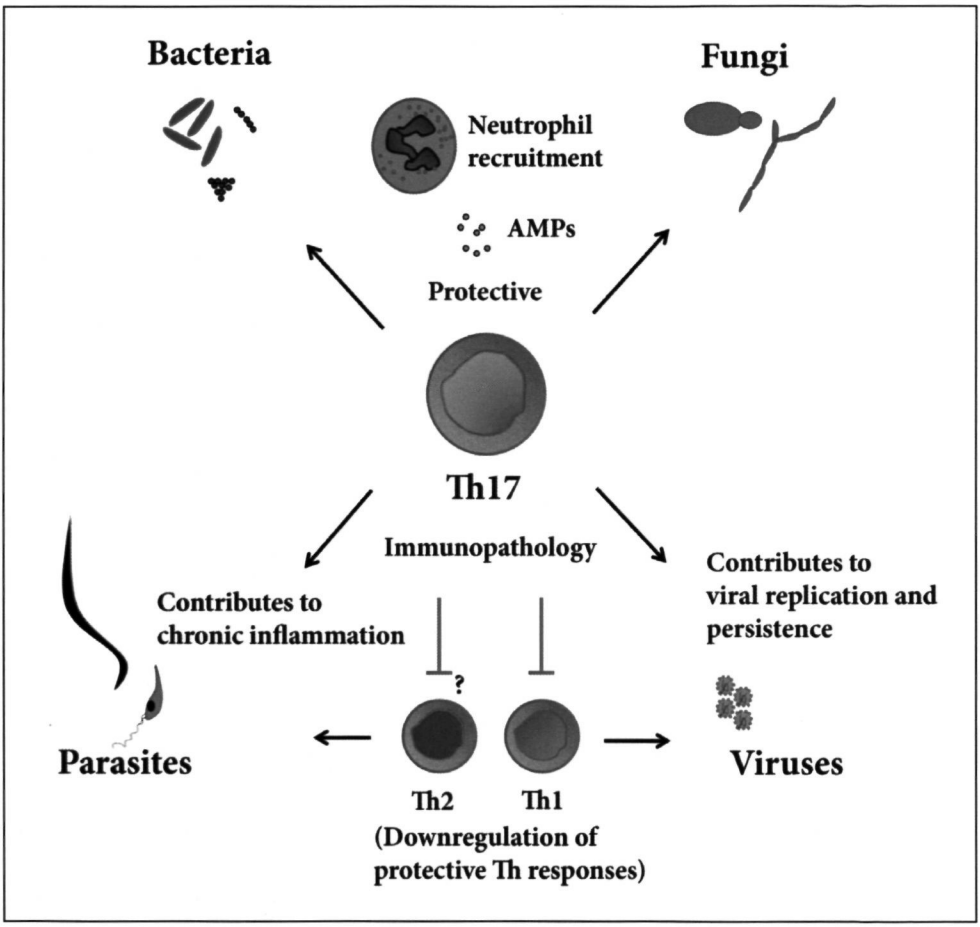

Figure 2. Th17 responses in infection: the balance between protection and immunopathogenesis.
Th17 responses are important for the host defense against extracellular bacteria and fungi by orchestrating neutrophil recruitment and stimulating antimicrobial peptides (AMPs). However, Th17 responses can also contribute to chronic inflammation and can be favorable for the pathogen, as has been shown for parasites and viruses.

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Table 1. Overview regarding the role of Th17 responses in relation to viral, bacterial, fungal and parasitic infections.

	Pathogen	Infection	Th17 response
Fungi	*Candida albicans*	Systemic	Increased susceptibility in IL-17RA deficient mice (7)
		Mucosal	Deficient Th17 response in humans and mice leads to increased susceptibility to candidiasis (6, 11, 63, 64) IL-17 deficiency increases susceptibility to oropharyngal candidiasis in mice (8)
		Intra gastric	Th17 responses are detrimental to the host (10)
	Aspergillus fumungatus	Intranasal	IL-23 deficiency reduces fungal burden, IL-17 inhibits protectiveTh1 responses (9)
		Pulmonary	Deficient ROS results in increased Th17 responses detrimental for the host (17)
	Pneumocystis carinii	Pulmonary	IL-17 neutralization increases disease severity (18)
	Cryptococcus neoformans	Brain	IL-23 deficiency leads to impaired IL-17 expression and higher mortality in mice (19)
	Paracoccidioides braziliensis	Pulmonary	Mice deficient for TLR2 show increased protective Th17 responses (20)
Bacteria	*Borrelia burgdorferi*	Synovium	Neutralization of Th17 responses prevents *Borrelia* induced arthritis (25)
	Helicobacter pylori	Gastric	STAT3 induction resulting in sustained IL-17 expression and persistent inflammation (27)
	Klebsiella pneumoniae	Pulmonary	Th17 deficiency leads to lethal infection in mice, IL-17 administration restores normal host defence (30, 32) Induction of AMPs by Th17 cytokines IL-17 and IL-22 (33, 34)
	Salmonella enterica Typhimurium	Intestinal mucosa	IL-17 deficiency results in impaired neutrophil recruitment (43)
	Salmonella enterica Enteritidis	Intestinal mucosa	IL-22 crucial in protection against dissemination of infection (42) IL-17 deficiency causes higher bacterial load in liver and spleen (42)
	Salmonella enterica Typhi	Intestinal mucosa	viaB capsule encoding locus inhibits IL-17 expression (40)
	Mycobacterium bovis BCG	Pulmonary	Induction of IL-17 expression in CD4 T cells (21) IL-23 crucial for Th17 response (46)
	Mycobacterium tuberculosis	Pulmonary	IL-17RA deficiency gives no difference in bacterial clearance (47)
	Bordetella pertussis	Pulmonary	Host response is skewed towards Th17 response and neutralization of IL-17 before challenge showed reduced vaccine protection(51) Pertussis toxin induces IL-17 responses (52)
	Bacteroides fragilis	Abdominal	IL-17 is associated with abscess formation (61)
	Porphyromonas givinalis	Periodontal	Elevated IL-17 in patients with severe PD (54) Th17 responses prevent bone destruction in mice (55)
	Pseudomonas aeruginosa	Pulmonary	High levels of IL-23 and IL-17 associated with severe inflammation during infection in CF patients (58) IL-23 deficiency causes decreased inflammation but unchanged bacterial dissemination in CF mice (59) IL-17 critical in vaccine induced protectivity (60)

	Mycoplasma pneumoniae	Pulmonary	IL-23 depletion results in reduced neutrophil recruitment in mice (56)
	Citrobacter rodentium	Intestinal mucosa	Reduced survival rates in IL-23 deficient mice (35) Induction of AMPs by Th17 responses in mice (36, 37)
	Listeria monocytogenis	Liver	IL-17 contributes to protection, mainly derived from γδ T-cells (67, 68)
	Escherichia coli	Intra peritoneal	IL-17 contributes to protection, mainly derived from γδ T-cells (69)
	Staphylococcus aureus	Epithelium (lung, skin)	Deficient Th17 response in humans and mice leads to increased susceptibility infection (6, 62-64) Induction of AMPs by Th17 responses in mice (36)
	Haemophilus influenzae	Pulmonary	Deficient Th17 response in humans and mice leads to increased susceptibility infection (62)
Virusses	Vaccinia Virus	Skin	localized Th17 responses are associated with increased viral loads (74)
	Theilers murine encephalomyelitis virus	Brain	IL-17 contributed to viral persistence (76)
	Herpes Simplex Virus	Eye	IL-17R deficient mice have less corneal pathology but unaffected viral growth (78)
	Human Immunodeficiency Virus	Systemic	Increased IL-17 production in early HIV infection (80) Induction of acute-phase proteins by IL-22 (81)
	Hepatitis B Virus	Liver	Chronic hepatitis show increased Th17 and decreased Th1 responses (83) IL-22 is protective in conA induced hepatitis (84)
	Rhino virus	Pulmonary	Increased induction of Th17 and pulmonary neutrophil infiltration (87)
	Influenza virus		Protective Th17 responses in IL-10 deficient mice (88)
Parasites	*Toxoplasma gondii*	Intestinal	IL-17 contributes to host defense, but also inflammatory damage (89)
		Brain	IL-27 deficiency results in higher Th17 responses and neuroinflammation (90)
	Trypanosoma cruzi	Systemic	IL-27 mediated suppression of Th17 responses is beneficial (91)
	Cryptosporidum parvum	Intestinal	IL-23 and IFN-γ reduced parasite shedding (100)
	Leishmania braziliensis	Systemic	Protection by IFN-γ and Th17 producing T-cells (95)
	Leishmania donovani	Systemic	IL-22 and IL-17 shown to be protective (92)
	Leishmania major	Systemic	Less pathology when Th17 response is inhibited by IL-27 (93)
	Schistosoma mansoni	Liver	Less Th17 responses resulted in absence of pathology (96, 97)
		Lung	TGF-β mediated upregulation of Th17 responses resulted in protection (98)
	Trichuris muris	Intestine	Downregulation of Th2 responses resulted in immunopathology through Th1 and Th17 responses (99)

Chapter 10

The macrophage mannose receptor induces IL-17 in response to *Candida albicans*

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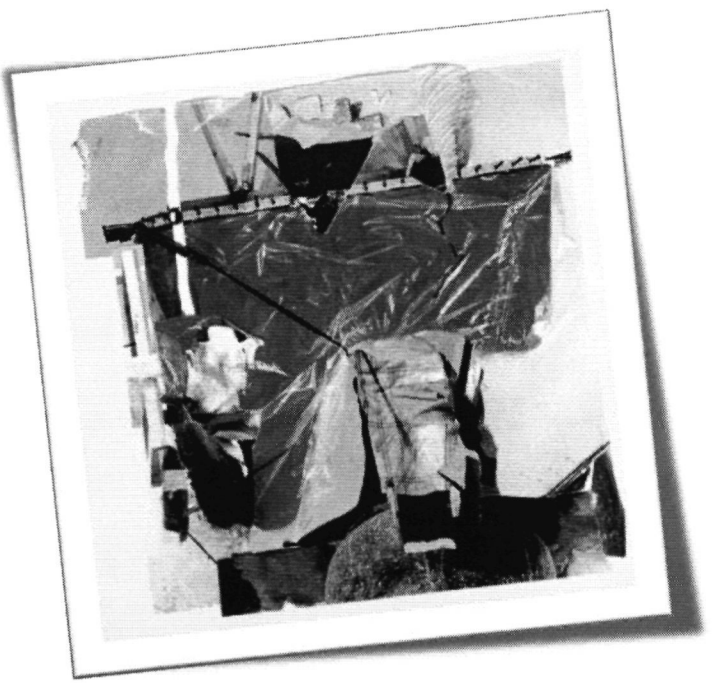

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Summary

IL-17 controls neutrophil-mediated responses in inflammation. Little is known about the pattern recognition receptors that induce Th17 responses during infection models, in the absence of artificial mitogenic induction of IL-17 with anti-CD3/anti-CD28 antibodies. The fungal pathogen *Candida albicans* induced significantly more IL-17 compared to Gram-negative bacteria, and the mannose receptor (MR) played a central role in this process. *Candida* mannan, but not zymosan, Toll-like receptor (TLR) agonists or the NOD2 ligand MDP, induced IL-17 production in the absence of anti-CD3/anti-CD28 antibodies. The TLR2/dectin-1 pathway, but not TLR4 or NOD2, is involved in the amplification of MR-induced IL-17 production. This is the first pathway described that can trigger the Th17 response mediated by a specific pathogen in the absence of anti-CD3/anti-CD28 stimulation.

Introduction

The recently described T helper 17 cells (Th17 cells) produce interleukin-17A (IL-17), a cytokine that is important in the host defense against various pathogens (1), including Gram negative bacteria such as *Kleibsiella pneumonia* (2) and *Citrobacter rodentium* (3), the spirochetal infection *Borrelia burgdorferi* (4), and the fungal pathogen *C. albicans* (5). *Candida*-specific Th17 responses have also been observed in peripheral blood in humans (6). IL-17 induces infiltration of polymorphonuclear leucocytes (PMN) at the site of infection, activation of tissue neutrophils and macrophages, and the synthesis of antimicrobial peptides (1). These data suggest an important role for the Th17 response in the host defense against pathogens. However, overproduction of IL-17 can also lead to autoimmune processes (7-9), underscoring the delicate balance in regulation of the Th17 response.

Recently, much has been learned about the cytokine profiles that are needed to differentiate naïve T cells into Th17 cells, and how to enable central memory T cells to secrete IL-17. Whereas IL-1β alone is capable of inducing IL-17 secretion in human central memory T cells (10), IL-21 and TGFβ seem to be needed for driving differentiation of naïve T- cells into Th17 cells (11). More controversial are the receptor pathways that direct the immune response towards a Th17 profile, with each of the TLRs (12), dectin-1 (13) and NOD2 (14) pathways individually being implicated in the induction Th17 cells by different researchers. However, these studies have used simultaneous stimulation of these receptors with proliferation cocktails such as anti-CD3/anti-CD28, a model with limited relevance for the in-vivo situation. Little is known about the interplay between the various receptor pathways of the innate immune system that will eventually lead to a Th17 response during infection with human pathogens. In the present study, we have focused on the induction of IL-17 by human microbial pathogens, and tried to clarify which innate immune receptors and pathogen-associated molecular patterns (PAMPs) are involved in triggering a Th17 response during the pathogen-specific host defense. Surprisingly, we have identified the prototypic fungal pathogen *C. albicans* as a much more potent inducer of IL-17 production compared to Gram-negative bacteria. The most important pathway of IL-17 induction was represented by the engagement of the macrophage mannose receptor (MR), with the TLR2/dectin-1 pathway having a secondary amplification effect on MR-induced IL-17 production.

Results

Receptor-vs proliferation-dependent induction of IL-17 production

Several receptor-dependent pathways have been described to induce IL-17 induction, such as dectin-1 (6), NOD2 (14) or TLRs (12). However, all of these studies have used combinations of receptor ligands and proliferation stimuli such as anti-CD3/anti-CD28 antibodies (aCD3/aCD28). We wanted to differentiate the relative importance of the receptor-specific and proliferation-dependent induction of IL-17 by stimulating cells with a known IL-17 inducer, zymosan, in the presence or absence of aCD3/aCD28. Zymosan alone was able to induce only very limited amounts of IL-17 in the presence of human serum, and anti-CD3 as a co-stimulatory signal was needed in order to potentiate the zymosan induced IL-17 production (Fig. 1a). The non-specific stimuli aCD3/aCD28, in the presence of serum, induced the highest amount of IL-17 secretion (Fig. 1a). To investigate whether TLR and NLR ligands alone were able to induce IL-17 production, PBMCs were stimulated with a TLR2 ligand (Pam3Cys 10 μg/ml), TLR4 ligand (LPS 10 ng/ml), TLR3 ligand (PolyI:C 10 μg/ml), TLR5 ligand (flagellin 10 μg/ml), TLR9 ligand (ODN 10 μg/ml) and the NOD2 ligand MDP (10 μg/ml) in the presence of human serum, without additional co-stimulatory factors. No single ligand was able to induce IL-17 production, although all ligands were able to induce IL-6 production (Fig. 1b). Several combinations of these ligands (TLR2 with TLR4, TLR2 with NOD2 and TLR4 with NOD2) were also not able to induce IL-17 production in human PBMCs (data not shown). Thus, stimulation of TLRs and NOD2 pathways alone cannot induce production of IL-17.

Pathogen induced production of IL-17

IL-17 is important in the host defense against extracellular bacterial and fungal infections (2, 15). We investigated the capacity of whole microorganisms to induce IL-17 production. Both Gram-negative and fungal pathogens induced IL-17 secretion. However, *C. albicans* was by far the most potent inducer of IL-17 production, in concentrations comparable to those induced by anti-CD3/anti-CD28 antibodies. In contrast, comparable amounts of monocyte derived IL-1β, a cytokine known to be important for the induction of IL-17, were induced by Gram-negative bacteria and *C. albicans* (Fig. 2a). Because bacteria and fungi differ in size, and therefore in surface area and antigen load, we compared high dosages of Gram-negative bacteria with low dosages of fungi. Even a 1000 fold higher concentration of Gram-negative bacteria was still less potent in the induction of IL-17 production (data not shown). RT-PCR showed that the induced IL-17 mRNA was accompanied by upregulation of *RORγt* (Fig. 2b).

The conidial form of *C. albicans* was the most potent for the induction of IL-17 (Fig. 2a), and both heat-killed and live *C. albicans* induced significant amounts of IL-17 (Fig. 2c). Cytokine kinetics during these stimulations showed that IL-17 increased steadily during the 7 day period, while IL-23 production was maximal in the first 24 hours and decreased thereafter (Fig. 2d). IL-10 was present in the first 48 hours and decreased to undetectable limits at day 3 and stayed undetectable (Fig. 2d). IFNγ was present at 48 hours and reached its maximum at 4-5 days, while IL-1β reached its maximum level at 24 hours after which a plateau was attained (Fig. 2d). At day 5 of stimulation, flow cytometry of PBMCs stimulated with *C. albicans* yeast cells showed a subset of CD4 positive cells that was capable of producing IL-17 alone or in combination with IFNγ (Fig. 2e,f).

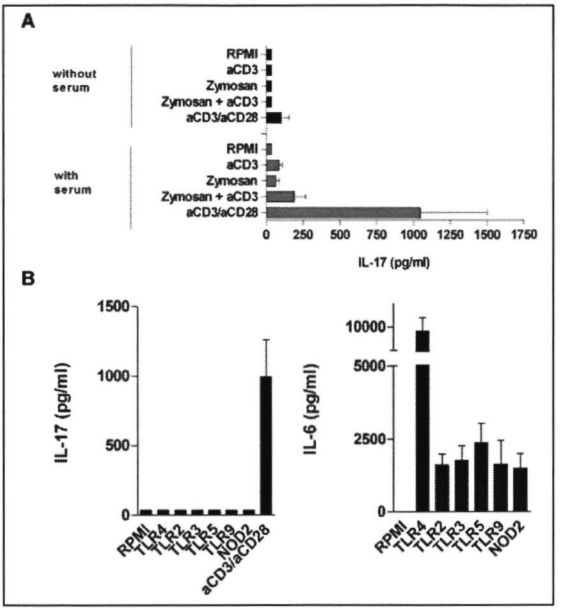

Figure 1. Receptor vs proliferation-dependent induction of IL-17 production.
(a) Human PBMCs were stimulated for 5 days with RPMI, zymosan 100 μg/ml, beads coated with anti-CD3, anti-CD28 or various combinations, in the presence or absence of human serum. Production of IL-17 in the supernatants was measured by ELISA. (b) Human PBMCs were stimulated for 5 days in the presence of human serum with either TLR ligands (LPS 10 ng/ml, Pam3Cys 10 μg/ml, PolyI:C 10 μg/ml, Flagellin 10 μg/ml and ODN M362 10 μg/ml) or the NOD2 ligand MDP (10 μg/ml), or anti-CD3/anti-CD28 (cell:bead ratio of 2:1). IL-6 and IL-17 were measured by ELISA. All experiments were performed at least twice with a total of 5 healthy volunteers. Data are pooled and expressed as mean ± SEM.

The mannose receptor is crucial for the induction of IL-17 production by C. albicans

The structure of the *C. albicans* cell wall is very different from that of bacteria, being composed of polysaccharides such as mannans, β-glucans and chitin (16). Single fungal components were tested for the induction of IL-17. *C. albicans* mannan, but not *S. cerevisiae* mannan, was able to induce IL-17 production (Fig. 3a). Surprisingly, the other fungal components, β-glucan and chitin, did not induce IL-17 production. Although zymosan was able to induce IL-1β, it did not lead to a significant production of IL-17 (Fig. 3a). *C. albicans* mannan showed a dose-dependent induction of IL-17 production (Fig. 3a). *C. albicans* mannan did not stimulate IL-17 production in the absence of serum (data not shown). Subsequently, we assessed the receptors leading to IL-17 production in human PBMCs, by blocking them with specific antibodies. Blocking of the mannose receptor resulted in the strongest inhibition of IL-17 production and quantitative RT-PCR showed that mRNA for IL-17A and *RORγt* were downregulated in the presence of the mannose receptor inhibitor (Fig. 3b,c). Blocking of the dectin-1/TLR2 pathway also lead to partial inhibition of IL-17 production (fig. 3c). Blocking TLR4 did not alter mannan-induced IL-17 secretion (Fig 3c), while blocking of TLR4 decreased LPS induced IL-1β on day 5 was still effective (data not shown). Furthermore, *C. albicans* was able to induce pro- and anti-inflammatory cytokines (Fig. 3d). Interestingly, in the presence of the anti-MR there was no effect on IL-12, IL-23, IL-2, IL-1β, TNFα and IFNγ production, but there was a significant reduction in IL-10 (Fig. 3d). Next, we determined if the observed lower IL-17 production was in line with a lower

induction of IL-17 producing cells. We observed that blocking the mannose receptor inhibited the percentage of CD4+ IL-17 producing cells (Fig. 3e,f). Since *S. cerevisiae* mannan itself cannot induce IL-17 production, but is able to interact with the MR, we preincubated PBMCs with an excess of *S. cerevisiae* mannan (Fig. 4a). This inhibited the IL-17 production induced by *C. albicans* mannan and *C. albicans*. To further establish that *C. albicans* mannan induces a Th-17 response specifically through the MR, we investigated the effect of siRNA depletion of the MR from monocytes. The IL-17 production induced by *C. albicans* mannan was reduced by roughly 50 % in PBMCs treated with MR siRNA (Fig. 4b).

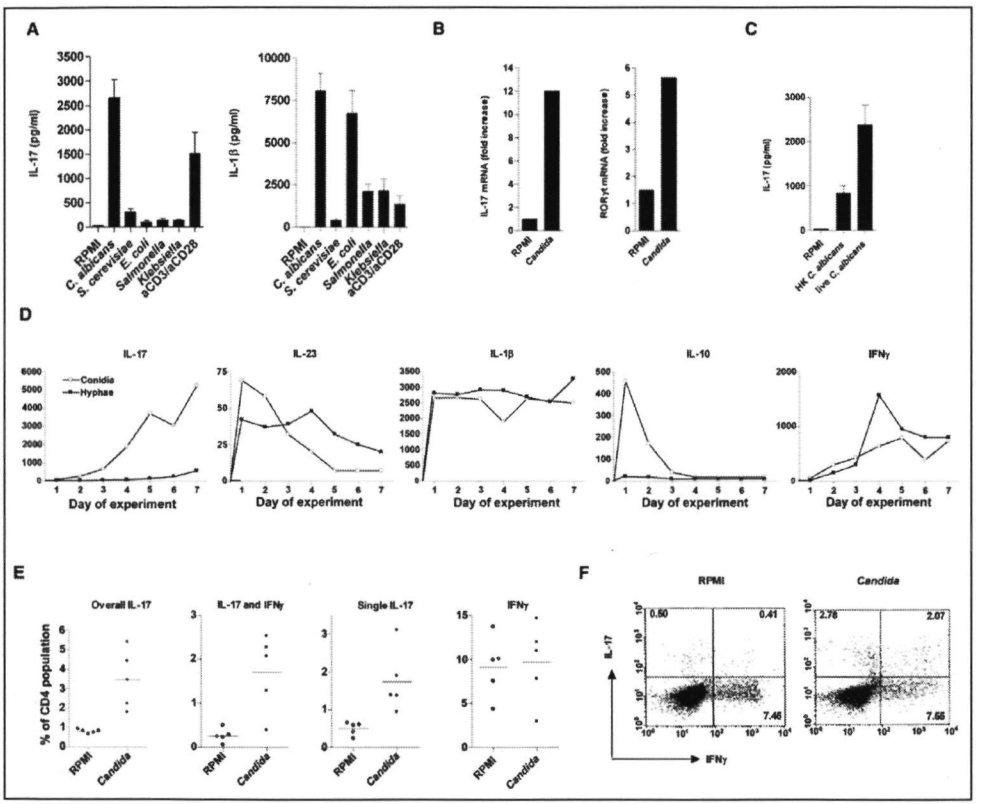

Figure 2. Pathogen induced production of IL-17.

(a) Human PBMCs were stimulated for 5 days with RPMI or several pathogens. IL-1β and IL-17 were measured by ELISA. (b) RT-PCR of the expression of RORγt and IL-17A in PBMCs stimulated with RPMI or *C. albicans* for 3 days. Data is given as relative mRNA expression ($2^{-\Delta CT}$ x 1000). (c) PBMCs stimulated for 5 days with either live *C. albicans* (10^5/ml) or heat-killed (HK) *C. albicans* (10^5/ml). (d) Time course of IL-17 production in human PBMCs stimulated with heat-killed *C. albicans* yeasts or pseudohyphae. Cyokines were measured by ELISA. (e) Intracellular cytokine staining of IL-17 and IFNγ in human PBMCs (n=5) stimulated for 5 days with RPMI or *C. albicans*, and than stimulated for 4 h with PMA and ionomycin. Cells were gated for CD4 and dat is given as % of total gated CD4 positive cells. (f) Representative intracellular cytokine staining of IL-17 and IFNγ for data given in (e). (a,c) n=10; data are pooled and expressed as mean ± SEM. (d,f) Data from one healthy volunteer, that represents the pattern observed in two separate experiments with a total of n=5. (b) Data is expressed as median from two separate experiments n=3.

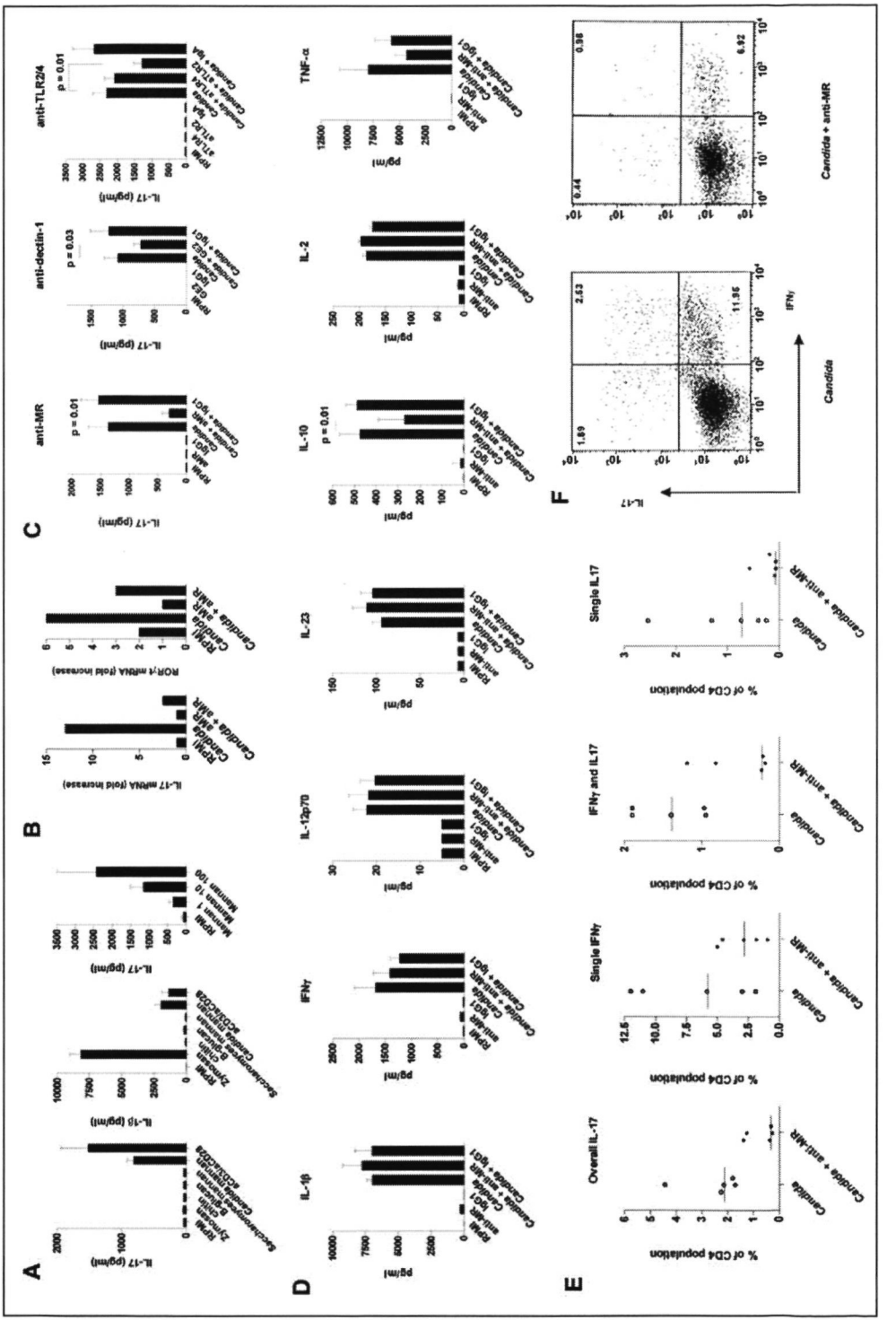
A
B
C
D
E
F
IL-17 (pg/ml)
IL-1β (pg/ml)
IL-17 mRNA (fold increase)
RORγt mRNA (fold increase)
anti-MR
anti-dectin-1
anti-TLR2/4
IL-10
IL-2
TNF-α
IL-23
IL-12p70
IFNγ
IL-1β
pg/ml
RPMI
Candida
Candida + anti-MR
Candida + anti-TLR2/4
Candida + anti-dectin-1
Candida + IgG1
Mannan 1
Mannan 10
Mannan 100
Overall IL-17
Single IFNγ
IFNγ and IL17
Single IL17
% of CD4 population
IL-17
IFNγ
Candida
Candida + anti-MR
0.44
0.38
1.89
2.53
11.35
6.92

Figure 3. The mannose receptor is the main receptor pathway for the induction of IL-17 production by *C. albicans*.
(a) Human PBMCs were stimulated for 5 days with RPMI or several fungal components (10 μg/ml), zymosan 100 μg/ml. Mannan was given in different concentrations (μg/ml). IL-1β and IL-17 were measured by ELISA. (b) Human PBMCs were stimulated for 5 days with heat-killed *C. albicans* yeast cells in the presence or absence of specific receptor inhibitors and isotype controls. (c) Human PBMCs were stimulated for 48 h with RPMI as a control or heat-killed *C. albicans* yeast cells in the presence or absence of blocking antibodies and and isotype controls. (d) RT-PCR of the expression of RORγt and IL-17A in PBMCs stimulated with RPMI or *C. albicans* at 3 days in the presence or absence of the blocking antibody for MR (aMR). Data is given as relative mRNA expression ($2^{-\Delta CT}$ x 1000). (e) Intracellular cytokine staining of IL-17 and IFNγ in human PBMCs (n=5) stimulated for 5 days with *C. albicans* in the presence or absence of the aMR, and than stimulated for 4 h with PMA and ionomycin. Cells were gated for CD4 and data is given as % of total gated CD4 positive cells. (f) Representative intracellular cytokine staining of IL-17 and IFNγ for data given in (d). (a,b,c,) Data are pooled from at least 2 separate experiments with a total of 6 healthy volunteers (mean ± SEM). (d) Data is expressed as median; two separate experiments n=3. (f) Data from one healthy volunteer, that represents the pattern observed in (e).

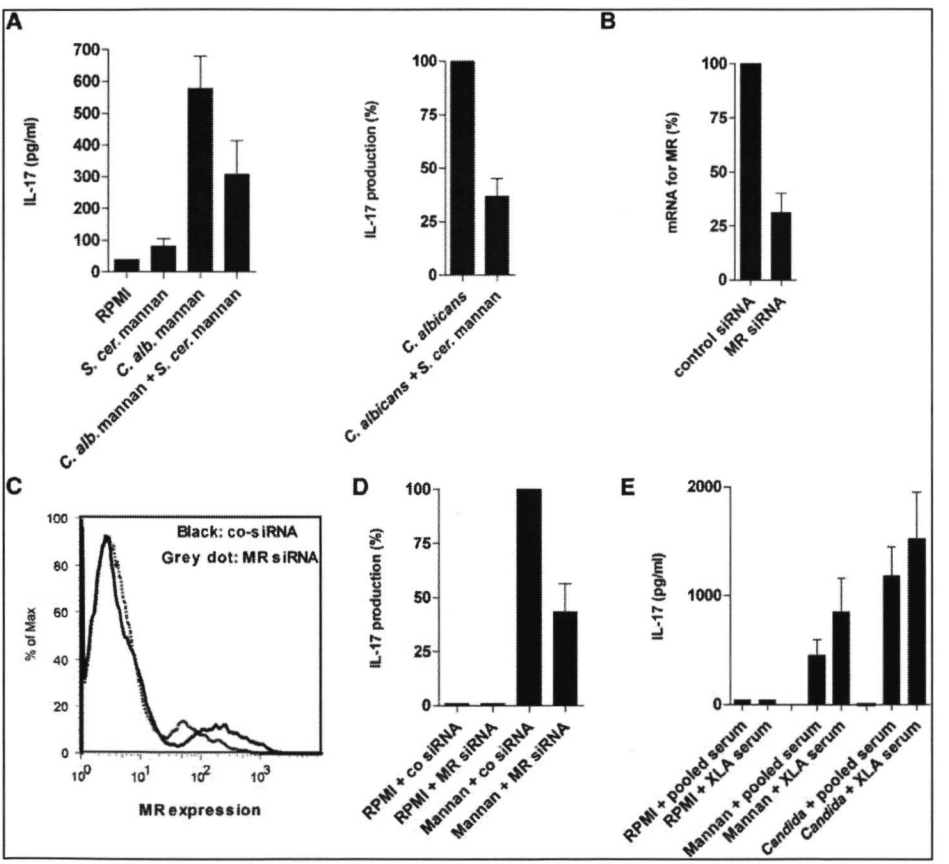

Figure 4. Blocking the MR with *S. cerevisiae* mannan and inhibiting MR expression using siRNA transfection.
(a) Human PBMCs were preincubated for 1 h with 100 μg/ml of *S. cerevisiae* mannan and stimulated for 5 days with RPMI, *C. albicans* mannan (10 μg/ml), or heat killed *C. albicans*. (b) RT-PCR for the expression of MR in cells transfected with control siRNA and MR siRNA. (c) Flow cytometry: expression of the MR on the cell surface of cells stimulated for 5 days with 10 μg/ml of *C. albicans* mannan that were treated with control siRNA (red) or MR siRNA (green). (d) IL-17 production in cells transfected with control siRNA or MR siRNA and stimulated with RPMI or *C. albicans* mannan. (a,b,d) A total of 5 healthy volunteers. Data are pooled and expressed as mean ± SEM. (c) Data are shown from one volunteer which is representative for all volunteers.

IL-17 production induced by C. albicans and mannan is monocyte dependent

It is known that fungal mannans contain immunodominant T cell epitopes (17) and that *Candida* can directly interact with lymphocytes (18). We therefore investigated whether *C. albicans* or purified *C. albicans* mannan alone were able to directly activate T cells and would lead to the production of IL-17. Neither naïve nor memory CD4+ T cells were able to produce IL-17 when stimulated in the absence of antigen-presenting cells (APC) (Fig. 5a). We therefore explored the role of APCs for the induction of IL-17. Monocytes were isolated and stimulated with *C. albicans* or *C. albicans* mannan, in the presence or absence of naïve or memory T cells. We demonstrate that monocytes alone were not able to produce IL-17, but the co-culture of monocytes with memory CD4+ T cells induced a strong Th-17 response (Fig 5b).

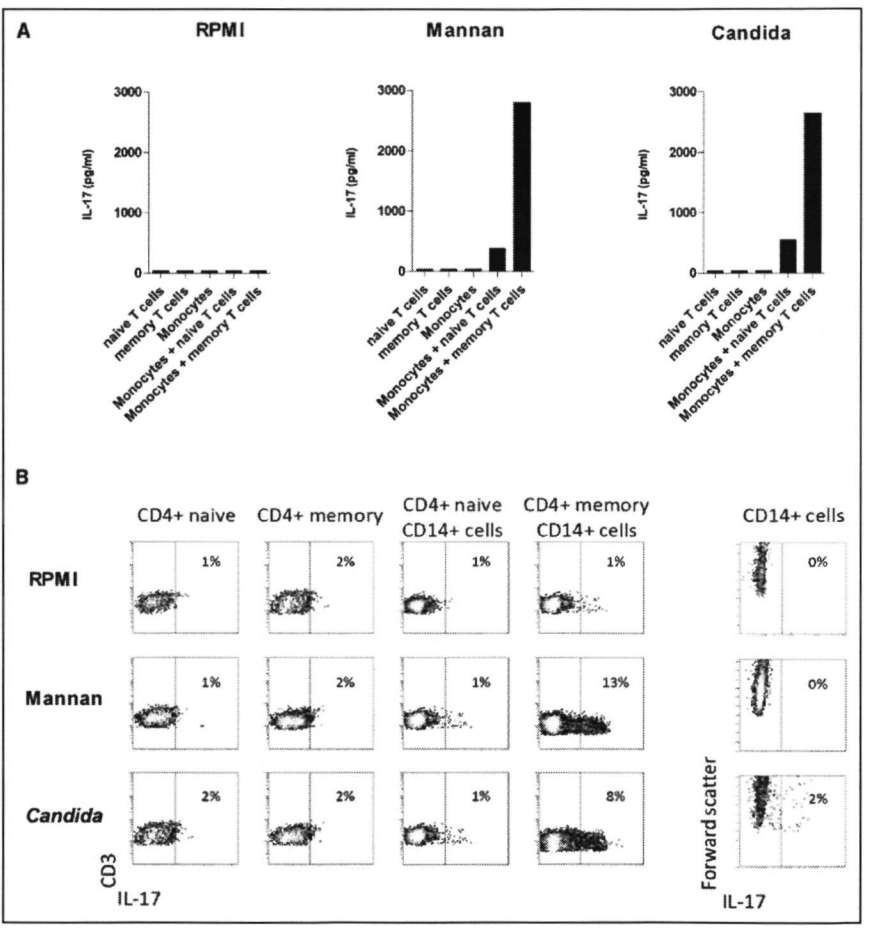

Figure 5. IL-17 production induced by *C. albicans* and mannan is monocyte dependent.

(a) CD4+ naïve (CD25-CD45RA+) and CD4+ memory (CD25-CD45RA-) T cells (2.5×10^4) were cultured with or without autologous monocytes (1.0×10^5) and stimulated for 8 days with culture medium, *C. albicans* mannan or heat killed *C. albicans*. IL-17 was measured in supernatants by ELISA. (b) Density plots show intracellular IL-17 staining of the same experiment as shown in (a). T cells were gated on CD3, CD14+ monocytes were CD3 negative. Data are representative for 2 identical separate experiments.

Dectin-1 deficient PBMCs have a relatively lower Th17 reponse to C. albicans yeasts

The in-vitro stimulations described above suggest that although β-glucan by itself cannot stimulate IL-17 production, the dectin-1/TLR2 pathway can still contribute to the IL-17 induction by *C. albicans,* possibly by potentiating other routes of stimulation. To assess whether this is the case in patients, we investigated IL-17 production in 3 patients with a complete defect in dectin-1 expression due to an early stop codon mutation (Tyr238Stop) (Netea et al., personal communication). FACS analysis confirmed that the cells of the three patients used in these experiments expressed no dectin-1 on their surface (Fig. 6a). Dectin-1 deficient PBMCs showed a lower IL-17 production compared to healthy controls when stimulated with *C. albicans* yeasts (Fig. 6b), but not in pseudohyphae that do not express β-glucans on their surface (19). The normal pattern of a higher IL-17 production induced by *C. albicans* yeast cells compared to pseudohyphae was lost in PBMCs that were dectin-1 deficient. IL-1β and IL-6 induction by *C. albicans* was also defective in these patients (data not shown).

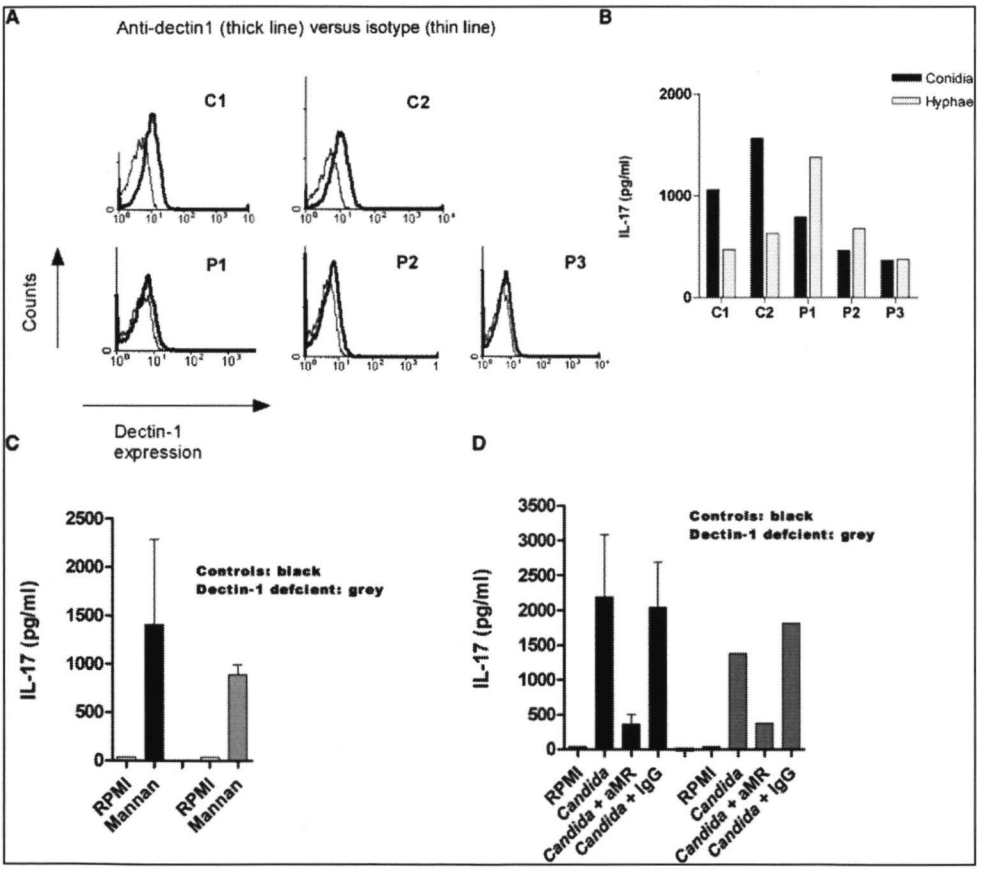

Figure 6. Dectin-1 deficient PBMCs have a relatively lower Th17 reponse to *C. albicans* yeasts.
(a) Flow cytometry of the expression of dectin-1 on the surface of PBMCs isolated from healthy volunteers (C1 and C2) and from three individuals homozygous for the Tyr238 stop mutation (P1-P3). (b) PBMCs described in (a) were stimulated for 5 days with heat-killed *C. albicans* yeasts or pseudohyphae. IL-17 was measured by ELISA.

TLR2 is synergistic with the mannose receptor for IL-17 production

Dectin-1 is known to associate with TLR2, and can induce synergistic effects on TLR2 responses (20, 21). In order to assess whether stimulation of both receptors could induce IL-17, we tested the combination of β-glucan with a TLR2 ligand (Pam3Cys). Although β-glucans synergized with Pam3Cys for the production of IL-1β, IL-10, IFNγ and TNFα, no IL-17 production was induced by this combination of stimuli (Fig. 7a). Preparations of β-glucans (β-glucan or curdlan) were not able to stimulate IL-17 production either alone or in combination with *C. albicans* mannan (data not shown). This is most likely due to the requirement of spacial presentation of the β-glucans in the fungal cell wall, stimulating dectin-1 more efficiently compared to the purified β-glucan preparations. Surprisingly, although Pam3Cys alone was not capable of inducing IL-17, combining Pam3Cys to *C. albicans* mannan resulted in strong synergism for IL-17 production (Fig. 7a). RT-PCR demonstrated that upregulation of mRNA for IL-17 was also synergistic (Fig. 7b). Interestingly, the mannan-induced IFNγ and IL-1β was significantly inhibited by stimulating the TLR2 pathway, while the production of IL-10 and TNFα by *Candida* mannan was unaffected by Pam3Cys (Fig. 7c).

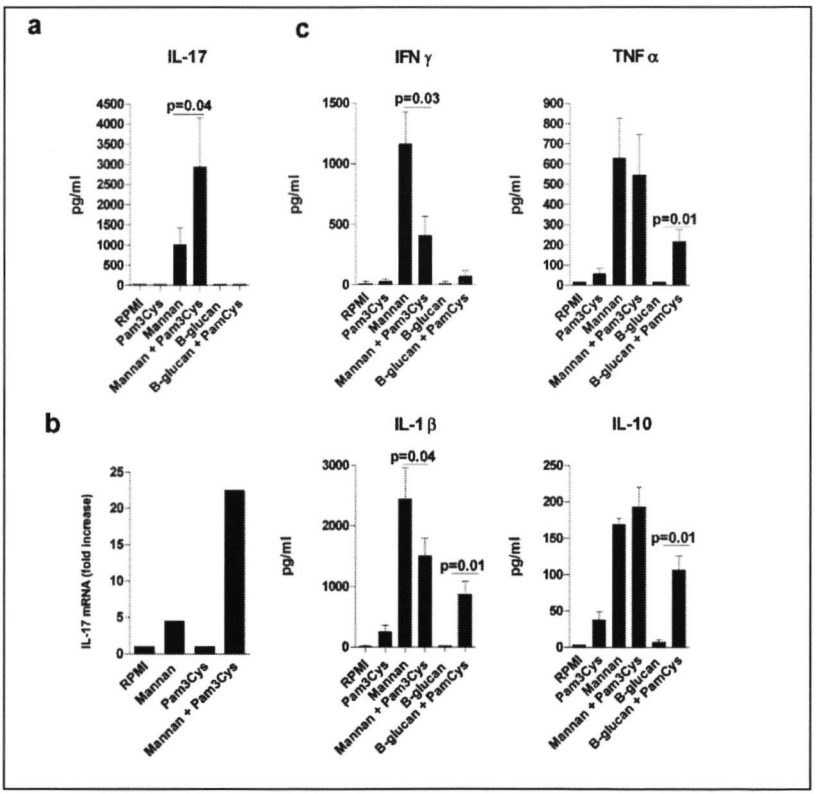

Figure 7. TLR2 is synergistic with the mannose receptor for IL-17 production.
(a) Human PBMCs were stimulated for 5 days with RPMI, fungal components or/and the TLR2 ligand Pam3Cys. IL-17 was measured by ELISA. (b) RT-PCR of the expression of IL-17A in PBMCs stimulated with RPMI, Pam3Cys and mannan or combinations for 3 days. Data is given as relative mRNA expression ($2^{-\Delta CT}$ x 1000). (c) Human PBMCs were stimulated for 48 h with the same set of stimuli used in (b) and IL-1β, IL-10, IFNγ and TNFα were measured by ELISA. (a,c) Data are pooled from at least 2 separate experiments with a total of 6 healthy volunteers (mean ± SEM). (b) Data is expressed as median; two separate experiments n=3.

Discussion

IL-17 has emerged as one of the most important pro-inflammatory cytokines, due to its modulation of neutrophil mediated responses, with important roles for both host defense (2, 5, 15) and autoimmunity (7-9). While most studies to date have focused on the conditions (cytokine profiles) necessary for generating Th17 cells in vitro (10, 11), or the generation of Th-17 cells in mice (22, 23), very little is known about the pathways regulating IL-17 upon the encounter of pathogenic microorganisms by human cells. In addition, in order to stimulate IL-17 production, these studies have used anti-CD3/anti-CD28 antibodies for inducing T-cell proliferation, artificial conditions obviously not encountered during in-vivo infections or inflammation. In the present study, we have investigated the pathways of IL-17 stimulation by human primary cells stimulated with whole microorganisms, conditions that mimic real life infections. In addition, we deciphered the receptor-ligand pathways responsible for IL-17 induction by the fungal pathogen *C. albicans*. We demonstrate that *C. albicans* is the most potent pathogen in eliciting a pathogen-mediated Th17 response in human PBMCs in the absence of anti-CD3 and/or anti-CD28 antibodies. The mannose receptor (MR) is the main pathway through which *C. albicans* induces the Th17 response. While no single TLR or NOD2 ligand was able to induce IL-17 in the absence of anti-CD3/anti-CD28 antibodies, mannan from *C. albicans* was the only component capable of inducing IL-17 secretion. Interestingly, the dectin-1/TLR2 pathway was able to synergize with the MR-induced IL-17 secretion, revealing a novel interaction between a C-type lectin receptor and TLRs. These data imply that direct recognition through the mannose receptor is sufficient for inducing a Th17 response, and that this ability is unique to the mannose receptor within the family of pattern recognition receptors.

Using anti-CD3 or a combination of anti-CD3 and anti-CD28 antibodies in order to investigate the pathways leading to the generation of Th17 cells, several studies suggested that either dectin-1 (13), NOD2 (14) or TLR (12) receptors specifically induce Th17 generation. However, these results are potentially biased by the use of anti-CD3 and anti-CD28 antibodies that can by themselves induce IL-17, as clearly demonstrated by our results. In this study we did not use anti-CD3/anti-CD28 antibodies to investigate the IL-17 responses to specific pathogens or ligands, but instead optimized the conditions necessary for the induction of IL-17 in human cells by pathogens alone. Human serum alone did not induce IL-17 secretion in human PBMCs, but was able to optimize the conditions needed for the induction of a Th17 response. Recently, it has been speculated that serum contains small amounts of TGF-β that may be responsible for the observed differences between IL-17 production of cells cultured in the absence or presence of human serum (24). Furthermore, we have addressed the role of serum antibodies in our experiments, by using serum from patients with X-linked agammaglobulinemia (XLA). These patients cannot produce antibodies because they lack functional B-cells. No differences were observed between pooled serum and XLA serum devoid of antibodies, concluding that IL-17 production was not antibody dependent (data not shown). Notably, in the absence of anti-CD3/anti-CD28, we show that no single TLR or NOD2 ligand was able to induce IL-17 production in human PBMCs.

IL-17 is important for the host defense against human pathogens (2, 5). Although both Gram-negative bacteria and the fungus *S. cerevisiae* were able to induce IL-17 production in human PBMCs, *C. albicans* was by far the most potent inducer of IL-17 secretion. The IL-17

production corresponded with the appearance of a subset of CD4+ cells that contained cells capable of producing IL-17, or both IFNγ and IL-17. Since *C. albicans* was the most potent inducer of IL-17 production, we wanted to further dissect the pathways responsible for inducing the Th17 response by this fungal pathogen. When purified fungal components were tested, we observed a strong IL-17 production in PBMCs upon stimulation with *C. albicans* mannan. This is in line with the fact that live *Candida* was even more potent in the induction of IL-17 production compared to heat-killed yeasts, since mainly mannoproteins are exposed when live *Candida* is used as a stimulus. Interestingly, *S. cerevisiae* mannan was not able to induce IL-17, and this is in line with the absent Th17 response to zymosan, as zymosan is derived from *S. cerevisiae* cell wall. The differential recognition of mannans from *C. albicans* and *Saccharomyces* is most likely due to the different branching of these structures: while *Candida*-derived mannan has a highly branched structure, *Saccharomyces* mannan mainly contains short linear chains of mannose polymers (25). These data argue that the MR is able to coordinate the different responses to fungal pathogens, particularly regarding the Th17 response. To investigate further the receptor pathways responsible for IL-17 induction by *Candida*, the main receptors involved in fungal recognition were blocked with specific antibodies, before stimulation of IL-17 was triggered. In line with the effects of *Candida* mannan, blocking of the MR resulted in the strongest inhibition of IL-17 production, confirming its important role in the Th17 response to *C. albicans*. Blockade of MR did not only result in a lower amount of IL-17, but also lead to a lower percentage of CD4+ cells that were able to produce IL-17 alone, or the combination of IFNγ and IL-17. Furthermore, blocking the MR with an excess of *S. cerevisiae* mannan or decreasing the MR expression with siRNA also inhibited IL-17 production.

It has been demonstrated in the literature that mannanoproteins contain immunodominant T cell epitopes (17). The mannoprotein 65 kDa (MP65) stimulates both T cell proliferation through its protein part, with the mannan component being responsible for the cytokine induction (26). Indeed, recombinant MP65 devoid of its mannan structure was unable to stimulate cytokine production (17). We have measured the amount of protein in our highly purified *C. albicans* mannan preparation. Despite the extensive purification procedures, we have measured a quantity of 0.77 µg/ml protein in the *C. albicans* mannan preparation with a concentration of 100 µg/ml. Although this amount is far lower than protein content in the MP65 used in the literature, which has a protein-to-polysaccharide ratio of 1.8:1 (27), we cannot exclude that these protein components induced TCR –dependent cell proliferation. These effects due to *Candida*-specific epitopes may in fact explain why the memory T cells were by far more effective for the release of IL-17, in comparison with the naïve T cells. Therefore we envisage a model in which TCR dependent effects are responsible for T cell activation/proliferation, while the mannan/MR interaction induces the skewing of this population towards a Th-17 phenotype. *Saccharomyces* mannan also contained these low amounts of protein. However, it did not stimulate IL-17 release, most likely due to the important differences in the branching of the mannan structures that does not allow proper stimulation of MR by *Saccharomyces* mannan (25).

Blockade of the TLR2/dectin-1 pathway partially inhibited IL-17 production, while TLR4 blockade had no effect. Stimulation of cells with both TLR2 and dectin-1 ligand alone or in combination did not result in IL-17 production, although synergism for the production of other cytokines has been previously reported (21) and confirmed in our study. Interestingly,

TLR2 showed a specific synergistic effect on the IL-17 production induced by the MR, while no such effect was apparent on IL-1β production. The role of dectin-1 was further investigated by stimulating PBMCs isolated from individuals homozygous for an early stop codon mutation in the dectin-1 gene, that leads to a total loss of dectin-1 activity (data not shown). When compared to cells isolated from healthy volunteers, the dectin-1-deficient PBMCs released less IL-17 after stimulation with *C. albicans*. The role of dectin-1 for the amplification of IL-17 production is supported by an additional line of evidence. *C. albicans* pseudohyphae do not express β-glucan on their surface (19), and this is likely why we observed a stronger Th17 response induced by *C. albicans* yeast cells, that stimulate both MR and dectin-1 trigger a stronger IL-17 response when compared to *C. albicans* pseudohyphae. This pattern was lost in PBMCs lacking dectin-1 expression on their membrane, again suggesting an amplification of β-glucan/dectin-1 on IL-17 production induced by other *C. albicans* components (e.g. mannan). It has been recently shown that the pathway of IL-17 induction by dectin-1 involves Syk and CARD9 (13). In contrast, when cells were stimulated with a combination of TLR ligands, or TLR ligands with the NOD2 ligand MDP, no IL-17 production was observed in human PBMCs. These data imply that the MR is the only receptor that can directly lead to IL-17 production in human PBMCs, and this pathway is amplified by dectin-1/TLR2 signaling.

In the present study we report a new methodology to investigate the pathogen-specific stimulation of Th17 cells, in conditions mimicking the in-vivo situation. We demonstrate the strong capacity of *C. albicans* to induce IL-17 production, and we have deciphered the receptor pathways involved. However, the data presented here also bring new questions into light and open new avenues for future research. Firstly, one of the most interesting observations regards the capacity of TLR2 ligation to amplify MR-induced IL-17 on the one hand, but to inhibit IFNγ production on the other hand. We and others have previously shown that TLR2 engagement is not able to induce Th1 cytokines (28-30). The data presented here demonstrate that the TLR2 pathway shifts the immune response towards a Th17 type, with important consequences for understanding pathophysiology of disease. In addition, previous studies have shown the capacity of TLR2 to induce Treg proliferation (31). It has been recently shown that FOXP3+ cells control the secondary induction of RORγt, and T_{REG} cells can be directed to induce differentiation of Th17 cells in the presence of IL-6 (32), or even differentiate themselves into Th17 cells (33). Our data suggest that TLR2 may play an important role in this process, but future studies are needed to assesss this hypothesis in more detail. Secondly, an important aspect to be investigated regards the pathways through which MR induces IL-17. Indeed, a very recent study has demonstrated that MR is recruited to the phagosome and has a crucial role for cytokine induction by *C. albicans* (34). However, the intracellular signaling induced by MR is yet unknown, and the cytokine profile induced by MR that is responsible for IL-17 induction is the first aim of future studies in our laboratory. All cytokines known to be required for the induction of IL-17, e.g. IL-23, IL-1β and IL-6 are stimulated by MR engagement by *C. albicans* or mannan, fulfilling a first set of conditions necessary for IL-17 production. However, none of these cytokines has been specifically induced only by or especially by MR, implying that an additional factor important for IL-17 production is induced by MR recognition. Thirdly, one has to acknowledge that although *C. albicans* was by far the most potent inducer of IL-17 production, the Gram-negative bacteria *E. coli*, *K. pneumonia* and *S. enteritidis* also stimulated small amounts of IL-17. TLR and NOD2 ligands alone or in combination did not stimulate IL-17 release, and therefore the precise

pathways through which Gram-negative bacteria stimulate IL-17 production remain to be elucidated.

In conclusion, we have analyzed for the first time the innate receptors and PAMPs responsible for inducing a Th17 response during a specific pathogen-host interaction. In anti-*C. albicans* host defense, the mannose receptor takes centre stage in the induction of the Th17 response, and this pathway can be augmented by TLR2/dectin-1. These data reveal a novel pathway leading to the induction of the Th17 response in human PBMCs. The essential position played by the mannose receptor warrants further investigation for its role in the Th17 response during infection or autoimmune diseases, and might lead to the design of novel immunotherapeutic strategies.

Experimental procedures

Volunteers
Blood was collected from 10 healthy, nonsmoking volunteers who were free of infectious or inflammatory disease, and from three patients that were defective in dectin-1 expression (Netea et al., personal communication), after informed consent. This was due to a SNP at position 238, leading to a premature STOP codon, and a defective transport of the truncated dectin-1 protein to the cell surface. Blood was collected by venipuncture into 10 ml EDTA syringes (Monoject). Serum was obtained from a patient with XLA after informed consent.

Reagents and ligands
Bartonella LPS was used as a potent TLR4 inhibitor (anti-TLR4) (35). TLR2-blocking antibody (anti-TLR2) and its control IgA1 (Catalog # maba-htlr2 and maba1 -ctrl) were purchased from InvivoGen (San Diego, CA). Anti-dectin-1 antibody GE2 (anti-Dectin-1) was a kind gift of Dr. Gordon Brown (University of Capetown, South Africa) (36). Mannose macrophage antibody (anti-MR) (catalog # 555953, clone 19.2) was purchased from BD biosciences (Dendermonde, Belgium). Matching mIgG1 isotype control (anti-IgG) was purchased from R&D systems (Minneapolis, MN). *E. coli* LPS (serotype 055:B5) was purchased from Sigma (St. Louis, MO) and re-purified as previously described and was used as an ultra pure TLR4 ligand (29). TLR ligands Pam3Cys, PolyI:C, Flagellin and ODN M362 were from InvivoGen (San Diego, CA). Synthetic MDP was purchased from Sigma (St. Louis, MO). Chitin was kindly provided by Prof. Neil AR Gow (School of Medical Scienses, Aberdeen, UK) and prepared according to previous protocols (37). *Candida* mannan and particulated β-glucan were prepared as previously described (38, 39). Briefly, for *Candida* mannan: 100 g wet wt of yeast biomass was suspended in 400 ml 2% (wt/vol) KOH and heated for 1 hour at 100°C. Insoluble residues were separated by centrifugation, and mannan was precipitated from supernatant with Fehling's reagent. The sedimented mannan-copper complex was dissolved in a minimum volume of 3 M HCl and added dropwise to methanol/acetic acid at a ratio of 8:1 (vol/vol). The procedure of dissolution and precipitation was repeated twice. Finally, the sediment was separated, dissolved in distilled water, and dialysed for 24 hours. The purified mannan was considered to be maximally deproteinized. Protein analysis by BCA kit (Pierce) measured 0.77 μg/ml of protein in the preparation of 100 μg/ml. Curdlan (WAKO) is a dectin-1 ligand consisting of linear β-1,3-glucan polymers derived from *Alcaligenes faecalis* (40). Zymosan and mannan (Lot 16H3842 from *Saccharomyces cerevisiae*) were from Sigma (St. Louis, MO). All stimulations with fungal ligands were in a concentration of 10 μg/ml, unless indicated

otherwise. Beads were coated with anti-CD3a alone or in combination with anti-CD28 and were prepared according to the manufacturer's instructions (Miltenyi Biotech, Utrecht, the Netherlands).

Microorganisms

C. albicans ATCC MYA-3573 (UC 820), a strain well described elsewhere (41) was used. *C. albicans* was grown overnight in Sabouraud broth at 37°C, cells were harvested by centrifugation, washed twice, and resuspended in culture medium (RPMI-1640 Dutch modification, ICN Biomedicals, Aurora, OH) (42). To generate pseudohyphae, *C. albicans* blastoconidia were grown at 37°C in culture medium, adjusted to pH 6.4 by using hydrochloric acid. Pseudohyphae were killed for 1h at 100°C and resuspended in culture medium to a hyphal inoculom size that originated from 10^6/ml blastoconidia (referred to as 10^6/ml pseudohyphae). All stimulations *with C. albicans* were with a concentration of 10^6/ml unless indicated otherwise. Clinical isolates of *Salmonella enteritidis*, *Kleibsiella pneumonia* and *Escherichia coli* were heat-killed and used in a dosage of 10^7/ml. A heat-killed clinical isolate of *S. cerevisiae* was kindly provided by Prof. Neil A.R. Gow and used in a dosage of 10^6/ml (School of Medical Sciences, Aberdeen, UK).

In vitro cytokine production

Separation and stimulation of PBMCs was performed as described previously (43). Briefly, the PBMCs fraction was obtained by density centrifugation of diluted blood (1 part blood to 1 part pyrogen-free saline) over Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden). PBMCs were washed twice in saline and suspended in culture medium supplemented with gentamicin 1%, L-glutamine 1%, and pyruvate 1%. The cells were counted in a Bürker counting chamber, and their number was adjusted to 5×10^6 cells/ml. 5×10^5 PBMCs in a volume of 100 µl per well were incubated at 37°C in round-bottom 96-wells plates (Greiner, Nuremberg, Germany), in the presence of 10% human pooled serum (unless indicated otherwise), with stimuli or culture medium alone. In blocking experiments, PBMCs were preincubated for 1 h with various inhibitors (anti-TLR4 100 ng/ml, anti-TLR2 10 µg/ml, anti-Dectin-1 10 µg/ml, anti-MR 5 µg/ml, anti-IgG1 10 µg/ml) before stimulation with *C. albicans*. After 48 h or 5 days of incubation, supernatants were collected and stored at –20°C until assayed.

Cytokine assays

IL1β, IL-17, and TNFα concentrations were measured by commercial ELISA kits (R&D systems); IL-6, IFNγ, IL-10 (Pelikine Compact, Sanquin, Amsterdam, The Netherlands); IL-23 (eBioscience) according to the manufacturers instructions. IL-12p70 and IL-2 were measured using the Bioplex (Luminex) cytokine assays from Bio-Rad (Hercules, CA), following the manufacturer's instructions.

Intracellular cytokine staining

PBMCs cells were stimulated for 4-6 hours with PMA (50 ng/ml; Sigma) and ionomycine (1 ug/ml; Sigma) in the presence of Golgiplug (BD biosciences) according to manufacturers protocol. Cells were first extracellularly stained using an anti-CD4 APC antibody (BD biosciences). Subsequently the cells were fixed and permeabilized with Cytofix/ Cytoperm solution (BD biosciences) and then intracellularly stained with anti-IFNγ PE (eBiosciences)

and anti-IL-17 FITC (eBiosciences). Samples were measured on a FACS Calibur and data were analyzed using the CellQuest-Pro software (BD biosciences).

Cell isolation, stimulation and flow cytometry

Isolation of CD4+ cells was performed as described previously (33). Purified CD4 positive T cells were labeled with CD25-PEconjugated mAb-(MA251; BD Biosciences), and CD45RA-ECD conjugated mAb (2H4; Beckman-Coulter, Mijdrecht, The Netherlands); thereafter CD25negCD45RApos cells and CD25negCD45RAneg were isolated by high-purity flow cytometric cell sorting using an Altra cell sorter (Beckman-Coulter). A rerun was performed to analyze the cell purity of the sorted cells; sorted cells were always more than 98% pure. Monocytes were positively isolated from PBMCs with magnetic bead isolation using CD14 microbeads (Miltenyi-Biotec, Utrecht, The Netherlands) according to the manufacturers' instruction. Cells were cultured in culture medium (RPMI-1640 with glutamax supplemented with pyruvate [0.02 mM[, 100 U/mL penicillin, 100 µg/mL streptomycin [all from Gibco, Paisley, United Kingdom], and 10% human pooled serum [HPS]) at 37°C, 95% humidity, and 5% CO2, in 96-well round-bottom plates (Greiner, Frickenhausen, Germany). Sorted naïve (CD25-CD45RA+) and memory (CD25-CD45RA+) cells (2.5×10^4) populations were cultured with or without autologous monocytes (1.0×10^5) in 200 µL culture medium in the absence or presence of stimuli. All stimuli were added at the start of the cultures. Cells were phenotypically analyzed by flow cytometry. The following conjugated mAbs were used: CD3-(UCHT1) ECD-labeled (Beckman Coulter). Intracellular analysis of cytokines was performed following stimulation for 4 hrs with PMA (12.5 ng/mL) plus ionomycin (500 ng/mL) in the presence of BrefeldinA (5 µg/mL; Sigma-Aldrich). Cells were fixed and permeabilized using Fix and Perm reagent (eBioscience) and subsequently stained with anti-IL-17-alexa fluor 647 -(64DEC17; eBioscience). Cell samples were measured on a FC500 flow cytometer (Beckman-Coulter), and flow cytometry data were analyzed using CXP software (Beckman-Coulter).

Quantitative PCR

PBMCs were cultured for 3 days and RNA was extracted using TRI-reagent (Sigma). The isolated RNA was treated with DNase to remove genomic DNA and subsequently reverse transcribed with oligo(dT) primers in a reverse transcriptase procedure with a total volume of 20 µl. Quantitative real-time PCR was performed using the ABI/PRISM 7000 Sequence Detection System, with primer pairs and SYBR Green PCR Master Mix (Applied Biosystems). The sequences used, were as follows: IL-17A, forward 5'-TTGATTGGAAGAAACGATGA-3' reverse 5'-CTCAGCAGCAGTAGCAGTGACA-3'; *RORγT*, forward 5'TGAGAAGGACAGGGAGCCAA 3' reverse 5'-CCACAGATTTTGCAAGGGATCA -3'; MR, forward 5'-CTCAAGACAATCCACCAGTTACTGA-3' reverse 5'-CTTCTCTTTGCTGAAATAATACTGGTAGTC -3'. Quantification of the PCR signals was performed by comparing the cycle threshold value (Ct) of the gene of interest with the Ct values of the reference gene GAPDH. All primers were developed using Primer Express 2.0 (Applied Biosystems) and validated according to the protocol. Values are expressed as fold increases of mRNA relative to that in unstimulated cells.

siRNA transfection

PBMCs were transfected by electroporation with the Amaxa Human Monocyte Nucleofector kit (Amaxa Inc.) in accordance with the manufacturer's instructions. In brief, PBMCs (2×10^7) were harvested and resuspended in 100 µl of nucleofector solution. After addition of MR

siRNA (Dharmacon Inc., 011730) or control GFP siRNA (Amaxa Inc., VSC-1001) at final concentration of 125 nM, cells were electroporated with Amaxa program Y-001 and recovered for 24 h before further stimulation.

Statistical analysis

The differences between groups were analyzed by a two-tailed paired t-test. Differences were considered statistically significant when $P \leq 0.05$, and the actual P value is given for each test. All experiments were performed at least twice, and the data presented as the cumulative result of all experiments performed, unless otherwise indicated.

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Chapter 11

The *Candida* Th17 response is dependent on mannan- and β-glucan-induced Prostaglandin E2

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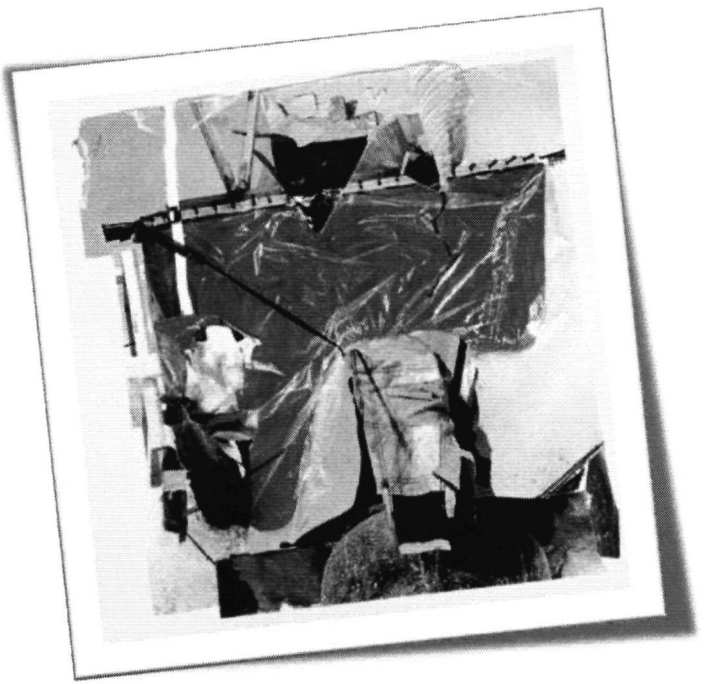

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* Both authors contributed equally to this work.

Summary

Background: The fungus *Candida albicans* is a potent inducer of the Th17 response. Prostaglandin E2 (PGE2) is a strong proinflammatory mediator, which has proven to be essential for the Th17 response. In this study, we wanted to investigate the role of PGE2 in the Th17 response induced by *C. albicans* in humans.

Methods: Peripheral blood mononuclear cells (PBMCs) were stimulated with *Candida albicans* in the absence or presence of a non-steroidal anti-inflammatory drug (NSAID). In separate experiments, PGE2 or the agonists of prostlaglandin receptors butaprost or misoprostol were added to the cells. PBMCs were also stimulated with fungal components and siRNA for mannose receptor (MR) was performed. PGE2 and cytokines were measured by ELISA or luminex.

Results: Blocking *Candida*-induced PGE2 production by an NSAID resulted in decreased IL-17 and IL-22 production and inhibited the expression of RORγT mRNA. Furthermore when PGE2 production was blocked, IL-6, IL-23 and IL-10 were decreased, while TNFα increased. Stimulation with PGE2 or EP2/EP4 agonists restored IL-17 production. *C. albicans* mannan was the only fungal component that was able to directly induce PGE2 and silencing of the MR resulted in a reduction of *Candida* induced PGE2. β-glucan engagement of dectin-1 synergistically increased TLR2-induced PGE2 production.

Discussion: These data provide evidence that the PGE2 pathway is important for the Th17 response induced by *C. albicans,* and that PGE2 is induced by the fungal components mannan and β-glucan that are recognized by the MR and the dectin-1/TLR2 pathway, respectively.

Introduction

Prostaglandin E2 (PGE2) is the most widely produced prostanoid in the body in response to proinflammatory cytokines (1), and plays an important role in the regulation of inflammatory responses. It is primarily involved in the induction of the classical signs of inflammation, namely: erythema, increased vascular permeability, edema, pain and fever (2-4). Furthermore, PGE2 alters the capacity of antigen presenting cells (APCs) and T cells to produce certain cytokines, and may therefore influence the functional phenotype of T cells during priming (5). PGE2 favors a Th2 response, by inhibiting the production of the Th1 cytokine IFN-γ, and by up regulating the production of the Th2-associated cytokines IL-4 and IL-5 (6).

The recently discovered T helper subset called Th17 cells plays an important role in the protection against extracellular bacteria and fungi (7). Th17 cells are characterized by the production of IL-17A (IL-17), IL-17F, IL-21 and IL-22 (7). IL-17 is capable of initiating and maintaining inflammation, and plays an important role in auto-immune diseases like rheumatoid arthritis, multiple sclerosis and psoriasis (8). The early differentiation of Th17 cells is initially regulated by IL-1β signaling (9), while IL-23 plays an important in role in the amplification and late stage of Th17 development (10). Interestingly, PGE2 induces IL-23 production (11-13), and together with IL-23 synergistically favors human Th17 expansion (1,

14). PGE2 has also been reported to be necessary for the production of the Th17 effector cytokine IL-17 (15). Furthermore, it has been shown that human memory T cells induce a robust Th17 response in reaction to the fungus *Candida albicans* (16, 17).

In the present study, we investigated the mechanisms through which *C. albicans* induces PGE2 production, and whether this influences the *Candida*-induced Th17 response. It is shown that *C. albicans* is a potent inducer of PGE2, and that the *Candida* induced Th17 response is dependent on the induction of prostaglandins. *C. albicans* mannan was the only fungal component that was able to directly induce PGE2 production, while β-glucan exerted synergistic effects on TLR2-induced prostaglandins.

Material and Methods

Volunteers
Blood was collected from healthy volunteers who were free of infectious or inflammatory disease, after informed consent was given. Blood was collected by venipuncture into 10 ml EDTA syringes (Monoject, s-Hertogenbosch, The Netherlands).

Reagents
The following study materials were used: anti-CD3 and anti-CD28 coated (αCD3αCD28) beads prepared from a T cell activation/expansion kit (MACS milteny biotec, Bergisch Gladbach, Germany); Pam3Cys (EMC microcollections, Tuebingen, Germany). Chitin was kindly provided by Prof. Neil AR Gow (School of Medical Scienses, Aberdeen, UK) and prepared according to previous protocols (18). *Candida* mannan and particulated β-glucan were are a kindly provided by Prof. David Wiliams and were prepared as previously described (19, 20). Curdlan was purchased from (WAKO, Richmond, USA) and diclofenac from Novartis Pharma (Arnhem, the Netherlands). PGE2, butaprost and misoprostol were commercially purchased (Sigma-Aldrich, Zwijndracht, the Netherlands). Culture medium used in the experiments was RPMI 1640 Dutch modifications (Sigma-Aldrich) supplemented with 1% gentamicin, 1% L-glutamine and 1% pyruvate (Life Technologies, Nieuwekerk, the Netherlands).

Microorganism. C. albicans
ATCC MYA-3573 (UC 820) (21) was grown overnight in Sabouraud broth at 37°C, cells were harvested by centrifugation, washed twice, and resuspended in culture medium. *C. albicans* was heat-killed for one hour at 100°C.

In vitro cytokine production
Separation and stimulation of PBMCs was performed as described previously (22). Briefly, the PBMCs fraction was obtained by density centrifugation of diluted blood (1 part blood to 1 part pyrogen-free saline) over Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden). PBMCs were washed twice in saline and suspended in culture. The cells were counted in a hemocytometer, and their number was adjusted to 5×10^6 cells/ml. 5×10^5 PBMCs in a volume of 100 µl per well were incubated at 37°C in round-bottom 96-wells plates (Greiner, Nurnberg, Germany), in the presence of 10% human pooled serum, with *C. albicans* or culture medium alone. After 1,2 or 7 days of incubation, supernatants were collected and stored at –20°C until assayed.

Cytokine assays
The concentration of IL-1β, IL-17, IL-22, IL-23 and TNF-α (Sanquin, Amsterdam, the Netherlands) and IL-6, IL-10 and IFN-γ (R&D Systems, Abingdon, UK) were measured in cell culture supernatants using enzyme-linked immuno sorbent assay (ELISA), according to the instructions of the manufacturer. IL-2, IL-4 and IL-12p70 were measured by using Luminex technology, according to instructions of the manufacturer (BioRad, Veenendaal, the Netherlands).

RT-PCR
Two million freshly isolated PBMC were incubated with the various stimuli. After 24 hours of incubation at 37°C, total RNA was extracted in 800 µl of TRIzol reagent (Invitrogen, Breda, the Netherlands). Isolated RNA was being reverse transcribed into complementary DNA using oligo(dT) primers and MMLV reverse transcriptase. PCR was performed using a 7300 realtime PCR system (Applied biosystems, Lennik, Belgium). Primer sequences for human RORγT: sense: 5'- TGA-GAA-GGA-CAG-GGA-GCC-AA-3' and antisense: 5'- CCA-CAG-ATT-TTG-CAA-GGG-ATC-A-3'. B2M was used as a reference gene, for which the primers were: 5- ATG-AGT-ATG-CCT-GCC-GTG-TG-3 (forward) and 5- CCA-AAT-GCG-GCA-TCT-TCA-AAC-3 (reverse). PCR conditions were as follows: 2 minutes at 50°C and 10 minutes at 95°C, followed by 40 cycles of PCR reaction at 95°C for 15, and 60°C for 1 minute.

siRNA transfection
PBMCs were transfected by electroporation with the Amaxa Human Monocyte Nucleofector kit (Lonza, Cologne, Germany) in accordance with the manufacturer's instructions. In brief, PBMCs (2×10^7) were harvested and resuspended in 100 µl of nucleofector solution. After addition of MR siRNA (Dharmacon, Epson, UK, 011730) or control GFP siRNA (Lonza, VSC-1001) at final concentration of 125 nM, cells were electroporated with Amaxa program Y-001 and recovered for 24 hours before further stimulation. Both MR expression on the cell surface and mRNA expression for MR were decreased after MR siRNA transfection compared to GFP siRNA transfection (data not shown).

Statistical analysis
Experiments were performed in duplicate, and supernatants were pooled. The differences between groups were analyzed using the Wilcoxon signed rank test. The level of significance between groups was set on $p < 0.05$. Data are presented as mean ± SEM.

Results

Candida albicans-induced IL-17 and IL-22 is dependent on PGE2
To determine whether *C. albicans* could induce PGE2 production, PBMCs from 8 healthy volunteers were stimulated for 48 hours with *C. albicans* blastoconidia. Stimulation of *C. albicans* resulted in PGE2 production by PBMCs (Figure 1a). In addition, when PBMCs were stimulated with *C. albicans* in the presence of the prostaglandin inhibitor diclofenac, the PGE2 production was completely blocked (Figure 1a). To investigate if the Th17 reponse induced by *C.albicans* is PGE2 dependent, PBMCs were stimulated for 7 days with *C. albicans* or beads coated with αCD3αCD28, in the absence or presence of diclofenac. Both IL-17 and IL-22 were produced upon stimulation with *C. albicans*, and both cytokines were lower when PCMCs were cultured in the presence of diclofenac (Figure 1b). Mitogenic T cell stimulation

with αCD3αCD28 beads resulted in IL-17 production, but not IL-22 production. The IL-17 induced production by beads was not influenced by PGE2 inhibition (Figure 1b). In addition, RORγT mRNA expression was decreased in the presence of addition of an NSAID to the system (Figure 1c). To assess whether the decrease in IL-17 production caused by the NSAIDs was due to the absence of PGE2, we added exogenous PGE2 to assess whether this could restore IL-17 production. Adding PGE2 to the cell culture partially restored IL-17 production (Figure 1d). Also butaprost, which is a selective EP2 receptor agonist, and misoprostol, a non selective-agonist with the highest affinity for the EP4 and EP3 receptor, restored the NSAID-inhibited IL-17 production (Figure 1d). PBMCs in the presence of PGE2, butaprost or misoprostol alone did not produce IL-17 (data not shown).

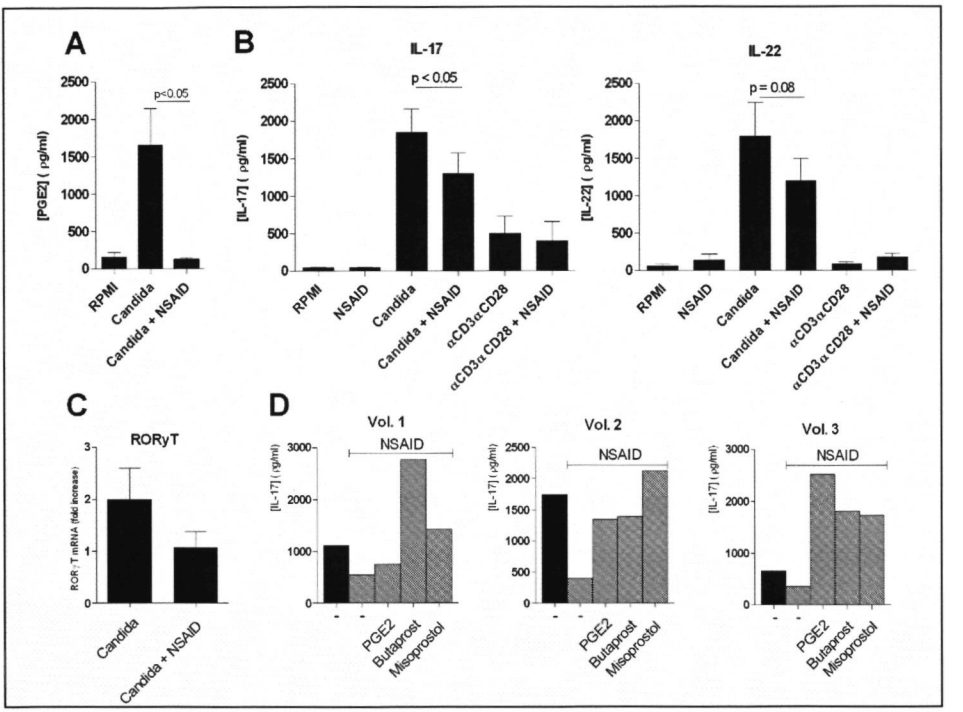

Figure 1. *Candida albicans*-induced IL-17 and IL-22 is dependent on PGE2.
(a) Human PBMCs were stimulated for 48 hours, in the presence of human serum, with RPMI or *C. albicans* and in the absence or presence of NSAIDs (n=11). (b) Human PBMCs were cultured with human serum for 7 days with RPMI, *C. albicans* or αCD3αCD28 beads, in the presence or absence of NSAIDs. Production of IL-17 (n=20) and IL-22 (n=7) in the supernatants was measured by ELISA. (c) qPCR of RORγt mRNA expression in human PBMCs that were stimulated for 24 hours, in the presence of human serum, with RPMI or *C. albicans* and in the absence or presence of NSAIDs (n=5). (d) Human PBMCs were stimulated for 7 days, in the presence of human serum, with *C. albicans* in the absence or presence of NSAIDs and in the absence or presence of PGE2 (10 mM), butaprost (10 μM) or misoprostol (35 μM) (n=3). Cytokines and PGE2 were measured by ELISA. (a-c) Data are pooled and expressed as mean ± SEM.

C. albicans mannan is the only fungal component that directly induces PGE2 production
To elucidate which fungal components were responsible for the PGE2 production induced by *C. albicans*, PBMCs were cultured with RPMI, mannan, β-glucan or curdlan, or chitin and PGE2 production was measured in cell culture supernatants. Interestingly, *C. albicans*

mannan was the only component able to induce PGE2 production (Figure 2a). In line with this, when the transcription of the MR was inhibited using siRNA, PGE2 production decreased (Figure 2b). β-glucan and curdlan alone were not able to induce PGE2. Also, the TLR2 ligand Pam3Cys alone was not able to induce PGE2 production. However, β-glucan and curdlan were able to synergize with the TLR2 ligand Pam3Cys and induce PGE2 production (Figure 2c).

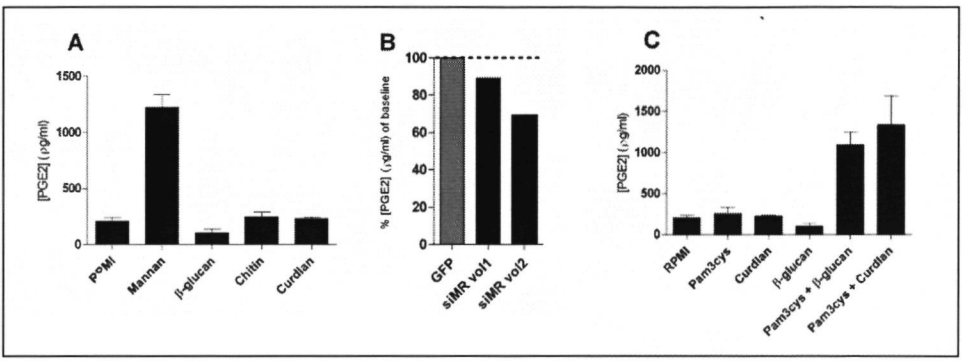

Figure 2. *C. albicans* mannan is the only fungal component that directly induces PGE2 production.
(a) Human PBMCs were stimulated for 48 hours in presence of human serum with RPMI, mannan, β-glucan, chitin, curdlan. (n=4) (b) PGE2 production in cells from two healthy volunteers transfected with control siRNA (GFP) or MR siRNA and stimulated with RPMI or *C. albicans*. (c) Human PBMCs were stimulated for 48 hours in presence of human serum with RPMI, mannan, β-glucan or curdlan, in the absence or presence of Pam3Cys (n=6). Production of PGE2 in the supernatants was measured using ELISA. Data are pooled and expressed as mean ± SEM.

Prostaglandin E2 skewes the cytokine profile favoring a Th17 response
To determine the effects of prostaglandins on the cytokine profile induced by *C. albicans*, we stimulated human PBMCs with *C. albicans* in the presence or absence of the prostaglandin inhibitor diclofenac. Strikingly, IL-6 and IL-23 decreased when PBMCs were preincubated with diclofenac. In addition, IL-10 decreased when PGE2 was blocked, while TNF-α increased (Figure 3). IL-1β was not affected by inhibition of PGE2 production.

Discussion

In the present study we provide evidence for an important role of PGE2 in the *Candida* induced Th17 response. PGE2 contributes to the *Candida*-induced Th17 response by inducing the Th17 polarizing cytokines IL-6 and IL-23. Furthermore, the fungal components mannan and β-glucan were shown to be responsible for the induction of PGE2 production.

In line with previous studies, we show that *C. albicans* is capable of inducing a strong PGE2 production in human PBMCs (23). A notable difference is that most studies used viable *C. albicans*. It has been reported that live *C. albicans* can produce PGE2 (24, 25), this is why we used heat-killed *C. albicans* to investigate the induction and functional role of PGE2 produced by the host. Boniface *et.al.* found that PGE2 is necessary for the production of IL-17 in the presence of antigen presenting cells (APCs), and that PGE2 directly promotes differentiation and proinflammatory functions of Th17 cells, while differentially regulating

IFN-γ production (15). In line with this, we found that blocking PGE2 production resulted in reduced RORγT mRNA expression, IL-17 and IL-22 production, and increased TNFα production. This indicates that PGE2 favours a Th17 response. In line with these findings, the addition of PGE2 partially restored the IL-17 production. Furthermore, PGE2 signals through the EP2 and EP4 receptors and both receptors are important for mediating Th17 responses (15, 26, 27). Our observations suggest that PGE2 induced by *Candida* also signals through the EP2 and EP4 receptor, since stimulation of these receptors in the absence of PGE2 partially, and sometimes completely, restored the *Candida*-induced Th17 response. These data further support the concept that the Th17 response induced by *C. albicans* is dependent on the PGE2 signaling pathway.

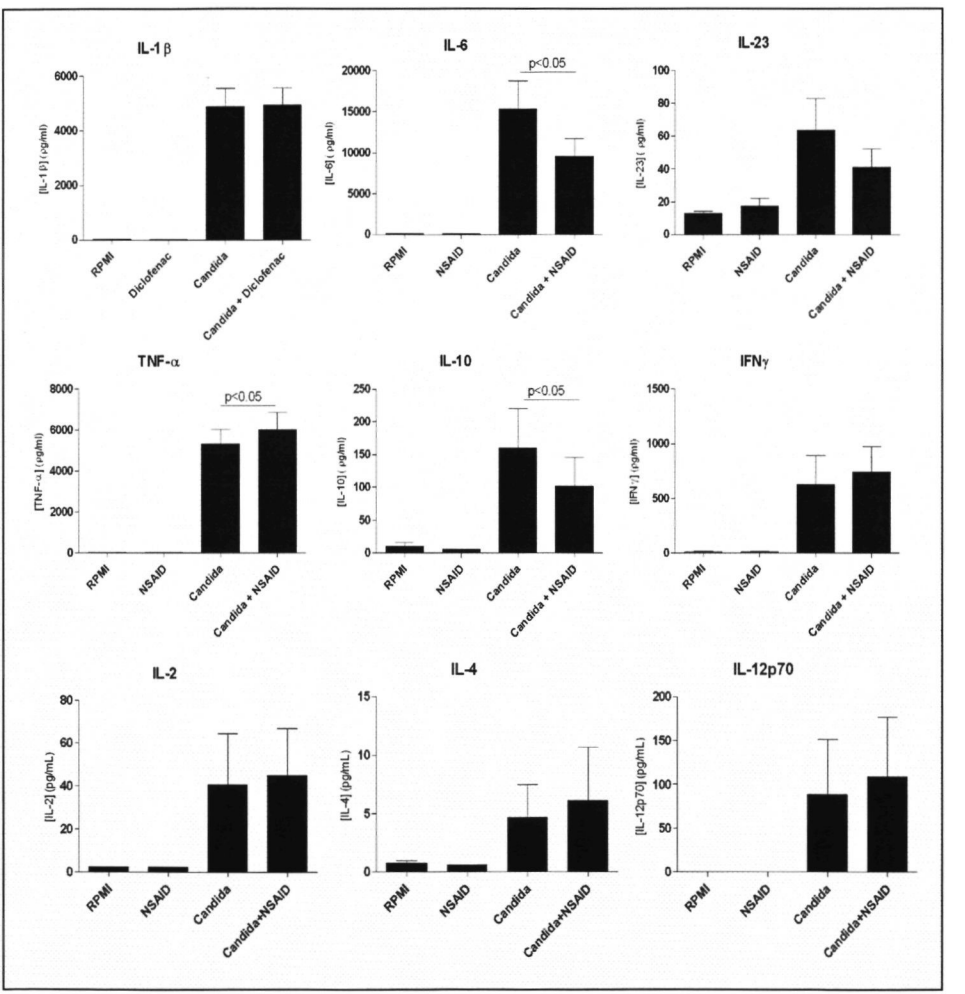

Figure 3. Prostaglandin E2 skewes the cytokine profile favoring Th17 response.
Human PBMCs were stimulated for 24 or 48 hours with RPMI or *C. albicans*, in the presence or absence of diclofenac. IL-1β, IL-6, IL-23, TNF-α, IL2, IL4 and IL-12p70 (24 hours stimulations), IL-10 and IFN-γ (48 hours stimulations) were measured in the supernatants by ELISA or Luminex (n=6). Data are pooled and expressed as mean ± SEM.

However, although in certain conditions the PGE2 release is completely blocked, the production of IL-17 and IL-22 is not completely inhibited. This indicates that other mechanisms contribute to the fungal-induced Th17 responses. Importantly, blocking PGE2 did not effect IL-1β, and there was still IL-6 and IL-23 production. Since IL-1β is essential for the early development of the Th17 response, the IL-1 pathway is most likely responsible for the IL-17 production present in the absennce of PGE2. In addition, NSAIDs are not associated with mucosal candidiasis, while a defect in the Th17 response has been linked to mucosal candidiasis (28, 29). The residual production of IL-17 is thus most likely to be sufficient to maintain adequate host defense against mucosal candidiasis when NSAIDs are used.

In a subsequent set of experiments, we tried to elucidate which pathogen recognition receptors were involved and which components of *C. albicans* were responsible for the induction of PGE2. Mannans were the only fungal components that were able to directly induce PGE2 production. We have previously reported that mannan from *C. albicans* was the only component capable of inducing IL-17 production (17). Furthermore, blocking of the MR with siRNA lead to inhibition of PGE2 production, suggesting that the MR receptor plays an important role in the induction of PGE2 production by PBMCs. This is supported by the previous observation that overexpression of the MR in HeLa cells which are challenged with mannan results in a significant induction of COX-2 expression (30). Another C-type lectin, the β-glucan receptor dectin-1, also plays an important role in anti-*Candida* host defense, and prostaglandin production can be enhanced by overexpressing dectin-1 in a mouse macrophage cell line (31). However, β-glucans alone did not stimulate PGE2 production (31). In line with the known and previously reported synergism of dectin-1 and TLR2 (32, 33), β-glucans strongly increased the production of PGE2 induced by TLR2. Furthermore, dectin-1 and TLR2 have also been linked to the induction of the Th17 response (17). In contrast, we previously found that β-glucans or curdlan did not augment the mannan induced IL-17 response (17). Therefore, β-glucans contribute through the dectin-1/TLR2 pathway to the *Candida* specific Th17 response by inducing the production of PGE2 by the host.

To understand how PGE2 polarizes the immune response towards a Th17 profile, we investigated the changes in cytokine profiles when PGE2 production was inhibited. PGE2 is known to have a potent IL-6 inducing effect on monocytes (34). We observed that blocking of PGE2 by diclofenac reduced the production of IL-6 induced by *Candida*. IL-6 has been reported to be essential for the induction of Th17 memory cells in humans (35). This suggests that skewing of PGE2 towards a Th17 response is dependent on IL-6. When PGE2 production was blocked, the induction of IL-23 by *Candida* was lower. The role of IL-23 in the Th17 response has been well established and it is now generally accepted that it IL-23 is needed to maintain the Th17 response (10, 36). Furthermore, it has been suggested in an experimental inflammatory bowel disease model that the proinflammatory effects of PGE2 are due to the induction of dendritic cells derived IL-23 that subsequently supports the Th17 response (13). Interestingly, we found no effect on IL-1β when we blocked PGE2 production. Since IL-1β is one of the most important cytokines in the induction of an early Th17 response (9), it is therefore notable that PGE2 does not induce the Th17 response directly through IL-1β. We found an increase of TNF-α when PGE2 was inhibited, which is in line with the literature which has reported that PGE2 inhibits TNF-α (37). However, the exact role of TNF-α in the Th17 response is not clear and still has to be elucidated (38). Finally, we found that IL-10 production was lower in the presence of a PGE2 inhibitor. Veldhoen *et al.* already

speculated that IL-10 production, that suppresses IL-12, might aid the development of IL-17 producing T cells, and subsequently reported that anti-IL-10 caused a reduction in the proportion of T-cells that were able to produce IL-17 (39). Therefore, the loss of the suppressive effects of IL-10 could be a possible explanation for the observed lower IL-17 production when PGE2 production was blocked. This would ultimately result in a stronger Th1 response that is able to subvert the Th17 response. In line with this hypothesis, we observed that IL-12p70 and IFNγ were higher in the absence of PGE2, although this did not reach statistical significance.

In conclusion, prostaglandins were shown to play an important role in the *Candida albicans* induced Th17 response. Prostaglandin E2 (PGE2) production induced by *C. albicans* skewes the cytokine profile towards a Th17 response, by decreasing IL-6, IL-23, IL-17 and IL-22. Interestingly, *C. albicans* mannan, which is the main inducer of the Th17 response in *C. albicans*, was the only fungal component that was able to directly induce PGE2. Furthermore, dectin-1, the other major pathway important for the the induction of a Th17 response, was shown to act synergistically on the TLR2 induced PGE2 production. These data provide evidence that the Th17 response induced by the MR and dectin-1/TLR2 is enhanced by prostaglandins, which in turn skew the cytokine profile favoring Th17 polarization.

Acknowledgements

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Chapter 12

Differential effects of IL-17 pathway in disseminated candidiasis and zymosan-induced multiple organ failure

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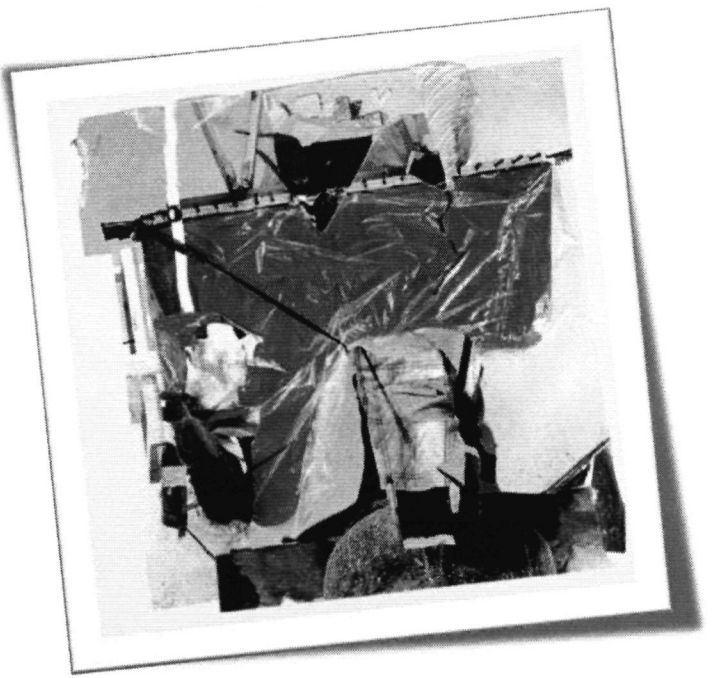

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Summary

The role of the IL-17 pathway in antifungal host defense is controversial. Several studies suggested that IL-17 is crucial for the protection against *Candida* infection, while other studies reported that IL-17 may contribute to inflammatory pathology and worsening of fungal disease. To address these discrepancies, we assessed the differential role of IL-17 pathway in two models of fungal sepsis: intravenous infection with live *C. albicans*, in which fungal growth is the main cause of mortality, and zymosan-induced multiple organ failure in which the inflammatory pathology drives the mortality. Firstly, IL-17 receptor deficient (IL-17RA-/-) mice showed increased mortality and higher fungal loads in the kidneys in the model of disseminated candidiasis, partly due to lower neutrophil recruitment in the IL-17RA-/- mice. Secondly, IL-17RA-/- mice were not protected against the multi-organ failure induced by zymosan. These data demonstrate that IL-17 does not have a major contribution to the inflammatory pathology leading to organ failure in fungal sepsis, and support the concept that the IL-17 pathway is protective in antifungal host defense.

Introduction

T helper 17 cells (Th17 cells) which are characterized by the production of interleukin-17A (IL-17), IL-17F, IL-21 and IL-22 have been shown to be crucial in host defense against several pathogenic microorganisms (1-3). IL-17 induces infiltration of neutrophilic granulocytes at the site of infection, and is also able to stimulate activation of macrophages (2). In systemic candidiasis, the main host defense mechanism is phagocytosis and killing of *C. albicans* by neutrophilic granulocytes, monocytes, and macrophages (4, 5).

IL-17 has been linked to neutrophil recruitment in several fungal infection models. Neutrophil influx was demonstrated to be decreased in the kidneys of IL-17RA-/- mice during disseminated candidiasis (6). Furthermore, mobilization of the neutrophils in the blood during systemic challenge with *C. albicans* was also impaired (6). Similarly, IL-17RA-/- showed a reduced recruitment of neutrophils to the local site of inflammation in an oral model of candidiasis (7). Intratracheal challenge with *Aspergillus fumigates* in dectin-1-/- mice resulted in insufficient neutrophil recruitment to the lung and uncontrolled fungal outgrowth (8). IL-17 was shown to be dependent on dectin-1 and neutralization of IL-17 resulted in decreased fungal clearance in the lungs, linking IL-17 to neutrophil recruitment in pulmonary fungal infection. In addition, a recent study demonstrated that IL-17 plays an important role in neutrophil recruitment to the lungs in a pulmonary fungal infection model with *Paracoccidioides brasiliensis* (9). Taken together, these data point to a crucial protective role for IL-17 in the recruitment of neutrophils during fungal infection. However, others have proposed that the Th17-induced activation of neutrophils may also result in an overwhelming inflammatory tissue response which impairs antifungal immune resistance and leads to defective pathogen clearance (10). The cause of the discrepancies between these studies is not known.

In the present study, the role of the IL-17 pathway was investigated in two experimental models, using IL-17RA-/- and control mice. The role of the IL-17 pathway in anti-*Candida* host defense was assessed in a mouse model of disseminated candidiasis, while the contribution of IL-17 to an overwhelming inflammatory tissue response was investigated in a

septic multiple organ failure model based on the injection of the fungal component zymosan. The latter model is characterized by neutrophil influx and severe tissue inflammation that leads to organ failure and ultimately death (11). By using these two models we dissected the inflammatory mechanisms during fungal sepsis, in order to discern between the potential harmfull and protective effects of the IL-17 pathway that have been described in *Candida* infections.

Materials and methods

Animals

Experiments were performed using IL-17 receptor knockout (IL-17RA-/-) mice (kindly provided by dr. J.J. Peshon, Amgen, Seattle) and wild-type mice, 7 to 9 weeks old, weighing 20-25 g. The IL-17RA-/- mice were characterized elsewhere (1). The animals were fed standard chow (Hope Farms RMB-H, Woerden, The Netherlands) and acidified water *ad libitum*. The day/night cycle was 12 h/12 h. Before use, the animals were allowed to acclimatize for 5 days. The experiments were approved by the Animal Ethics Review Committee of the Radboud University Nijmegen Medical Centre.

Candida albicans infection model

C. albicans ATCC MYA-3573 (UC 820), a strain well described elsewhere (12), was used in all experiments. *Candida* was grown overnight in Sabouraud broth at 37°C, cells were harvested by centrifugation, washed twice, and resuspended in culture medium in culture medium (RPMI-1640 Dutch modification, ICN Biomedicals, Aurora, OH) (13). *Candida* yeasts were killed for 1h at 100°C and resuspended in culture medium. IL-17 RA-/- mice and their control littermates were injected intravenously with *C. albicans* blastoconidia (2×10^5 CFU/mouse) in a 100 µl volume of sterile pyrogen-free phosphate-buffered saline (PBS). Survival was assessed daily for three weeks. Subgroups of 5 animals were killed on days 3 and 7. To assess the tissue outgrowth of the microorganisms, the kidneys of the sacrificed animals were removed aseptically, weighed, and homogenized in sterile saline in a tissue grinder. The number of viable *Candida* cells in the tissues was determined by plating serial dilutions on Sabouraud dextrose agar plates as previously described (5). The CFU were counted after 24h of incubation at 37°C, and expressed as log CFU/g tissue.

Zymosan-induced generalized inflammation

The zymosan-induced generalized inflammation model for multi-organ failure has been described previously (14). To improve reproducibility and to limit mortality in the first phase, a small modification to the initial model was introduced, consisting of an aseptic intraperitoneal injection of 40 µg lipopolysaccharide (LPS;*Escherichia coli*, Sigma Chemical Co., St. Louis, MO) dissolved in 200 µL of phosphate-buffered saline 6 days before zymosan administration [13]. Zymosan A (Sigma) was sterilized by gamma radiation (5 kGray) and homogeneously suspended in sterile paraffin oil (25 mg/mL) by high frequency vibration for 1 h. After sonification, the suspension was sterilized again in a waterbath at 100°C for 80 min. All suspensions were freshly made before use. Zymosan was given intraperitoneally in a dose of 1 mg/g body weight.

Recruitment and in vitro cytokine production

Groups of five IL-17RA−/− mice and their control littermates were sacrificed four hours and 72 hours after intraperitoneal injection with heat-killed *C. albicans* 1 × 10^7 microorganisms/mL. Peritoneal cells were harvested by injecting 4 mL of sterile phosphate-buffered saline (PBS) containing 0.38% sodium citrate (15). After washing, the cells were resuspended in RPMI 1640 containing 1 mM pyruvate, 2 mM L-glutamine, 100 μg mL^{-1} gentamicin and 2% fresh mouse plasma (culture medium). Cells were cultured in 96-well microtiter plates (Greiner, Alphen, The Netherlands) at 10^5 cells per well, in a final volume of 200 μL. The cells were stimulated with either control medium or heat-killed (1 h, 100 °C) *C. albicans* at 1 × 10^7 microrganisms/mL. After 24 h of incubation at 37 °C, the plates were centrifuged (500 *g*, 10 min), and the supernatant was collected and stored at −80 °C until cytokine assays were performed.

To assess IFN-γ, IL-17 and IL-10 production, primed spleen cells from mice on day 3 of infection with *C. albicans* 2 × 10^5 CFU/mouse were stimulated in vitro with heat-killed *Candida* yeast cells (1 × 10^7 microorgansims/mL). Spleen cells were obtained by gently squeezing spleens in a sterile 200 μm filter chamber. The cells were washed and resuspended in RPMI1640, counted in a Bürker counting chamber and the number was adjusted to 5 × 10^6 mL^{-1}. One milliliter of the cell suspension was stimulated with 1 × 10^7 heat killed *C. albicans* yeasts. Measurement of IFN-γ, IL-17 and IL-10 concentrations was performed in supernatants collected after 48 h of incubation at 37 °C in 5% CO_2 in 24-well plates.

Phagocytosis and killing of C. albicans

Phagocytosis and killing of *C. albicans* yeast cells was assessed according to a method described elsewhere (16, 17). Exudate peritoneal phagocytes from groups of five IL-17RA-/- mice and their control littermates were elicited by an intraperitoneal injection of 1 × 10^7 heat killed *C. albicans* yeasts. After 4 hours, cells were collected in separate sterile tubes by washing the peritoneal cavity with 4mL of ice-cold PBS that contained 50 U/mL heparin. Peritoneal cells were centrifuged (for 10 min at 2250 g), counted in a Bürker counting chamber, and resuspended in culture medium. The processes of phagocytosis and intracellular killing were studied in an adherent monolayer of phagocytes, as described (16). Briefly, 5 x 10^5 cells were dispensed into the wells of a 96-well flatbottom plate (Costar), allowed to adhere for 2 h, and washed to remove nonadherent cells. Subsequently, the cells were incubated with 1 x 10^4 CFU *C. albicans*, which were opsonized for 45 min at 24°C in modified Eagle's medium (MEM; Gibco Life Technologies) containing 2.5% fresh mouse serum (effector:target ratio, 40:1). After 15min, supernatants were aspirated, and monolayers were gently washed with MEM to remove noningested microorganisms. The supernatant and well washings that contained the noningested *Candida* blastoconidia were plated in serial dilutions on Sabouraud agar plates. The percentage of phagocytized microorganisms was defined as [1 − (number of uningested cfu/cfu at the start of incubation)] x 100. Killing of *C. albicans* by phagocytes was assessed in the same monolayers. After removal of the nonphagocytized *Candida* blastoconidia, 200 mL of culture medium, consisting of Sabouraud in MEM (50% vol/vol), was added to the monolayers. After 3 h of incubation at 37°C in air and 5% CO2, the wells were scraped gently with a plastic paddle and washed with 200mL distilled H_2O to achieve lysis of phagocytes in 3 cycles, and 10-fold dilutions of each sample were spread on Sabouraud agar plates and incubated at 37°C for 24 h. The percentage of yeast killed by the phagocytes was determined as follows: [1 − (cfu

after incubation/ number of phagocytized cfu)] x 100. Phagocyte-free incubations of blastoconidia were included as a control for yeast viability. In earlier experiments we have shown that 90-97% of attached/internalized *C. albicans* are intracellular (18).

Cytokine assays
TNFα was determined by specific radioimmunoassay (detection limit 20 pg/ml), as previously described (19). IL-10, IFNγ and IL-17 concentrations were measured by a commercial ELISA (Biosource, Camarillo, CA; detection limit 16 pg/ml), according to the instructions of the manufacturer.

Statistical analysis
Statistical significance of differences in mortality between experimental groups was determined using the Log-rank (Mantel-Cox) Test. Statistical significance of differences in cytokines, phagocytosis and killing experiments and fungal outgrowth were determined using a two-sided Mann-Whitney U test. *P* values smaller than 0.05 were considered significant.

Results

The IL-17 pathway is protective in disseminated candidiasis
The role of the IL-17 pathway in anti-*Candida* host defense remains controversial. On the one hand IL-17RA-/- mice are reported to be more susceptible to *Canidida* infections (6, 7), while on the other hand IL-17 is said to be responsible for inflammatory pathology and defective clearance of *Candida* (10). To assess the role of the IL-17 pathway in anti-*Candida* host defense, we tested the susceptibility of IL-17RA-/- mice to disseminated *C. albicans* infection. Mice were infected intravenously with *Candida* yeasts and monitored for survival and fungal growth. IL-17RA-/- mice were more susceptible to disseminated *Candida* infection, as judged by the decreased survival (Fig. 1a) and the increased fungal burden in the kidneys (Fig. 1b) compared to the control mice.

IL-17RA-/- mice are susceptible to zymosan-induced multiple organ failure
IL-17 has been suggested to play a role in severe tissue inflammation that is detrimental for the host and impairs antifungal clearance. We investigated whether this is the case in an experimental model of zymosan-induced multi-organ failure. There was no significant statistical difference in survival between IL-17RA-/- and control mice (Fig. 1c).

Neutrophil recruitment and phagocytosis and killing in IL-17RA-/- mice
It has been reported in oral and disseminated candidiasis that a defect in IL-17 leads to lower neutrophil recruitment which causes disease susceptibility (6, 7). To investigate the dynamics of the influx of neutrophils and their function, we harvested cells from the peritoneal cavity at 4 hours and 72 hours after intraperitoneal injection with heat-killed *Candida* and addressed the number of monocytes, neutrophils and lymphocytes and the capacity of these cells to phagocytose and kill *C. albicans*. Although no difference in neutrophil recruitment between control mice and IL-17RA-/- mice was observed at 4 hours a significant decrease in neutrophil influx was apparent 72 hours after challenge with heat killed *Candida* (Fig. 2b). In addition, the capacity to phagocytose and kill *Candida* was not impaired in neutrophils from IL-17RA-/- mice (Fig. 2c).

A deficient IL-17 pathway is associated with lower production of tumor necrosis factor in response to C. albicans

The cytokine profiles induced by *C. albicans* were assessed in cells isolated from control mice and IL-17RA-/- mice. Splenocytes isolated three days after the infection of control and IL-17RA-/- mice with *C. albicans* showed no difference in the production of IL-10, IFNγ and IL-17 (Fig. 3a). Inflammatory peritoneal cells from IL-17RA-/- showed a significant lower production of TNFα in response to *C. albicans*, when compared to cells harvested from control mice (Fig. 3b).

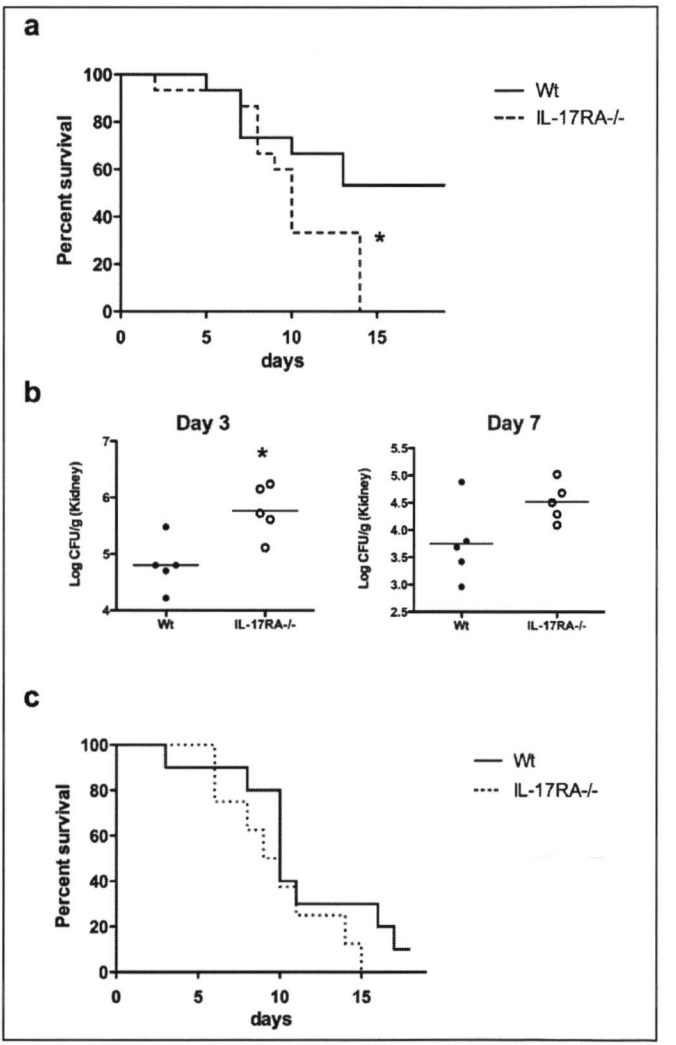

Figure 1. IL-17 pathway in disseminated candidiasis and zymosan-induced multi-organ failure.
(a) IL-17RA-/- and control mice were intravenously infected with live *C. albicans* yeast (2 x 10^5 CFU). (b) Control and IL-17RA-/- mice were sacrificed at 3 days and 7 days after infection. *Candida albicans* fungal outgrowth was determined in the kidneys. (c) Survival of IL-17RA-/- and control mice after administration of zymosan and LPS. (a,c) The disseminated candidiasis model was performed in a total of n=15 mice per group and the zymosan model with n=10 per group. Survival was assessed daily for three weeks and analyzed using the Log-rank (Mantel-Cox) Test. (b) The results are expressed as log CFU/g tissue (n=5 mice/group, means ± SD). * $P<0.05$.

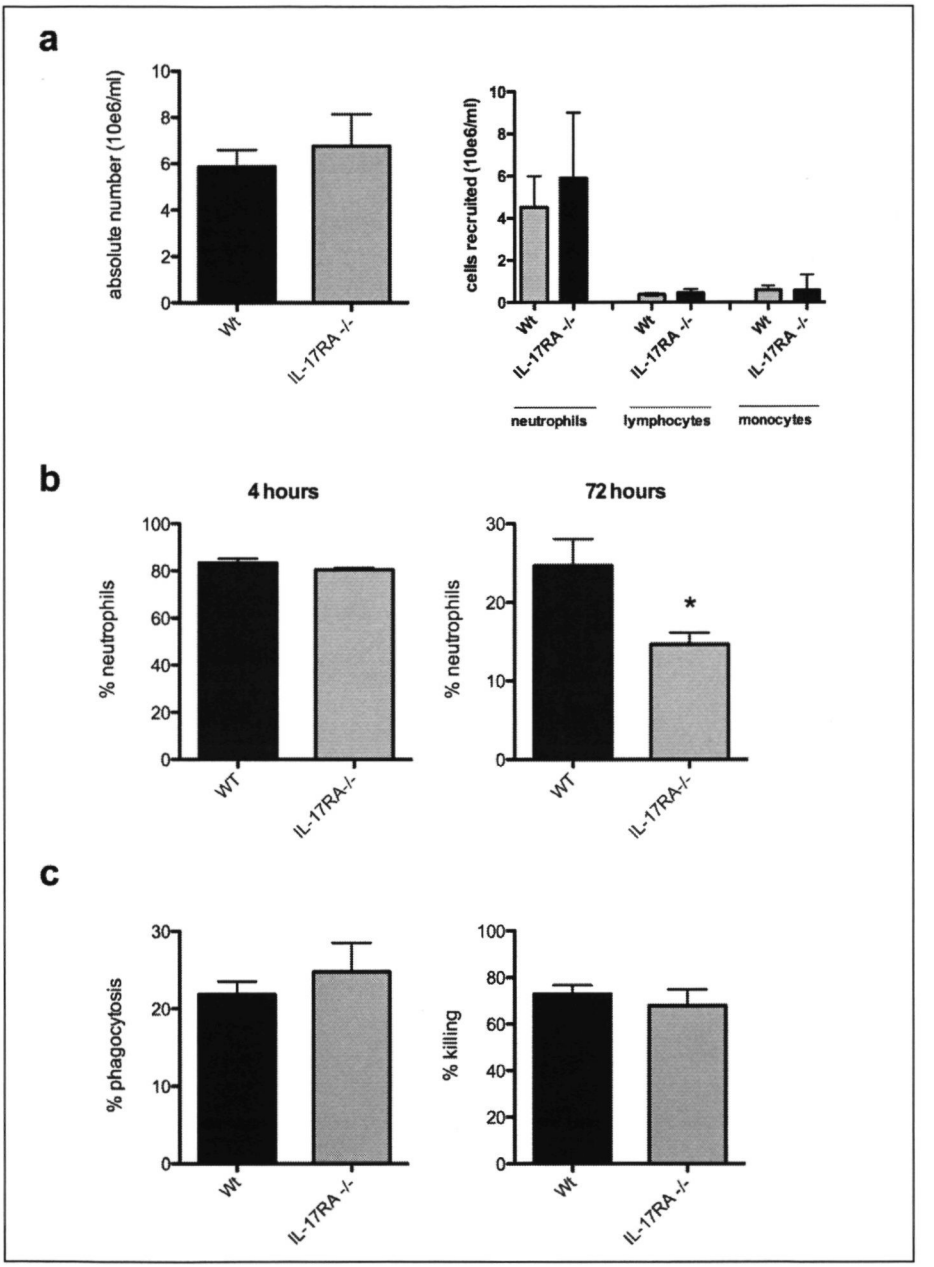

Figure 2. Neutrophil recruitment and phagocytosis and killing in IL-17RA-/- mice.
(a) Total cell recruitment and cell subpopulations harvested 4 hours after the intraperiteoneal injection with heat killed *C. albicans*. (b) Percentage of neutrophils in the peritoneal cavity at 4 hours versus 72 hours. (c) Phagocytosis and killing of *C. albicans* by neutrophils isolated from IL-17RA-/- and control mice at four hours. Percentage of *Candida* blastoconidia of the initial inoculum that was ingested after 15 min of phagocytosis and percentage of phagocytized *Candida* blastoconidia that was killed after incubation at 37 °C for 3 h is shown. All data are shown as median ± IQR. * $P<0.05$.

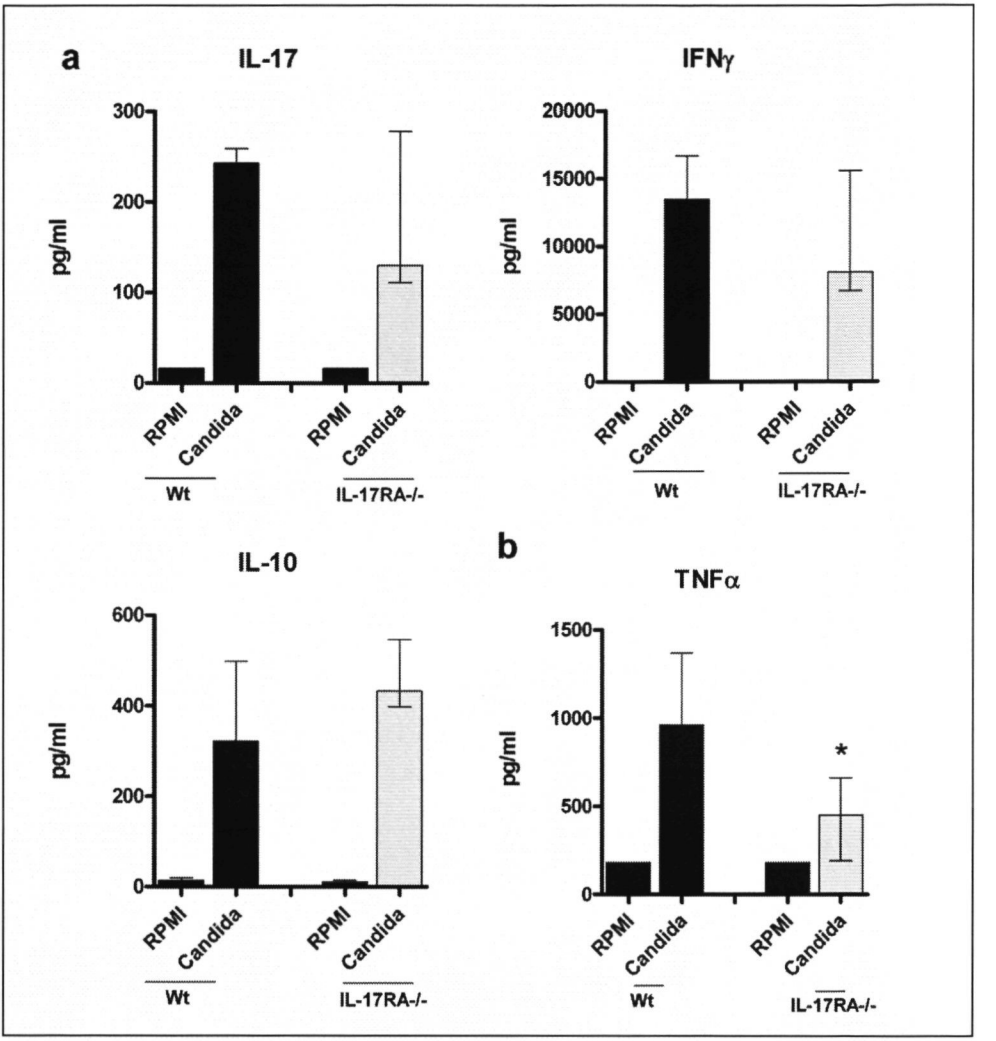

Figure 3. Cytokine production capacity in cells isolated from IL-17RA-/- mice.
(a) Mice were infected intravenously with *C. albicans* (2×10^5 CFU/mouse), and after 3 days splenocytes were harvested and stimulated *in vitro* with 1×10^7 heat-killed *C. albicans* (E : T ratio 2 : 1). Measurements of IFN-γ, IL-10 and IL-17 concentrations were performed in supernatants collected after 48 h of incubation at 37°C. (b) Peritoneal cells from IL-17RA-/- and control mice, harvested 4 hours after the intraperitoneal injection with heat-killed *Candida albicans,* were stimulated in vitro with additional heat-killed *C. albicans* yeasts. Production of TNF in the supernatants was measured by specific ELISA after 24 h of stimulation at 37°C. (a,b) The results are pooled data from two separate experiments with a total of 8 mice per group. Data are expressed as median ± IQR. * $P<0.05$.

Discussion

In the present study, we addressed the controversy surrounding the role of the IL-17 pathway in antifungal host defense and show that IL-17RA-/- mice have a decreased survival and display higher fungal loads in the kidneys in early stages of disseminated candidiasis

when compared to control mice. This demonstrates that the IL-17 pathway is non-redundant and protective in disseminated candidiasis. In line with previous studies, neutrophil recruitment to the peritoneal cavity was impaired in IL-17RA-/- mice challenged intraperitoneally with *Candida*. Interestingly, early neutrophil recruitment in the first 4 hours was not affected in these experiments. Furthermore, we found no defects in phagocytosis and killing of Canidida by the IL-17RA-/- neutrophils. Notably, we found a significantly lower TNF production in response to *Candida* in cells from IL-17RA-/- mice, which might contribute to susceptibility to disseminated candidiasis. IL-17 was not important for the severe tissue inflammation that leads to multiple organ failure in a sterile inflammatory fungal model using zymosan, which is in contrast with the suggestion that IL-17 is detrimental to the host by inducing severe tissue inflammation during infection.

Recently, the cytokine IL-17 has received a lot of attention since T cells producing IL-17 were characterized as a different lineage of T helper cells with an important role in autoimmunity (2). Interestingly, human memory T helper cells were found to have the capability of inducing a robust IL-17 production in response to *C. albicans* (20). In addition, it has been found that patients with primary immunodeficiencies such as hyperIgE syndrome and chronic mucocutaneous candidiasis which suffer from chronic mucosal fungal infections, displayed a defect in their *Candida* specific Th17 response (21, 22). Because these studies strongly indicate that the Th17 memory response and the IL-17 pathway is essential in host defense against mucosal *Candida*, it was surprising to notice that IL-17 was reported to be detrimental for the host during murine mucosal *Candida* infection (10). This could be due to the different model that was used in this latter study, namely a gastric model of mucosal *Candida* infection. Furthermore, differences between mice and humans in the development of Th17 responses have been described (23), and mice are not colonized by *Candida* in contrast to humans (24). However, Conti et al. have recently investigated the role of the IL-17 response in a murine oropharyngeal *Candida* infection model, and clearly demonstrate that IL-17 was needed to provide an effective host defense against mucosal candidiasis (7).

The controversial observation that IL-17 is counter-productive for protection in fungal infection has been explained by the fact that IL-17 induces neutrophil activation which causes excessive inflammation (10). To test this hypothesis, the role of IL-17 was addressed in a model that depends on neutrophil influx and leads to severe tissue inflammatory responses. The model is based on the injection of zymosan that will induce a strong inflammatory response eventually resulting in multi-organ failure (25). Notably, zymosan is a fungal component known to induce a predominant Th17 response in mice (26, 27). It has been previously demonstrated that there is a strong influx of inflammatory cells in the various tissues during the development of organ damage in this animal model (14, 25). For instance, there is an influx of macrophages and neutrophils in the liver and there are accumulations of lymphocytes, plasma cells and neutrophils in the lungs. We speculated that if the IL-17 pathway plays a crucial role in severe tissue inflammation and regulation of neutrophil function, IL-17RA-/- mice should be protected from the severe inflammation and multiple organ failure which is induced by zymosan. However, we observed no difference in survival between control mice and IL-17RA-/- mice in this model. This argues against the suggestion that the IL-17 pathway is detrimental to the host by causing a diversion of neutrophil function that leads to uncontrolled inflammation during infection.

The observation that a deficient IL-17 pathway results in higher mortality and fungal burden in disseminated candidiasis is in line with several studies arguing that the Th17 response is protective for the host during *Candida* infection. Huang et al. were the first to show that IL-17 was non-redundant for the host defense against disseminated candidiasis (6). They observed significant increases of fungal outgrowth in the kidneys at day 4 of infection. In line with this, we observed a 10 fold increase in fungal burdens at day 3 in the kidneys of IL-17RA-/- mice and a 6 fold higher burden on day 7. Notably, in the present study the IL-17RA-/- and wild type mice had a similar survival curve until day 10, and only after this time point a higher mortality rate could be seen in the IL-17RA-/- mice. Although it is most likely that the increase in fungal burden leads to renal failure subsequently causing the higher mortality rate observed in the IL-17RA-/- mice (28), other mechanisms could account or contribute to the increased susceptibility. Interestingly, a significantly lower production of TNF was observed in response to stimulation with *Candida albicans* in cells recruited from IL-17RA-/- mice. It has been reported that mice lacking the TNF receptor are highly susceptible to disseminated candidiasis (29), suggesting that the lower TNF response observed in IL-17RA-/- mice may contribute to the increased susceptibility to disseminated *Candida* infection. An important observation presented here is that neutrophils from IL-17RA-/- mice do not display an intrinsic defect in phagocytosis and killing. In line with reports that suggest IL-17 to be important for neutrophil recruitment, we found that at 72 hours after intraperitoneal injection with heat-killed *Candida* the neutrophils influx was lower in the IL-17RA-/- mice. This supports the previously reported finding that neutrophil recruitment is impaired in IL-17RA-/- mice during fungal infection. However, the initial influx of neutrophils at four hours was not disturbed, which indicates that IL-17 is especially important for the neutrophil recruitment after the initial hours/days of infection.

It has been reported that IL-23 driven inflammation promotes infection and impairs fungal resistance (10). Circulating IL-23 did not reach detectable concentrations in the disseminated candidiasis model or the multiple organ failure model, similar to other cytokines. Therefore, it is most likely that IL-23 exerts its effects locally in the inflamed tissue. As a consequence, IL-23 driven Th17 responses could be context dependent, and promote either protective or pathogenic responses depending on additional local factors.

In conclusion, host defense against systemic *Candida* infection critically depends on the IL-17 pathway, while IL-17 does not appear to be involved in enhancing the inflammatory potential of neutrophils in a model of severe tissue inflammation induced by zymosan. Our observations support the idea that in anti-*Candida* host defense the Th17 response is essential for protective antifungal immunity. This knowledge is crucial with respect to the focus for the development of novel strategies to prevent and treat *Candida* infections.

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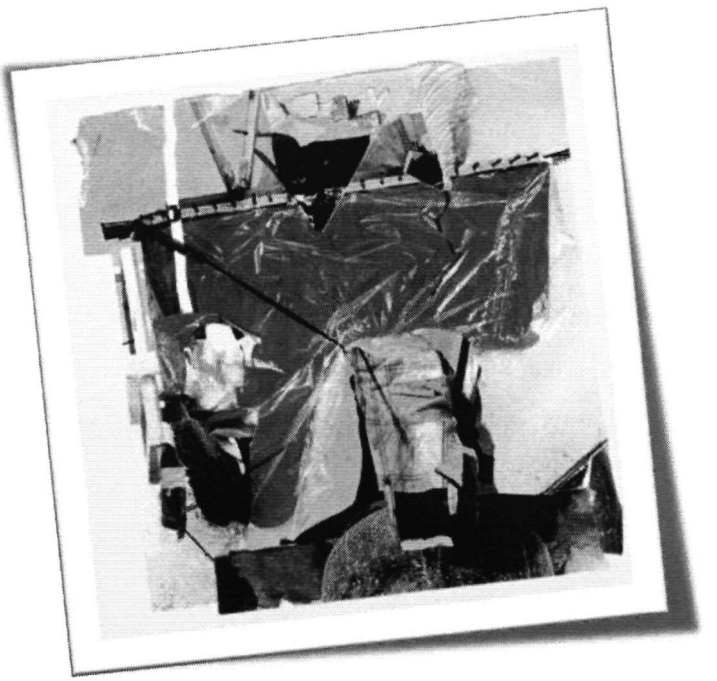
Anti-*Aspergillus* human host defence relies on type 1 T helper (Th1), rather than type 17 T helper (Th17), cellular immunity

Immunology. 2010 May;130(1):46-54



Chai LY, van de Veerdonk FL, Marijnissen RJ, Cheng SC, Khoo AL, Hectors M, Lagrou K, Vonk AG, Maertens J, Joosten LA, Kullberg BJ, Netea MG.

Summary

Both interferon-gamma-producing type 1 T helper (Th1)- and interleukin-17 (IL-17)-producing type 17 T helper (Th17) cells have been proposed to be involved in anti-fungal host defense. Although invasive aspergillosis is one of the most severe human fungal infections, little is known regarding the relative importance of the Th1 versus Th17 cellular immune pathways for the human anti-*Aspergillus* host defense. Using human peripheral blood mononuclear cells and a system consisting of monocyte-derived macrophages, we found that *Aspergillus fumigatus* is a weak inducer of human IL-17 but induces a strong Th1 response. These data were validated by the very low IL-17 levels in bronchoalveolar lavage fluid and serum of patients with invasive aspergillosis. Surprisingly, live *A. fumigatus* reduced IL-17 production induced by mitogenic stimuli. This effect was mediated through the propensity of *A. fumigatus* to metabolize tryptophan and release kynurenine that modulates the inflammatory response through inhibition of the IL-17 production. In conclusion, *A. fumigatus* does not stimulate production of IL-17 and human host defense against aspergillosis may not rely on potent Th17 responses.

Introduction

Aspergillus fumigatus, an opportunistic ubiquitous mold, causes invasive aspergillosis in immunocompromised hosts with high rates of mortality and morbidity [1, 2]. Until recently, the paradigm of the type 1 T helper (Th1) and type 2 T helper (Th2) cells was traditionally the model upon which knowledge of host immune response to fungal infection was based [3, 4]. These CD4+ T-cell subtypes mediate distinct immune responses. Th1 cells produce proinflammatory cytokines such as interferon-gamma (IFN-γ) that induces protective antifungal defense mechanisms, whereas Th2 cells release IL-4 and IL-10 which evoke a humoral response and in turn down regulate Th1-dependent mechanisms [5]. It is the balance between Th1 and Th2 responses that has been perceived as critical in determining outcome of invasive fungal diseases [6].

However, the recent discovery in both mice and man of a novel member of the CD4+ effector T-cell family (Th17 cells) producing IL-17 has provided novel insights into the immune mechanisms that are responsible for both protection from infections and immunopathology in autoimmune diseases [7-9]. Differentiation of murine and human naive CD4+ T cells into either Th1, Th2 or Th17 cells depends on the cytokine milieu present at the time of initial engagement of the T-cell receptor and co- stimulatory receptors [10]. Homology between human and murine IL-17 is high and the major function of IL-17 is to promote recruitment and activation of neutrophils [11]. As a result of these effects, IL-17 is generally perceived as having a protective role against fungal infection, as demonstrated by an increased susceptibility to disseminated candidiasis of mice lacking the IL-17 receptor [12]. However, human Th17 cells have a different origin than those in mice [13], and the cytokines that direct Th17 development exert their effect differently in the two hosts [14].

Few and conflicting data are available regarding the potential role of IL-17 for the host defense against *A. fumigatus*. On one hand, IL-17 was recently reported to hamper neutrophil-mediated killing of *A. fumigatus* and the in-vivo clearance of the organism in non-

immunosuppressed mice [15]. In contrast, other data suggest a protective role for IL-17 in host defense against *A. fumigatus*. Neutralization of IL-17 early in host defense against *A. fumigatus* infection in non-immunosuppressed mice resulted in an increased pulmonary fungal burden [16]. Very little is known regarding the role of IL-17 for the host defense against *Aspergillus* infections in humans.

On this background and with the understanding that regulation of Th17 pathway may differ between murine and human cells, we studied the IL-17 host response to *A. fumigatus* in human leukocytes and in patients with invasive aspergillosis.

Materials and methods

Microorganisms

The *A. fumigatus* strain V05-27 is a previously characterized clinical isolate [17]. Live conidia and heat-killed hyphae were obtained as previously described [18]. For the experiments involving *Aspergillus*-conditioned medium, live V05-27 *A. fumigatus* (at concentrations as specified) were grown in RPMI 1640 DM (ICN Biomedicals, Costa Mesa, CA) supplemented with 10 μg/ml gentamicin, 10 mM L-glutamine, and 10 mM pyruvate at 37°C. After 3 days, the culture suspension was centrifuged and the supernatant was passed through a 0.2 μm filter (Whatman GmbH, Dassel, Germany). In addition, conidia arrested at the different growth phases were prepared from *A. fumigatus* B-5233, another well-characterized strain [19]. After incubation on *Aspergillus* minimal agar media for one week, both resting and germinating conidia (following incubation for 4 hours at 37°C in liquid broth yeast nitrogen base, Difco, Franklin Lakes, NJ)) were harvested in 0.01% Tween-20/phosphate-buffered saline, washed with sterile distilled water and resuspended in Hanks' balanced saline solution without Ca^{2+} and Mg^{2+}. Conidia were heat-inactivated by incubation at 65°C [20]. The treatment was repeated until no viable conidia were detected on malt agar plate. All specimens were kept frozen at -20°C until use.

Heat-killed *Candida albicans* blastoconidia, strain ATCC MYA-3573 (UC820) were used as a positive control [21, 22]. Experiments involving heat-killed *C. albicans* blastoconidia at a concentration of 10^6 microorganisms/ml were performed in a similar manner to that described above.

Reagents

Anti-CD3/anti-CD28-coated beads (to mimic action of antigen-presenting cells for T-cell activation) were purchased from Miltenyi-Biotech (Utrecht, the Netherlands) and used according to manufacturer's instructions. Recombinant human IL-1β (rIL-1β; 50ng/mL) from Biosource (Etten-Leur, the Netherlands), IL-6 (rIL-6; 50ng/mL) and IL-23 (rIL-23; 50ng/mL) from R&D Systems (Abingdon, UK) were used. Rabbit anti-human anti-interferon-gamma (anti-IFN-γ) (10μg/mL) was purchased from U-CyTech (Utrecht, the Netherlands) and corresponding isotype control, rabbit immunoglobulin G (IgG), from Jackson ImmunoResearch Laboratories (West Grove, PA). Kynurenine and tryptophan were purchased from Sigma-Aldrich (Zwijndrecht, the Netherlands). The Dectin-1 receptor antagonist, laminarin, was kindly provided by Dr David Williams (University of Tennessee, Knoxville, TN). Anti-human monoclonal antibody against mannose receptor CD206 (α-MR)

and corresponding isotype control was purchased from BD Pharmingen (Breda, the Netherlands).

In-vitro cytokine production
Separation and stimulation of peripheral blood mononuclear cells (PBMC) was performed as described previously [23]. Briefly, venous blood was drawn into ethylenediaminetetraacetic acid tubes from healthy volunteers after informed consent. PBMC were isolated by density centrifugation on Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden). Cells were washed twice in saline, counted and the number adjusted to $5x10^6$ cells/mL. For experiments involving monocyte-derived macrophages (MDM) [24], PBMC were maintained in culture medium (RPMI 1640 DM supplemented with gentamicin, L-glutamine, and pyruvate) in the presence of 10% human pooled serum in humidified atmosphere (5% CO2) at 37°C for 6-7 days to permit differentiation with the lymphocyte component in-situ. Culture medium was refreshed after 3 days. Stimulation assays were performed in 96-well plates (Greiner, Alphen a/d Rijn, the Netherlands) using 100 µl volume of PBMC or MDM with lymphocytes (abbreviated as MDM/lymphocytes), and the various stimuli to a total volume of 200 µl/well. For investigation of Th17 responses, the cells were incubated with the pathogen at 37°C for 3 or 7 days [25]. After incubation, the supernatants were collected and stored at -20°C until assay.

Flow cytometry
MDM/lymphocytes were stimulated for 4-6 hours with 13-phorbol 12-myristate acetate (PMA) (50 ng/ml; Sigma) and ionomycine (1 µg/ml; Sigma) in the presence of Golgiplug (BD Biosciences, Breda, the Netherlands) according to manufacturer's protocol. Cells were first stained extracellularly using an allophycocyanin-conjugated anti-CD4 antibody (BD Biosciences). Subsequently the cells were fixed and permeabilized with Cytofix/ Cytoperm solution (BD Biosciences) and then intracellularly stained with fluorescein isothiocyanate-conjugated anti-IL-17 (eBiosciences, Halle-Zoersel, Belgium). Samples were measured on a FACS Calibur and data were analyzed using the Flow Jo software (version 7.2.5.).

Patients
Serum from hematology patients diagnosed with proven or probable invasive aspergillosis (IA) [26] and bronchoalveolar lavage (BAL) fluid of IA patients as per European Organization for Research and Treatment of Cancer (EORTC) criteria [27] were obtained before the initiation of appropriate treatment and in line with the respective institutional guidelines. Matched controls consisted of similar patient cohorts with underlying hematological malignancies who did not have IA.

Cytokine assay
Interleukin-6, IL-10 and IFN-γ were measured by commercial ELISA kit (Pelikine Sanquin Compact, Amsterdam, the Netherlands), according to the instructions of the manufacturer. IL-17 was measured by the appropriate commercial ELISA kit (R&D Systems). Detection limits were 8 pg/ml (IL-6 and IL-10), 20pg/ml (IFN-γ), and 16 pg/ml (IL-17) respectively.

Tryptophan and kynurenine measurement

Levels of tryptophan and kynurenine produced by *A. fumigatus* in *Aspergillus*-conditioned medium were quantified by UV detection with high performance liquid chromatography (HPLC). This was performed on a SpectraSYSTEM autosampler and pump (Thermo Separation Products, San Jose, CA). Chromatographic separation was performed using an Inertsil 5 ODS-2 column (100mm x 3.0 I.D.) (Varian Inc., Middelburg, the Netherlands). Absorbance was monitored with a diode-array detector (UV6000LP, Thermo Separation Products, San Jose, CA) at wavelength of 280nm for tryptophan and 360nm for kynurenine [28]. The mobile phase for isocratic elution was made by dissolving 40mM sodium acetate. The pH of the eluent was adjusted to pH 4.5 with a solution of 40mM citric acid and 2% acetonitrile of the total volume buffer was added. The continuous flow rate was 0.3 ml/min [29]. For calibration, the standard was diluted in RPMI in the concentration range of 0-72 μM for tryptophan, and 0-42 μM for kynurenine. 50 μL of the standard or sample was injected into the column for measurement.

Statistical analysis

Experiments were performed in duplicates. For the in-vitro experiments, results from 3 sets of separate experiments (involving 5 or more distinct healthy volunteers) were pooled and analyzed using SPSS 16.0 statistical software. Data given as means ± standard errors of the means (SEM) and the Wilcoxon signed rank test was used to compare differences between groups (unless otherwise stated). The level of significance was set at $p < 0.05$.

Results

Aspergillus fumigatus is a poor inducer of human IL-17 by PBMC and MDM

We investigated the capacity of *A. fumigatus* to induce production of IL-17 by human PBMC. Incubation of live *A. fumigatus* V05-27 conidia (at doses of 10^4 – 10^6 microorganisms/ml) with PBMC over 7 days induced little IL-17, in contrast to *C. albicans*, a known inducer of IL-17 (Fig. 1a) [25]. We also assessed the capacity of MDM/lymphocytes to mediate IL-17 induction. However, IL-17 production upon incubation with live *Aspergillus* conidia was also nearly undetectable in this system (Fig. 1b). Flow cytometric analysis further corroborated the low propensity of live *Aspergillus* to induce IL-17, in contrast to *C. albicans,* as illustrated by the absence of IL-17-producing CD4+ cells (Figs. 1c&d).

To validate these findings and to demonstrate that the observed phenomenon was not strain-specific, we repeated the experiments using either heat-inactivated conidia of another well-characterized *A. fumigatus* strain B-5233 that were arrested at the resting and germinating growth stage, or with heat-killed V05-27 *A. fumigatus* hyphae. All of the above *Aspergillus* preparations showed limited IL-17 responses across the concentration range of 10^5 to 10^7 microorganisms/ml (Fig. 1e). In contrast, the *Aspergillus* components induced a distinct dose-dependent trend in IL-6 production, excluding significant cellular toxicity as the cause of the limited IL-17 production (Fig. 1f). In line with this, lactate dehydrogenase levels in supernatants of cells stimulated with live *Aspergillus* were not elevated as compared to vehicle-stimulated controls. Hence, both live and heat-inactivated *A. fumigatus* at different stadia were poor inducers of IL-17 in human cells.

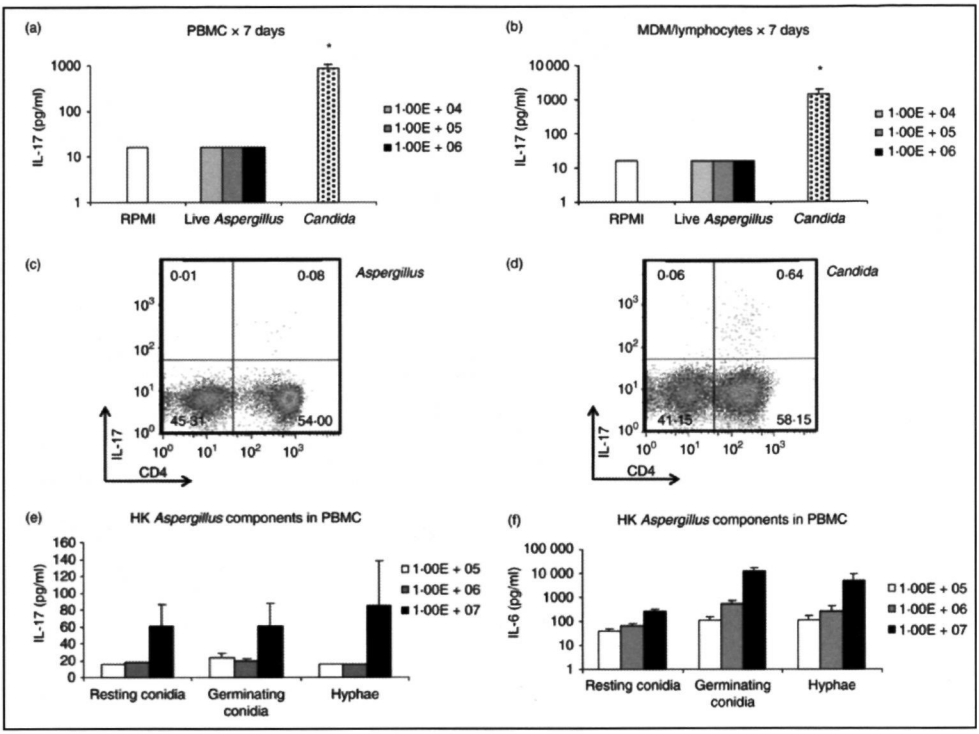


Figure 1. (a, b) Interleukin-17 (IL-17) -inducing capacity of 10^4–10^6 micro-organisms/ml *Aspergillus fumigatus* in peripheral blood mononuclear cells (PBMC) and monocyte-derived macrophages (MDM)/lymphocytes systems over 7 days with *Candida albicans* as positive control. (c, d) Flow cytometry of MDM/lymphocytes stimulated by *A. fumigatus* and *C. albicans*, respectively. The cells were labelled with anti-CD4-allophycocyanin and anti-IL-17-fluorescein isothiocyanate. Density plots show surface staining of CD4 (*x*-axis) and intracellular staining of IL-17 (*y*-axis). Numbers on the plots represent percentage of cells bearing positivity for the respective markers. Results from a representative experiment are shown. (e, f) IL-17 and IL-6 induced by 10^5–10^7 micro-organisms/ml of heat-killed *A. fumigatus* components: resting conidia, germinating conidia and hyphae in PBMC. Stimulation studies are cumulative from three sets of experiments. $^*P < 0{\cdot}05$ compared with control (RPMI-1640). *Aspergillus*: live *A. fumigatus* conidia (unless otherwise stated), *Candida*: heat-killed *C. albicans* blastoconidia (as positive control), HK: heat-killed.

Aspergillus fumigatus induces strong Th1 responses

It has been described that host immune response to *A. fumigatus* is primarily T-helper type 1 (Th1)-mediated, accompanied by the production of IFN-γ [30, 31]. We have also demonstrated the propensity of live *A. fumigatus* to induce a robust IFN-γ response in contrast to absence of IL-17, or the limited IL-6 and IL-10 production in the MDM/lymphocyte system (Fig. 2).

Live Aspergillus attenuates IL-17 production

As other studies have used mitogenic stimulation with anti-CD3/anti-CD28 antibodies to induce Th17 responses, live *Aspergillus* conidia were added to MDM/lymphocytes in the presence of anti-CD3/anti-CD28 beads. Surprisingly, IL-17 levels remained low in the presence of *A. fumigatus* compared with the mitogenic stimulation alone and even showed a dose-dependent inverse relationship to the concentration of the pathogen. Adding

recombinant IL-6, IL-1β and IL-23 to the anti-CD3/anti-CD28 T-cell activation failed to increase IL-17 induction by *A. fumigatus* (Figs. 3c&d). IFN-γ production showed a trend towards inhibition by *Aspergillus*, but this was not as markedly attenuated as the IL-17 response.

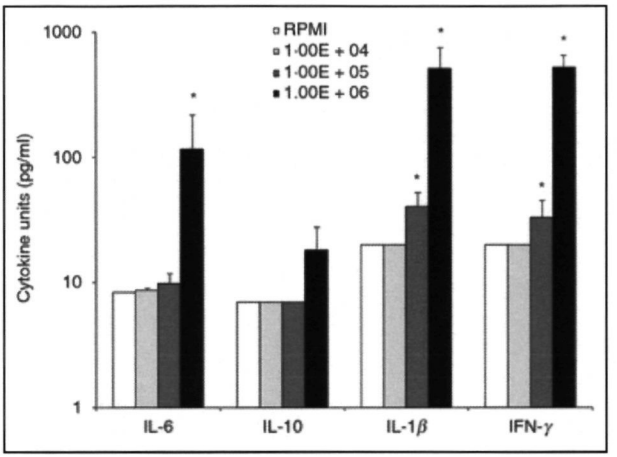


Figure 2. Interleukin-6 (IL-6), IL-10, interferon-γ (IFN-γ) and IL-1β production by monocyte-derived macrophages (MDM) following stimulation by live *Aspergillus fumigatus* at incremental concentrations. IFN--γ and IL-1β responses are robust compared with IL-6 and IL-10. *$P < 0.05$ compared with RPMI-1640 control, n = 8 subjects.

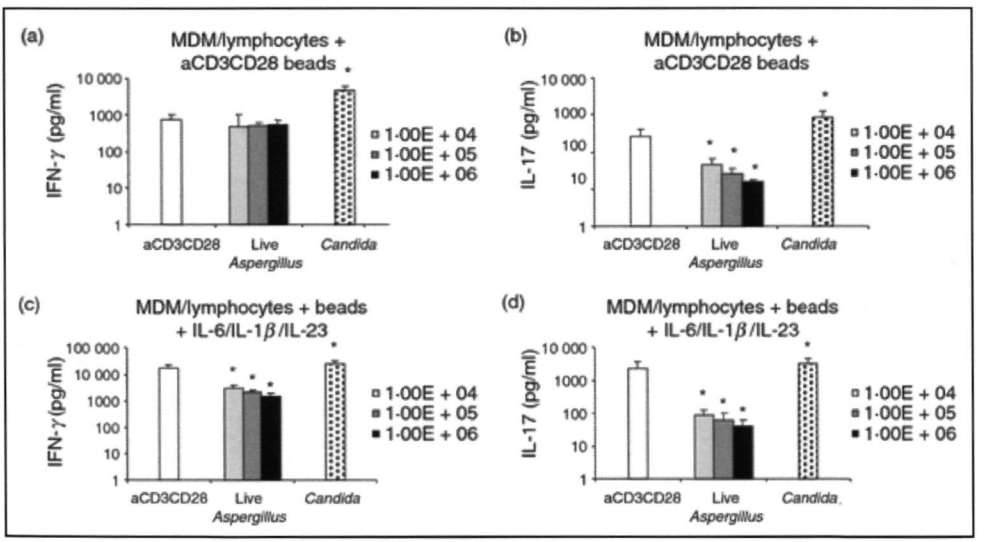


Figure 3. (a, b) Interferon-γ (IFN-γ) and interleukin-17 (IL-17) produced by monocyte-derived macrophages (MDM)/lymphocytes stimulated with anti-CD3/anti-CD28 beads alone and with incremental amounts of *Aspergillus fumigatus* or *Candida albicans* (positive control) over 3 days (c, d) In addition to anti-CD3/anti-CD28 beads, recombinant IL-6 (rIL-6) + rIL-1β + rIL-23 were added to MDM/lymphocytes stimulated with *A. fumigatus* or *C. albicans* (positive control) over 3 days. *$P < 0.05$ compared with stimulated MDM/lymphocytes but without pathogen, n = 6 subjects. *Aspergillus*: live *A. fumigatus* conidia, *Candida*: heat-killed *C. albicans* blastoconidia, aCD3CD28: anti-CD3/anti-CD28-coated beads.

Weak IL-17 response is not linked to the pro-Th1-propensity of A. fumigatus

IFN-γ can suppress Th17 responses and shift cellular responses towards a Th1 bias. We attempted to silence the Th1 response with anti-human IFN-γ antibodies and investigate its effect on IL-17 induction by *Aspergillus* (Figs. 4a&b), although IFN-γ production was effectively inhibited, there was no significant rescue of IL-17 production induced by anti-CD3/anti-CD28 antibodies in the presence of *A. fumigatus*. Hence, the weak IL-17 response seen in *A. fumigatus* is not related to its pro-Th1-inducing propensity.

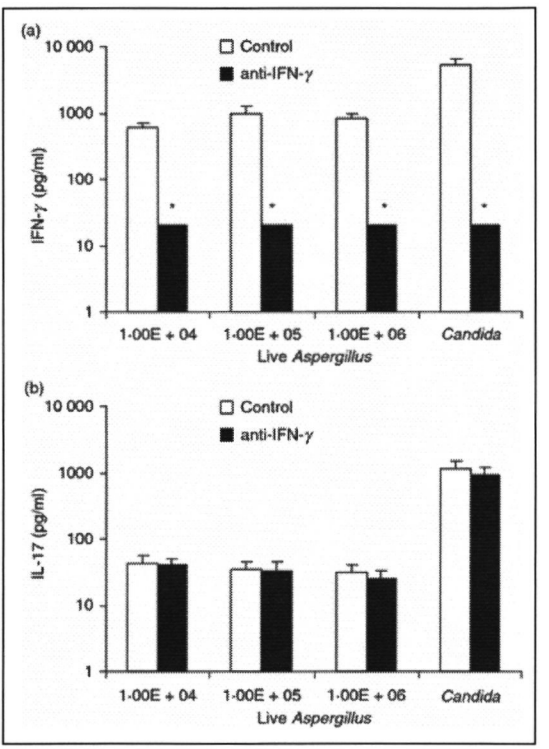


Figure 4. Interferon-γ (IFN-γ) and interleukin-17 (IL-17) from monocyte-derived macrophages (MDM)/lymphocytes stimulated with anti-CD3/anti-CD28 beads and incremental amounts of Aspergillus fumigatus or Candida albicans (positive control) in the presence of anti-IFN-γ (a, b). *P < 0·05 compared with respective control (white bar) without anti-IFN-γ, n = 6 subjects. Aspergillus: live A. fumigatus conidia, Candida: heat-killed C. albicans blastoconidia (as positive control).

Secreted products from live Aspergillus attenuate IL-17 production

The inhibitory effects of *Aspergillus* on the IL-17 response induced by mitogens may be exerted directly through cell-cell contact, or indirectly through products released by *Aspergillus.* To investigate this, *Aspergillus*-conditioned medium was added to the activated cells. As observed in Fig. 5, IL-17 production was inhibited in a dose-dependent manner by the conditioned media on which live *A. fumigatus* conidia had grown. The IL-17-attenuating effects induced by *Aspergillus*-conditioned medium could not be assigned to shed β-glucan or mannan-derivatives from the *Aspergillus* cell wall, as the addition of laminarin (inhibitor

of Dectin-1, the β-glucan receptor) and anti-MR antibodies (which block the mannose receptor) did not reverse the effects of the conditioned medium (data not shown).

The tryptophan metabolism pathway has recently been implicated for its role in regulating Th17 response to fungal infection [32]. Tryptophan and kynurenine concentrations were measured in the *Aspergillus*-conditioned media. Kynurenine concentrations in the *Aspergillus*-conditioned media were increased (as compared to control) across the concentration range of live *A. fumigatus*. This correlated with a drop in the concentration of the substrate, tryptophan, in the conditioned medium (Table 1).
The effect of kynurenine was assessed on the IL-17 production induced by anti-CD3/anti-CD28 antibodies. As depicted in Fig. 5c, kynurenine inhibited IL-17 production in a dose-dependent manner as compared to control stimulation.

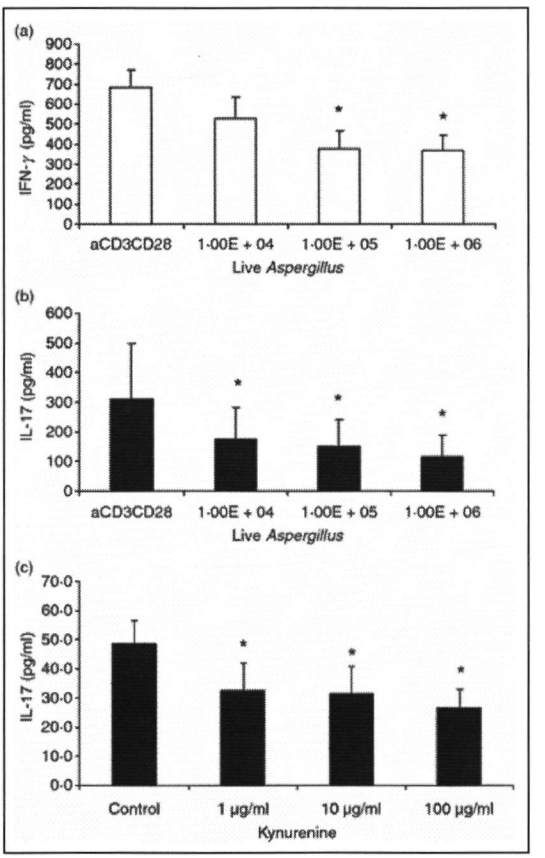


Figure 5. (a, b) Interferon-γ (IFN-γ) and interleukin-17 (IL-17) produced by anti-CD3/anti-CD28-stimulated monocyte-derived macrophages (MDM)/lymphocytes over 3 days in the presence of conditioned medium in which the respective concentrations of live *Aspergillus fumigatus* had previously been grown over 72 hr. **P* < 0·05 compared with anti-CD3/anti-CD28-stimulated MDM/lymphocytes in the presence of conditioned medium in which no *Aspergillus* had been grown (control; first bar), *n* = 6 subjects. (c) IL-17 production by anti-CD3/anti-CD28-stimulated MDM/lymphocytes over 3 days in the presence of increasing concentrations of kynurenine compared with control (without kynurenine). **P* < 0·05 compared with control, *n* = 6 subjects. *Aspergillus*: live *A. fumigatus* conidia, aCD3CD28: anti-CD3/anti-CD28-coated beads.

Table 1. Tryptophan and kynurenine levels of conditioned media in which live *A. fumigatus* (of incremental concentrations) had been grown over 3 days as compared to levels in conditioned medium incubated over 3 days without *Aspergillus* (RPMI Control). Tryptophan and kynurenine levels were measured via high performance liquid chromatography (HPLC). The analysis was performed on 3 sets of assays and is presented as means±SD.

	CONDITIONED MEDIUM			
	RPMI Control	Live *Aspergillus* (microorganism/ml)		
		10^4	10^5	10^6
Tryptophan (μM)	28.38 ± 0.17	20.14 ± 4.46	18.95 ± 4.10	17.98 ± 3.48
Kynurenine (μM)	0	0.12 ± 0.01	0.18 ± 0.05	0.26 ± 0.04

IL-17 concentrations are low in patients with invasive aspergillosis

To assess the capacity of *Aspergillus* to stimulate Th17 responses in vivo, we measured IL-17 concentrations in clinical specimens of patients with invasive aspergillosis. This consisted of BALs from 17 distinct patients diagnosed with proven/probable IA and another 20 BALs from corresponding control patients (i.e. patients with similar background underlying disease, but without invasive aspergillosis). The same analysis was performed on the serum of 19 patients with invasive aspergillosis (specimen obtained at the point of diagnosis of disease) and also 15 other control patients without IA. As seen in Fig. 6a, IL-17 levels are generally low in BALs of patients at risk of IA, and not higher than the concentrations found in patients without IA. Similarly, IL-17 concentrations were low in the circulation of patients with IA, and even decreased compared with controls (Fig. 6b).

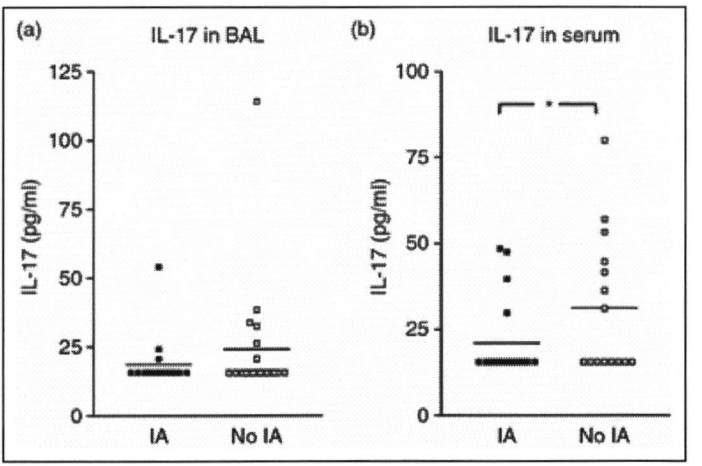


Figure 6. (a) Interleukin-17 (IL-17) levels in bronchoalveolar lavage of patients diagnosed with proven/probable invasive aspergillosis (IA) compared with a corresponding patient cohort without IA (No IA): $n = 17$ in IA group (eight proven IA cases and nine probable IA cases), $n = 20$ in No IA group. (b) IL-17 levels in serum of IA patients (IA) and corresponding patient controls (No IA): $n = 19$ in IA group (eight proven IA cases and 11 probable IA cases), and $n = 15$ in No IA group. $^*P < 0{\cdot}05$ (unpaired *t*-test).

Discussion

In the present study we investigated the activation of T-helper cellular responses activated by *A. fumigatus*. We demonstrate that *A. fumigatus* induces a very limited Th17 response in humans, both in-vitro and in-vivo. In contrast, *A. fumigatus* induces robust stimulation of IFN-γ production, implying that Th1 responses likely represent the cornerstone of T helper-dependent immunity against *Aspergillus* species.

Few studies have investigated the role of IL-17 in host defense against *Aspergillus* infection. The studies performed to date involved murine knock-out models, and reported contradicting results, with some studies suggesting a deleterious role of IL-17 [15, 33], while others have suggested that IL-17 is beneficial for anti-*Aspergillus* host defense [16]. In addition, induction of Th17 cells in humans may differ from that of mice [34, 35]. No studies have assessed the induction of Th17 responses in human cells. Moreover, patients with IA represent a unique susceptible cohort who have primarily hematological malignancies and are profoundly immunocompromised: being recipients of high-dose, long-term steroid therapy, chemotherapy, stem-cell transplantation and having prolonged agranulocytosis. Such mitigating conditions are difficult to simulate in the above-studied mice models. Therefore, studies in primary human cells are the only viable alternative for the study of Th17 responses in humans. Because host immune responses to live and killed *A. fumigatus* are known to be different [20, 36], and in order to closely mimic the physiological conditions in which the host immune cells encounter the pathogen, the stimulation of MDM/lymphocytes and live *A. fumigatus* conidia was investigated.

In the present study we report that *A. fumigatus* is a poor inducer of IL-17 in both PBMC and in a macrophage/T-cell system. Based on the current understanding of Th-17 activation pathway in other fungal infections such as *C. albicans* [25], this can be attributed to either the absence of specific memory T-cells against *A. fumigatus* or to a sub-optimal induction of the endogenous cytokines necessary for the induction of a Th17 response (e.g. IL-23, IL-1β, IL-6). However, even mimicking T-cell activation by the use of anti-CD3/anti-CD28 beads, as well as adding a 'pro-Th17 cytokine cocktail' consisting of IL-1β, IL-6 and IL-23 [34, 37, 38], failed to significantly augment IL-17 production in response to *Aspergillus*.

In contrast to the failure to induce IL-17 production, *A. fumigatus* induced robust Th1 IFN-γ responses [3, 30, 39]. It has been suggested by some studies that Th1 and Th17 differentiation can be mutually exclusive [10, 15]. If this would be indeed the case, the strong induction of IFN-γ by *Aspergillus*, may inhibit the Th17 responses and account for the lack of IL-17 induction in the presence of a predominant Th1 response. However, when we neutralized IFN-γ bioactivity with anti-human IFN-γ antibodies, this failed to stimulate IL-17, arguing for an intrinsic incapacity to stimulate Th17 responses, rather than indirect effects through Th1 induction. Similar to *Aspergillus*, the inhibition of IFN-γ during stimulation with *C. albicans* did not up-regulate IL-17 release, arguing against a major role of IFN-γ as a modulator of Th17 responses in fungal infections.

Interestingly, not only was *A. fumigatus* unable to induce much IL-17 production, it even actively inhibited the IL-17 release induced by mitogenic stimuli such as anti-CD3/anti-CD28

antibodies. The tryptophan metabolism pathway has recently been implicated for its role in regulating Th17 response to fungal infection [32]. Tryptophan can be metabolized by the enzyme indoleamine 2,3-dioxygenase (IDO) into the catabolite kynurenine. IDO and kynurenine have been shown to inhibit Th17 differentiation in mice [33]. IDO is also involved in modulation of Th1 differentiation [40, 41]. Interestingly, *A. fumigatus* also possess an IDO-family enzyme [42], and we show that *A. fumigatus* can induce metabolization of tryptophan into kynurenine. We investigated whether kynurenine is able to modulate Th17 also in human cells, and we have indeed observed inhibitory effects. Therefore, it seems rational to hypothesize that the observed inhibitory effect of *A. fumigatus* on the induction of IL-17 is at least in part mediated through its effects on tryptophan metabolites, via the generation of the inhibitory product kynurenine. However, besides kynurenine, we cannot exclude that additional factors may also mediate inhibition of Th17 induction by *Aspergillus*. Nonetheless, in our hands, these additional IL-17-modulating effects were probably not linked to innate signaling via the macrophage receptor [43] or Dectin-1 β-glucan receptor [16].

The importance of IL-17 in the pathogenesis of invasive aspergillosis remains to be proven. Recently it has been reported that IL-17 receptor-deficient mice, despite an initially elevated fungal burden, were eventually able to efficiently control aspergillosis [44]. This raises the question on the perceived non-redundancy of IL-17. From the clinical perspective, patients with hyper-IgE (HIE) syndrome are known to have deficiency of Th17 cells due to mutations in signal transducer and activator of transcription 3 (STAT-3) [45, 46] and are susceptible to secondary *Aspergillus* infection of pneumatoceles, but not to primary invasive aspergillosis [47]. Whilst colonization of pneumatoceles *with Aspergillus* in hyper IgE syndrome patients would seemingly support the notion of a protective role of IL-17 against fungal infections, one will need to keep in mind that the pathogenic mechanism behind an 'opportunistic colonization' of a lung cavity in a patient with hyper-IgE is perceivably distinct from that of invasive aspergillosis in a bone marrow transplant patient. Therefore, the clinical picture in Th17-defective patients with hyper-IgE syndrome does not dispute the notion that Th17 responses may not be essential in the host defense against invasive aspergillosis.

In conclusion, we demonstrate that *A. fumigatus* is a poor inducer of IL-17 response in humans, and this may be at least partly attributable to the pathogen's ability to metabolize tryptophan and secrete kynurenine which, in turn, down-regulates the Th17 pathway. The importance of IL-17 in the pathogenesis of invasive aspergillosis remains therefore in question, as Th1 response is likely the predominant T helper-dependent immunity mounted by the host against *Aspergillus* species. It is hence difficult to envisage a dominant role for IL-17 in host defense against *Aspergillus* at this juncture.

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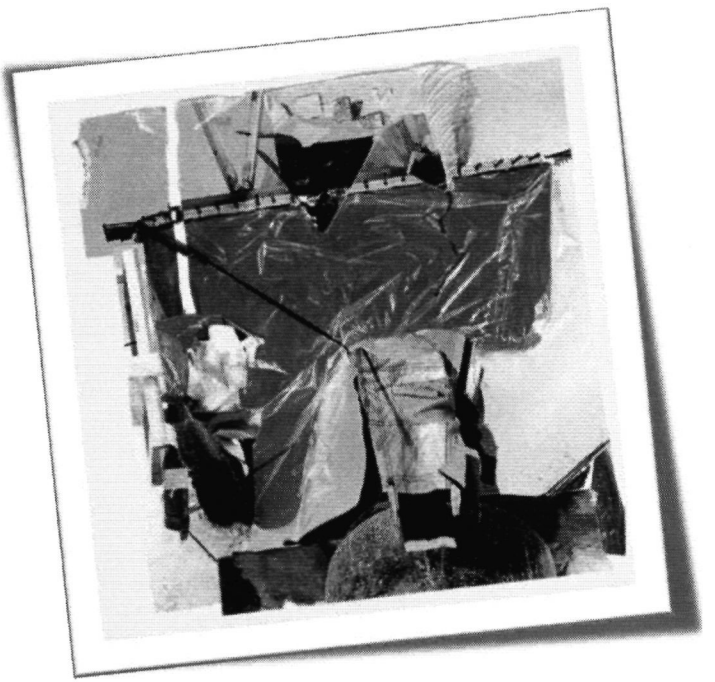
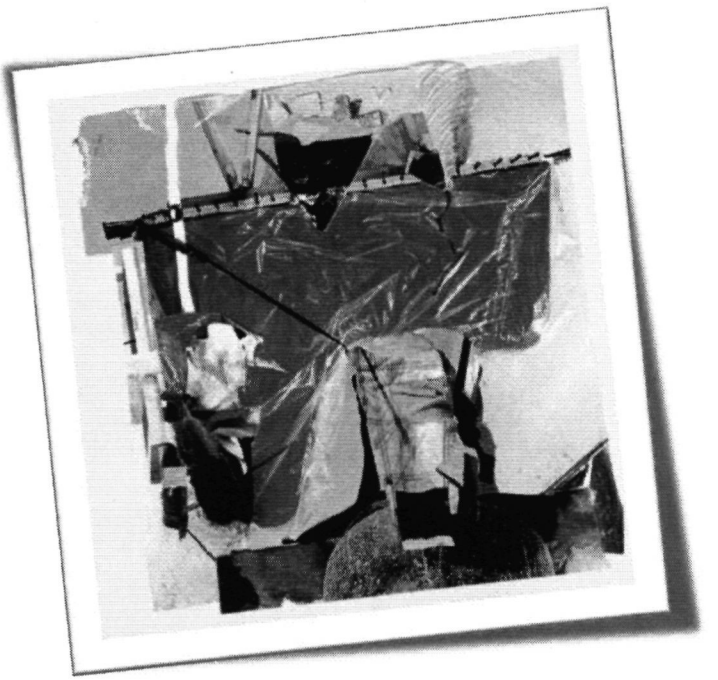
The inflammasome/Th17 axis in clinical syndromes



Reactive oxygen species-independent activation of the IL-1beta inflammasome in cells from patients with chronic granulomatous disease

Proc Natl Acad Sci USA. 2010 Feb 16;107(7):3030-3



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Summary

Humans with chronic granulomatous disease (CGD) due to mutations in p47-phox have defective NADPH activity and thus cannot generate NADPH-dependent reactive oxygen species (ROS). The role of ROS in inflammation is controversial; some in vitro studies suggest that ROS are crucial for secretion of IL-1β via inflammasome activation, whereas mice defective for ROS and patients with CGD have a proinflammatory phenotype. In this study, we evaluated activation of the IL-1β inflammasome in cells from CGD patients. In contrast to previous studies using the small molecule diphenylene iodonium (DPI) as a ROS inhibitor, we found no decrease in either caspase-1 activation or secretion of IL-1β and IL-18 in primary CGD monocytes. Moreover, activation of CGD monocytes by uric acid crystals induced a 4-fold higher level of IL-1β secretion compared with that seen in monocytes from unaffected subjects, and this increase was not due to increased synthesis of the IL-1β precursor. In addition, Western blot analysis of CGD cells revealed that caspase-1 activation was not decreased, but rather was increased compared with control cells. Examination of the effects exerted by the inhibition of ROS activity by DPI revealed that the decrease in IL-1β secretion by DPI was actually due to inhibition of IL-1β gene expression. Thus, inconsistent with the proinflammatory role of ROS, the present findings support the concept that ROS likely dampen inflammasome activation. The absence of ROS in CGD monocytes may explain the presence of an inflammatory phenotype characterized by granulomas and inflammatory bowel disease occurring in CGD patients.

Introduction

There is much recent interest in the processing and release of bioactive IL-1β, especially since the discovery that blockade of IL-1 receptors with the IL-1 receptor antagonist (IL-1Ra) is a very effective treatment for autoinflammatory disorders, such as familial Mediterranean fever (1), familial cold autoinflammatory syndrome (2), Muckle-Wells syndrome (3), neonatal-onset multisystem inflammatory disease (4), hyperimmunoglobulin D syndrome (HIDS) (5), and adult-onset Still's disease (6). Blood monocytes from patients with some of these disorders, especially cryopyrinopathies and HIDS, readily release more IL-1β than do monocytes from unaffected controls (7, 8).
Activation of caspase-1 by the protein complex known as the inflammasome leads to the conversion of pro-IL-1β to IL-1β (9). Several protein platforms/inflammasomes have been described for the activation of caspase-1, each of which includes members of the NOD-like receptor (NLR) family of proteins (10). The most intensely studied of these are the inflammasomes formed by the NLR family members NLRP3 and NLRP1. Several conditions are thought to be required for the activation of the inflammasome, including the interaction of "danger-signaling" molecules with NLRP components, the induction of K+ efflux through the P2X7 receptor, and the generation of reactive oxygen species (ROS) (9, 11).

The role of ROS in inflammation is controversial, however. On the one hand, ROS have been suggested to induce NF-κB activation (12, 13), and several in vitro studies have proposed that activation of the inflammasome is strictly dependent on ROS generation (11, 14). On the other hand, other studies have reported anti-inflammatory effects of the NADPH system and ROS (15), and a recent study in mice defective for the generation of ROS strongly suggested anti-inflammatory effects of oxygen species (16). In line with this, patients with chronic

granulomatous disease (CGD) with defects in the NADPH system and, consequently, defective ROS generation (17) display an inflammatory phenotype characterized by granulomas and Crohn-like colitis (18). To evaluate the role of ROS in inflammasome activation, we investigated the activation of caspase-1 and the production of IL-1β in the presence of NADPH inhibitors. We also assessed inflammasome activation in cells isolated from CGD patients.

Results

DPI inhibits transcription of proinflammatory cytokines

We found that LPS induced production of IL-1β and TNF-α from primary human peripheral blood mononuclear cells (PBMCs), as reported previously (19). ROS inhibition by diphenylene iodonium (DPI) decreased production of both IL-1β and TNF-α induced by LPS (Fig. 1A). Because TNF-α release is independent of caspase-1 activation, this argues for effects of DPI independent of the inflammasome. Indeed, the effect of DPI was exerted at a transcriptional level, because mRNA for both IL-1β and TNF-α was decreased by DPI (Fig. 1B), whereas active caspase-1 was still present (Fig. 1C).

Toll-like receptor–induced production of IL-1β is normal in CGD patients

The Toll-like receptor (TLR) ligands Pam3Cys (TLR2 ligand) and LPS (TLR4 ligand) induced a strong IL-1β response in PBMCs of both healthy volunteers and CGD patients (Fig. 2A). Although ROS have been specifically implicated in the activation of the inflammasome (11), when PBMCs from CGD patients who lacked ROS were primed with LPS and subsequently stimulated with the inflammasome activator ATP for 15 min, no difference in the release of IL-1β was seen between cells of healthy individuals and those of CGD patients (Fig. 2B). As expected, intracellular pro-IL-1β intracellular concentrations did not differ between healthy volunteers and CGD patients (Fig. 2B). IL-18 also is an important proinflammatory cytokine of the IL-1β family that is processed by caspase-1. No differences in IL-18 production were observed between healthy volunteers and CGD patients (5.8 pg/mL vs 4.9 pg/mL), although the very low amounts of IL-18 released by primary monocytes must be noted.

Inflammasome activation is increased in CGD patients

Despite defective NADPH-dependent ROS generation, more prominent activation of caspase-1 was apparent in monocytes isolated from CGD patients compared with those from healthy volunteers (Fig. 3A). The NLRP3 inflammasome has been reported to be activated by uric acid crystals (11,20), and priming with a TLR stimulus such as LPS is required to accomplish this activation (21). However, PBMCs from CGD patients were able to produce IL-1β when exposed to uric acid alone in the absence of LPS priming, a phenomenon not observed in normal volunteers (Fig. 3B), demonstrating increased activation of the inflammasome in cells from CGD patients. In addition, DPI decreased cytokine production in both cells from healthy controls and cells isolated from CGD patients (Fig. 4).

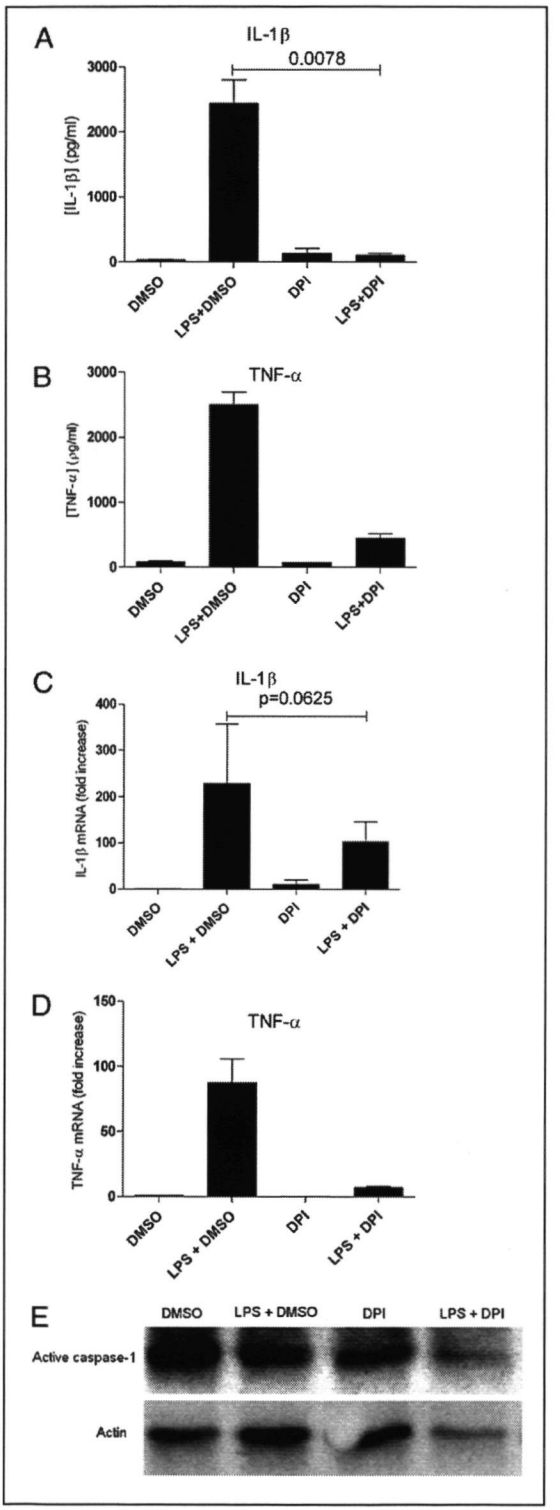

Figure 1. ROS inhibition decreases production and transcription of IL-1β and TNF-α.

(A) Monocytes isolated from eight healthy controls were stimulated with LPS in the absence or presence of the ROS inhibitor DPI. In the presence of DPI, IL-1β production was completely inhibited (n = 8). (B) TNF-α production also was decreased when LPS-stimulated PBMCs of healthy controls were cultured in the presence of DPI (n = 2). (C) PBMCs of healthy controls were stimulated for 4h with LPS in the absence or presence of DPI. mRNA was isolated from the cell lysates using TRIzol. DPI decreased mRNA expression of IL-1β (n = 6). (D) DPI also decreased mRNA expression of TNF-α (n = 2). (E) Active p10 caspase-1 was still expressed in cells cultured in the presence of DPI. Data are representative for four healthy volunteers.

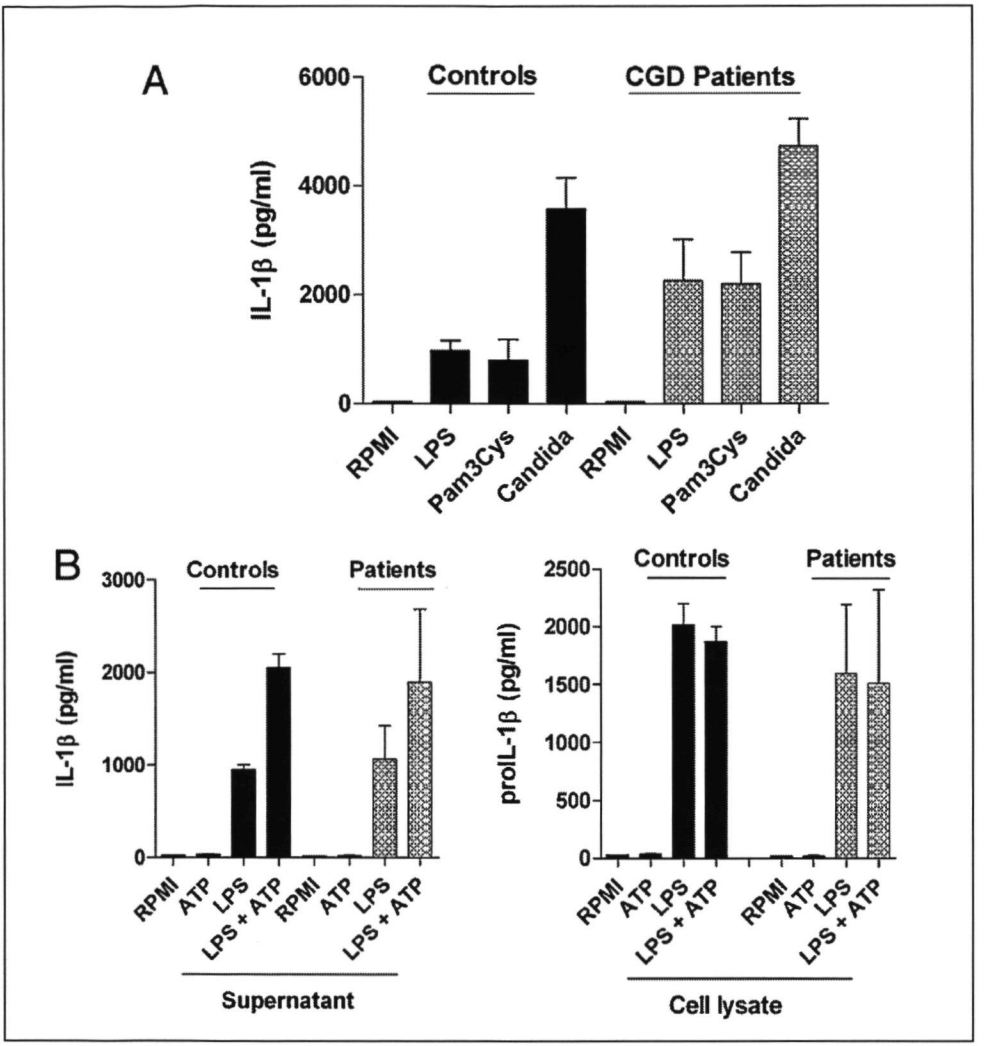

Figure 2. Inflammasome activation and IL-1β production in CGD patients.
(A) Monocytes isolated from CGD patients and healthy controls produced IL-1β on stimulation with LPS, Pam3cys, and *Candida*. (B) IL-1β stimulation by LPS and ATP was similar in CGD and control individuals. Data are presented as mean ± SEM of five healthy controls and of three CGD patients.

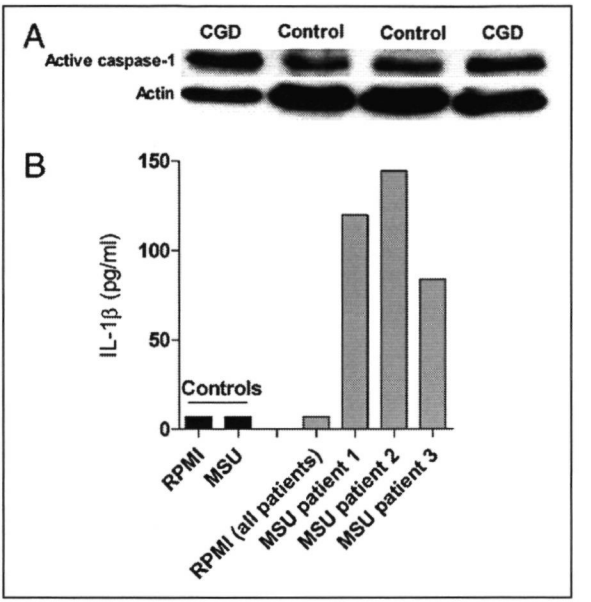

Figure 3. ROS inhibit inflammasome activation.
(A) Active p10 caspase-1 was expressed more strongly in unstimulated monocytes from CGD patients than in those from healthy controls. (B) NALP3 inflammasome stimulus uric acid crystals stimulated IL-1β release in monocytes isolated from CGD patients, but not in those from healthy controls. Data are presented as mean ± SEM of five healthy controls and of three CGD patients.

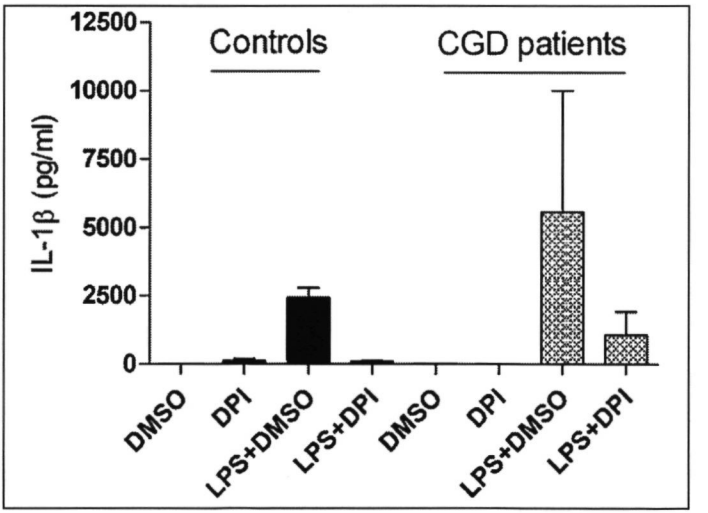

Figure 4. ROS inhibition decreased IL-1β production in both healthy controls and CGD patients.
In both healthy controls and CGD patients, DPI decreased IL-1β production in LPS-stimulated cells. Data are presented as mean ± SEM.

Discussion

Our findings indicate that ROS are not essential for inflammasome activation. This conclusion is based on several lines of evidence. First, treatment of human primary monocytes with the commonly used ROS inhibitor DPI did not inhibit generation of active caspase-1. Second, cells from CGD patients expressed more constitutively active caspase-1 activation. Third, ROS-deficient cells from CGD patients produced more IL-1β after stimulation with urate crystals, a classical inflammasome stimulus.

These findings differ from the results of other recently published studies suggesting that ROS induce inflammasome activation and IL-1β production (11, 14). Those studies were based on the use of ROS inhibitors such as DPI in cell lines or mouse macrophages, however (11, 14, 22). Thus, we investigated the effects of DPI on caspase-1 activation and IL-1β production in human PBMCs. We found that DPI can indeed inhibit IL-1β production in human PBMCs, as reported previously in THP-1 cells and mouse macrophages (11, 14). This inhibition was accompanied not only by lower IL-1β release, but also by decreased TNF-α production, suggesting that DPI can exert its effects independently of the inflammasome. These effects of DPI were exerted at the level of transcription, with repression of mRNA for both TNF-α and IL-1β, whereas the active caspase-1 p35 fragment was normally present. Our data demonstrating that the anti-inflammatory effects of DPI on IL-1β production are exerted at the transcriptional level are in line with the large body of literature reporting that ROS induce NF-κB activation (12, 23, 24). In addition, the clear inhibition of cytokine stimulation by DPI in cells of CGD patients, a system in which ROS production is defective, clearly demonstrates that the effects of DPI on cytokine production are largely independent of NADPH-oxydase generation of ROS. Possible additional mechanisms modulated by DPI include inhibition of nitric oxide synthetase and mitochondrial complex 1-dependent (25, 26). Thus, results based on DPI as the sole method of inhibition of ROS production should be interpreted with caution.

In addition to these arguments, it should be kept in mind that the concept that ROS induces inflammasome activation is at odds with the known proinflammatory phenotype of patients with CGD, characterized by the occurrence of sterile granulomas, colitis, and inflammatory skin and urogenital reactions, in which IL-1β is thought to play an important pathogenic role (15). In contrast, our findings of the inhibitory effects of ROS on caspase-1 activation agree with a proinflammatory state in CGD, because we found that the release of mature IL-1β was normal and after certain stimuli even increased in cells of CGD patients. Our data do not stand alone; they are in line with several previous studies that have demonstrated consistent up-regulation of various proinflammatory cytokines in cells isolated from CGD patients (27–30).

Unexpectedly, whereas we found that primary monocytes from healthy volunteers did not produce IL-1β in response to uric acid alone, monocytes from CGD patients secreted substantial amounts of IL-1β. In addition, we observed more constitutively activated caspase-1 in monocytes from CGD patients compared with healthy volunteers. In line with our data, there is additional evidence that increased ROS production inhibits caspase-1 activation. Superoxide dismutase 1 (SOD-1) degrades ROS, and thus SOD-1 deficiency results in increased endogenous ROS production. It was recently reported that the increased super-

oxide production in SOD-1–deficient macrophages specifically inhibits caspase-1 activation by oxidation and glutathionylation (31). SOD-1–deficient mice produced less IL-1β in vivo and were less susceptible to LPS-induced shock (31). Another recent study demonstrated that silencing SOD-1 in human monocytes results in a reduction of IL-1β secretion on stimulation with zymosan (32). Taken together, these data are in agreement with our findings and strongly suggest that ROS inhibit inflammasome activation and, subsequently, IL-1β production.

In conclusion, our findings of the present study settle the controversy regarding the role of ROS in inflammasome activation in human cells by providing evidence that oxygen radicals have an inhibitory effect on caspase-1 activation and IL-1β release. These data explain the proinflammatory clinical phenotype seen in patients with CGD.

Methods

Patients and Controls

Eight healthy volunteers with no known infectious or inflammatory disorders donated blood as a control group for the assessment of cytokine production capacity. In addition, PBMCs were isolated from three patients with CGD harboring homozygous mutations in the NCF1 gene (p47-phox), in which defective ROS production has been demonstrated. After informed consent was obtained, blood was collected by venipuncture from both patients and volunteers into 10-mL EDTA tubes [BD, Plymouth, UK (art. no. 367525)].

In Vitro Cytokine Production

Separation and stimulation of PBMCs was performed as described previously (33). In brief, the PBMC fraction was obtained by density centrifugation of diluted blood (1 part blood to 1 part pyrogen-free saline) over Ficoll-Paque (Pharmacia Biotech). PBMCs were washed twice in saline and suspended in culture medium supplemented with gentamicin 1%, L-glutamine 1%, and pyruvate 1%. The cells were counted in a Bürker counting chamber, and their number was adjusted to 5x10e6 cells/mL. Then 5x10e5 PBMCs in a volume of 100 μL per well were incubated at 37 °C in round- bottomed 96-well plates (Greiner). After 24h of incubation with the various stimuli as described below, supernatants were collected and stored at −80 °C until being assayed for IL-1β and TNF-α production. DPI in a concentration of 10 μM was used as a ROS inhibitor (34). Two methodologies were used to assess inflammasome stimulation. One assay used stimulation for 3h with LPS, followed by ATP-induced IL-1β release for 15 min (35), and a second assay used specific stimulation with LPS-free monosodium urate, also known as uric acid crystals, a putative NLRP3 ligand (20).

Cytokine Assays

IL-1β and TNF-α concentrations were measured with com- mercial ELISA kits (R&D Systems). Pro-IL-1β concentrations in the cell lysates were measured by specific ELISA (R&D Systems). The concentration of IL-18 was measured with a BioPlex kit (Bio-Rad).

RT-PCR

Two million freshly isolated PBMCs were incubated with the various stimuli. After 4h of incubation at 37°C, total RNA was extracted in 800 μL of TRIzol reagent (Invitrogen). Isolated RNA was reverse-transcribed into cDNA using oligo(dT) primers and M-MLV reverse

transcriptase. PCR was performed using an Applied Biosystems 7300 real-time PCR system. The primer sequences for human IL-1β were as follows: sense, 5′- GCC-CTA-AAC-AGA-TGA-AGT-GCT- C-3′; antisense, 5′- GAA-CCA-GCA-TCT-TCC-TCA-G-3′. β2M was used as a reference gene, for which the primers were as follows: 5-ATG-AGT-ATG-CCT- GCC-GTG-TG-3 (forward) and 5-CCA-AAT-GCG-GCA-TCT-TCA-AAC-3 (reverse). PCR conditions were 2 min at 50°C and 10 min at 95°C, followed by 40 cycles at 95 °C for 15 s and at 60°C for 1 min.

Immunoblotting for Caspase-1

For immunoblotting, 10x10e6 cells were lysed in 100 mL of lysis buffer [50 mM Tris (pH 7.4), 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 10% glycerol, 1% Triton X-100, 40 mM a-glycerophosphate, 50 mM sodium fluoride, 200 mM sodium vanadate, 10 mg/mL leupeptin, 10 mg/mL aprotinin, 1 mM pepstatin A, and 1 mM phenylmethylsulfonyl fluoride]. The homogenate was frozen, then thawed and centrifuged at 4°C for 10 min at "15,000 × g", and the supernatant was taken for Western blot analysis. Equal amounts of protein were subjected to SDS/PAGE using 10% and 15% polyacrylamide gels at a constant voltage of 100 V. After SDS/PAGE, proteins were transferred to nitrocellulose membrane (0.2 mm). The membrane was blocked with 5% (wt/vol) milk powder in PBS for 1h at room temperature, followed by incubation overnight at 4°C with a caspase-1 p10 antibody (SC-515; Santa Cruz Biotechnology) in 5% BSA/TBS/Tween 20. After overnight incubation, the blots were washed three times with TBS/Tween 20 and then incubated with HRP-conjugated goat anti-rabbit antibody at a dilution of 1:10 000 in 5% (wt/vol) milk powder in PBS for 1h at room temperature. After being washed three times with TBS/Tween 20, the blots where developed with ECL (GE Healthcare) according to the manufacturer's instructions.

Statistical Analyses

Differences between groups were analyzed using the Mann-Whitney U test for unpaired data and the Wilcoxon signed-rank test for paired data. Differences were considered statistically significant at $P \leq 0.05$. Data represent the cumulative results of all experiments performed and are presented as mean ± SEM.

Acknowledgements

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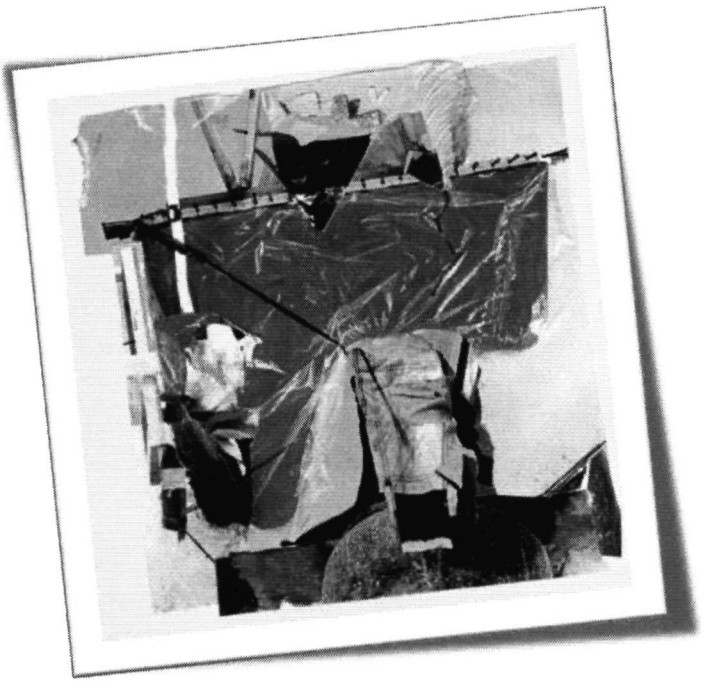
Chapter 15

The anti-CD20 antibody rituximab reduces the T helper 17 response

Arthritis and Rheumatism, accepted



van de Veerdonk FL, Lauwerys B, Marijnissen RJ, Timmermans K, Di Padova F, Koenders MI, Gutierrez-Roelens I, Durez P, Netea MG, van der Meer JW, van den Berg WB, Joosten LA.

Summary

B cell depleting therapy with rituximab is unexpectedly succesful in rheumatoid arthritis (RA) and multiple sclerosis (MS). The findings that B cells in MS and RA play an important role, raises the question of the mechanism of action of rituximab. In the present study, we demonstrate that rituximab specifically inhibits the Th17 response which correlates with an improved clinical outcome in rheumatoid arthritis. In agammaglobulinemia patients, that do not have functional B cells, the inhibitory effect of rituximab on the Th17 response was not observed. Thus supporting a direct effect of rituximab on B cells. Taken together, these data demonstrate an unexpected role of B cells for the development of Th17 responses, possibly leading to B cell-based strategies in patients with Th17-related immune diseases.

Introduction

Rituximab is a monoclonal antibody directed towards the CD20 antigen on B lymphocytes, which is nowadays a first line treatment in antibody-mediated autoimmune disorders such as autoimmune thrombocytopenia and autoimmune hemolytic anemia. More surprising and less easy to explain, is the ability of rituximab to reduce disease severity in patients with rheumatoid arthritis (RA) (1), as this effect cannot be attributed to a reduction in circulating autoantibodies (2). Also, it has been shown that rituximab remarkably reduces brain lesions in patients with multiple sclerosis (MS), a disease with a proven T-lymphocyte-mediated pathology (3). The effect of rituximab on disease activity in MS is rapid and since rituximab does not deplete mature plasma cells, it is unlikely that a reduction of pathogenic antibodies is responsible for these effects (4). Thus, mechanisms other than depleting (auto)antibodies should be sought for.

Recently, MS, RA, Crohn's disease and psoriasis have been linked to a subgroup of T helper (Th) lymphocytes, called Th17 cells (5). These lymphocytes are preferential producers of a series of cytokines (IL-17A, IL-21 and IL-22) (6) which are thought to contribute to the tissue damage in RA (7) and MS (8). The role of Th17 cells in the pathogenesis of these autoimmune diseases led us to hypothesize that rituximab leads to inhibition of Th17 cells. In the present study we assessed this hypothesis in synovial biopsies in RA patients treated with rituximab, and in in-vitro experiments.

Material and Methods

Ethics Statement
The clinical study was approved by the ethics committee of the Université Catholique de Louvain and informed consent was obtained from all patients.

Volunteer
Blood was collected from 21 healthy, non-smoking volunteers who were free of infectious or inflammatory disease, and from four patients with genetically proven X-linked agammaglobulinemia, after informed consent. Blood samples were collected in 10 ml EDTA tubes (BD Vacutainer®, United Kingdom).

RA Patients and synovial biopsies

12 patients with RA (9 women and 3 men, average age ± SEM: 54.6 ± 4.6 years) were included in the study. All patients met the American College of Rheumatology classification criteria for RA. All patients had active disease at the time of baseline tissue sampling and were resistant to TNF blockade. They all had erosive changes imaged on conventional x-rays of the hands and/or feet. All of them had a swollen knee at inclusion. Mean baseline C-reactive protein ± SEM was 33.08 ± 6.76 mg/l and mean baseline Disease Activity (DAS28)-CRP score (4 variables) ± SEM was 5.80 ± 0.38. Rituximab therapy was administered at a dose of 1,000 mg IV at baseline (T0) and week 2, together with 125 mg IV Methylprednisolone. Disease activity at baseline (T0) and 12 weeks after initiation of therapy (T12) was evaluated using DAS(28)-CRP (4-variables) scores (DAS-28 score). Synovial biopsies were obtained by needle-arthroscopy of the affected knee of all patients at T0 and T12. For each procedure, 4 to 8 synovial samples were kept overnight at 4°C in a RNA stabilizing solution and then stored at –80°C for later RNA extraction. The same amount of tissue was snap-frozen in liquid nitrogen and kept at -80°C for immunostaining experiments on frozen sections. The remaining material was fixed in 10% formaldehyde and paraffin embedded for conventional optical evaluation and immunostaining of selected markers. All the experiments (RNA extraction, histology, immunohistochemistry) were performed on at least 4 biopsies harvested during every procedure in order to correct for variations related to the potential heterogeneous distribution of synovial inflammation.

Microorganism

C. albicans ATCC MYA-3573 (UC 820)(9) was grown overnight in Sabouraud broth at 37°C, cells were harvested by centrifugation, washed twice, and resuspended in culture medium (RPMI-1640 Dutch modification, ICN Biomedicals, Aurora, OH) (10). *C. albicans* was heat-killed (1h at 100°C).

In vitro cytokine production

Separation and stimulation of PBMCs was performed as described previously (11). Briefly, the PBMCs fraction was obtained by density centrifugation of diluted blood (1 part blood to 1 part pyrogen-free saline) over Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden). PBMCs were washed twice in saline and suspended in culture medium supplemented with gentamicin 1%, L-glutamine 1%, and pyruvate 1%. The cells were counted in a hemocytometer, and their number was adjusted to 5×10^6 cells/ml. 5×10^5 PBMCs in a volume of 200 µl per well were incubated at 37°C in round-bottom 96-wells plates (Greiner, Nurnberg, Germany), in the presence of 10% human pooled serum, with *C. albicans* or culture medium alone. In blocking experiments, PBMCs were preincubated for 2 h with Rituximab 10 ug/ml. After 5 days of incubation, supernatants were collected and stored at –20°C until assayed.

Cytokine assays

IL-17A, IL-22, IFNγ, and TNFα concentrations were measured by commercial ELISA kits (R&D systems) following the manufacturer's instructions.

Intracellular cytokine staining

PBMCs were stimulated for 4-6 hours with PMA (50 ng/ml; Sigma) and ionomycine (1 ug/ml; Sigma) in the presence of Golgiplug (BD biosciences) according to manufacturer's protocol.

Cells were first extracellularly stained using an anti-CD4 APC antibody (BD biosciences). Subsequently the cells were fixed and permeabilized with Cytofix/ Cytoperm solution (BD biosciences) and then intracellularly stained with anti-IFNγ PE (eBiosciences) and anti-IL-17A FITC (eBiosciences). Samples were measured on a FACS Calibur and data were analyzed using the CellQuest-Pro software (BD biosciences).

Quantitative PCR

PBMCs were cultured for 3 days and RNA was extracted using TRI-reagent (Sigma). The isolated RNA was treated with DNase to remove genomic DNA and subsequently reverse transcribed with oligo(dT) primers in a reverse transcriptase procedure with a total volume of 20 µl. Quantitative real-time PCR was performed using the ABI/PRISM 7000 Sequence Detection System, with primer pairs and SYBR Green PCR Master Mix (Applied Biosystems). The sequences used, were as follows: *IL-22*, forward 5'-TGCAGGCTTGACAAGTCCAA-3' reverse 5'-CTAGCCTCCTTAGCCAGCATGA-3'; *RORγT*, forward 5'TGAGAAGGACAGGGAGCCAA 3' reverse 5'-CCACAGATTTTGCAAGGGATCA -3'; *TNF*, forward 5'-TCTTCTCGAACCCCGAGTGA-3'reverse 5'-CCTCTGATGGCCCCACCAG-3';*IFNγ*, forward 5'-GGAACTCTTTTCTTAGGCATTTTGA-3'reverse 5'-GATGGTCTCCACACTCTTTTGGA-3'. Quantification of the PCR signals was performed by comparing the cycle threshold value (Ct) of the gene of interest with the Ct values of the reference gene GAPDH. All primers were developed using Primer Express 2.0 (Applied Biosystems) and validated according to the protocol. Values are expressed as fold increases of mRNA relative to that in unstimulated cells.

Microarray hybridization

Total RNA was extracted from the synovial biopsies using the Nucleospin® RNA II extraction kit (Macherey-Nagel), including DNase treatment of the samples. At least 1 ug total RNA could be extracted from 12 paired samples at T0 and T12 for further processing. RNA quality was assessed using an Agilent 2100 Bioanalyzer and RNA nanochips (Agilent Technologies Inc). All samples had a 28s/18s ratio > 1.8. Labeling of RNA (cRNA synthesis) was performed according to a standard Affymetrix® procedure (One-Cycle Target Labeling kit, Affymetrix UK Ltd., High Wycombe, UK); briefly total RNA was first reverse transcribed into single-stranded cDNA using a T7-Oligo(dT) Promoter Primer and Superscript II reverse transcriptase. Next, RNase H was added together with E. Coli DNA polymerase I and E. Coli DNA ligase, followed by a short incubation with T4 DNA polymerase in order to achieve synthesis of the second-strand cDNA. The purified double-stranded cDNA served as the template for the in vitro transcription reaction, which was carried out overnight in the presence of T7 RNA polymerase and a biotinylated nucleotide analog/ribonucleotide mix. At the end of this procedure, the biotinylated complementary RNA (cRNA) was cleaned, and fragmented by a 35-minute incubation at 95°c. GeneChip® Human genome U133 Plus 2.0 Arrays (spotted with 1,300,000 oligonucleotides informative for 47,000 transcripts originated from 39,000 genes, Affymetrix UK Ltd, High Wycombe, UK) were hybridized overnight at 45°c in monoplicates with 10 ug cRNA. The slides were then washed and stained using the EukGE-WS2v5 Fluidics protocol on the Genechip® Fluidics Station (Affymetrix) before being scanned on a Genechip® Scanner 3000. For the initial normalization and analysis steps, data were retrieved on Affymetrix GCOS software. The frequency of positive genes (genes with a flag present) was between 40 and 50% on each slide. After scaling of all probe sets to a value of 100, the range of the reported amplification scales was between 0.7 and 6.7. The signals yielded by the poly-A RNA, hybridization and housekeeping controls (GAPDH 3'/5' ratio < 2.3

in all the slides) were indicative of the good quality of the amplification and hybridization procedures. Since values were labeled as "absent" we used these data only to support our observations. In the text and figures we use the microarray data, but mention that the data was only suggestive.

Immunolabeling experiments on paraffin-embedded sections

Fresh synovial biopsy tissue samples (n=12 at T0 and T12) were fixed overnight in 10 % formalin buffer at pH 7.0 and embedded in paraffin for immunohistochemical analyses. Immunolabeling experiments were performed using a standard protocol. After removal of paraffin and inactivation of endogenous peroxidases with 0.3% H_2O_2 for 30 minutes at room temperature, sections were incubated in 10 mM sodium citrate buffer, pH 5.8, and heated in a bain-marie at 98°C for 75 minutes to retrieve the antigenic sites. Non-specific binding was blocked by a 30-minutes incubation with 50 mM Tris-HCl, pH 7.4, containing 10% (volume/volume) normal goat serum and 1% (weight/volume) bovine serum albumin. Sections were then incubated overnight at 4°C with the primary antibody. The following antibodies were used: CD3 (Neomarkers, Westinghouse, CA), CD20 (Biocare medica, Walnut Creek, CA), CD68 (DakoCytomation, Glastrup, Denmark), CD138 (DakoCytomation), IL17 (eBioscience, San Diego, CA) and LRRC32 (Enzo Life Sciences, Farmingdale, NY, USA). After 3 washes in 50 mM Tris-HCl, pH 7.4, specifically bound antibodies were labeled for 1 hour at room temperature with Envision™ (DakoCytomation), and the activity of peroxidases was revealed by a 10-minutes incubation with 0.5 mg/ml diaminobenzidine in Tris-HCl buffer. As a final step, sections were washed in tap water and lightly counterstained with hematoxylin. Semiquantitative analyses were performed using a semiquantitative score on a scale of 0–3. Quantitative analysis of the CD20 and IL-17A antibody immunostained sections was performed using ImageJ software, according to the Digital Image Analysis process. Six digitalized pictures (400X magnification) were obtained for each slide by an operator who was blinded to the identity of the specimens. Each picture included lining and sublining regions when possible. When the distribution of the staining was heterogeneous, the pictures were taken in order to be representative of the globality of the slide. The surface staining (S) and the surface of the nuclei (N) were determined for each image, and the area of staining was then normalized by calculating the ratio of surface staining to nuclei staining.

Statistical analysis

The differences between groups were analyzed by the Wilcoxon rank test. Differences were considered statistically significant when $P \leq 0.05$. All experiments were performed at least twice, and the data is presented as the cumulative result of all experiments performed, unless otherwise indicated.

Results

Rituximab inhibits the Th17 response in patients with RA

To assess the influence of rituximab on inflammation and more specific, Th17 responses in vivo, 12 patients with refractory RA treated with rituximab were investigated. At day 0 and 12 weeks after rituximab treatment, disease activity scores (DAS-28) were calculated and synovial tissue biopsies were taken from the knee joints. This group of RA patients treated with rituximab showed a significant improvement of their DAS-28 score (Fig. 1a). Immunohistochemistry showed that rituximab had efficiently depleted the CD20+ B cells in

the synovium of inflamed knee joints at 12 weeks (Fig. 1b,c). The next step was to evaluate the effect of rituximab on Th17 responses in the joints of these patients before and after treatment of rituximab. Using immunostaining, we investigated the affected synovial tissue for IL-17A, before and after treatment with rituximab and found that rituximab therapy induced a significant decrease in synovial IL-17A (Fig 1d,e). In addition, the mRNA signals for the relevant Th17 cytokines IL-17A, IL-21 and IL-22 were measured in the inflamed joints, using microarray analysis. The mRNA for these three cytokines was lower in the affected joints of patients with RA at 12 weeks after treatment with rituximab (Supplemental data, Fig. S1). RT-qPCR confirmed the results of the microarray analysis, with mRNA for RORC and IL-22 being markedly reduced after treatment (Fig. 1f).

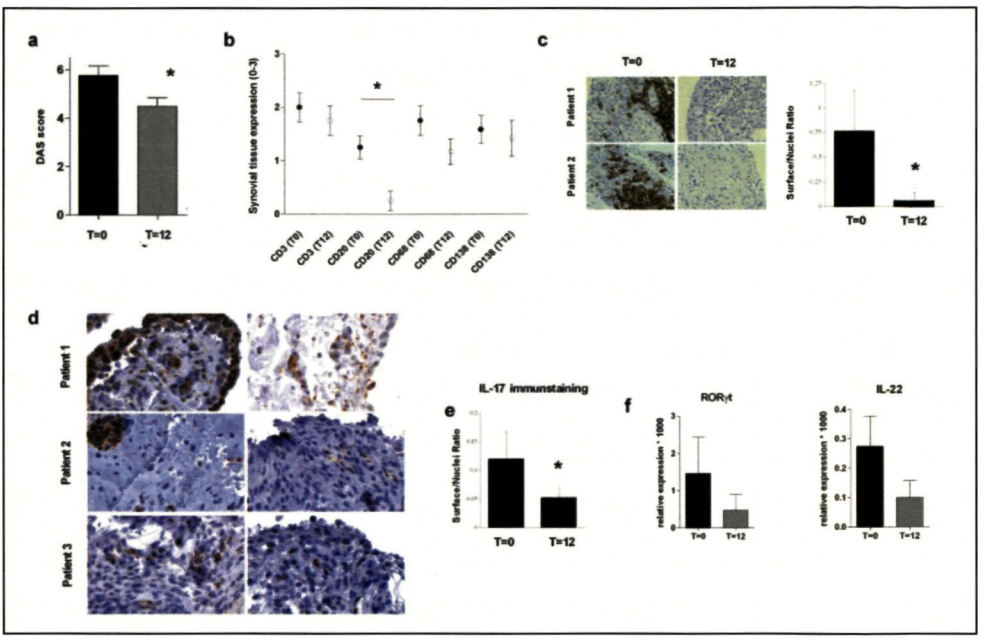


Figure 1. Rituximab inhibits the Th17 response in patients with RA.
(a) DAS-28 score of RA patients (n=12) treated with rituximab. (b) Histologic scores of CD3, CD20, CD68 and CD131 in synovial tissue obtained by biopsy of affected joints of patients with rheumatoid arthritis (n=12) before and after treatment. (c) Staining of CD20 in synovium before and after treatment with rituximab in two patients with a good clinical response (magnification 400x). Bars represent staining of CD20 in synovium before and after treatment with rituximab (n=12 patients). (d) IL-17A immunostaining in synovial biopsies of RA patients before and after rituximab therapy, characteristic images from three different patients at T0 and T12 (original magnification 400x). (e) IL-17A immunostaining in synovial biopsies from patients with RA before and after rituximab therapy in (n=12 patients) (f) qPCR of the expression of RORγt, IL-22 in inflamed synovial tissue from patients with RA before and after treatment with rituximab (n=4).

Rituximab specifically inhibits the Th17 response

Since the balance between T regulatory cells (Treg) and Th17 cells has been advocated to play an important role in autoimmune diseases (12, 13), it was investigated if the reponse to rituximab was Treg dependent. It has recently been shown that activated Tregs express mRNA for a transmembrane protein called glycoprotein A repetitions predominant (GARP, or LRRC32), and that expression of GARP on activated Tregs correlates with their

immunosupressive capacity (14). When GARP was stained in the knee joints of these patients, no significant difference was seen (Fig. 2a). Furthermore, to address if the response to rituximab was due to an overall effect on T cell responses CD3 positive cells were stained in the synovial tissue and the effects of rituximab on the Th1 response were studied. In line with previous studies (1, 3), there was no reduction seen in the amount of T cells after treatment with rituximab (Fig. 1b). In addition to the Th17 response, the proinflammatory Th1 response plays a critical role in autoimmunity. However, IFNγ was not affected by the treatment with rituximab in vivo (Fig. 2b). Inhibition of TNFα is known to be effective in RA (15). We therefore wanted to investigate if the effects seen in patients with RA that were treated with B cell depleting therapy was dependent on TNFα. Interestingly, the mRNA expression for TNFα was not influenced by treatment with Rituximab in the affected knee joints of patients with RA (supplemental data Fig S1, Fig 2b). These results demonstrate that rituximab exerts its effects in vivo specifically through the inhibition of the Th 17 response and not through T regulatory, Th1 or TNFα responses.

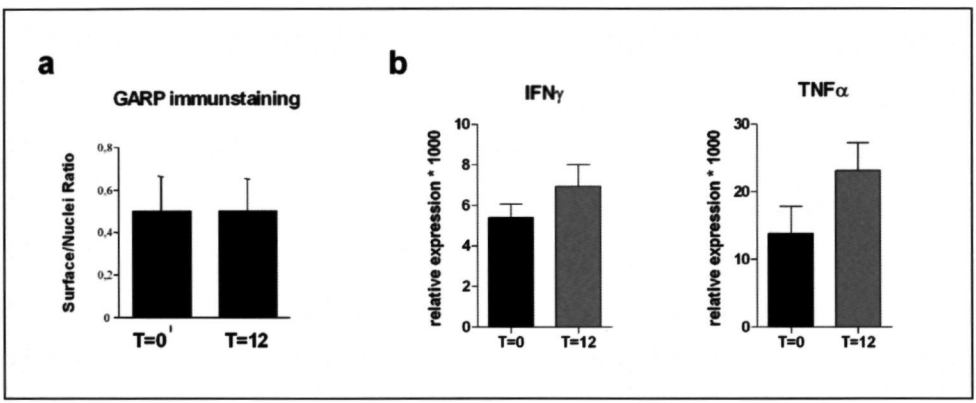


Figure 2. Rituximab inhibits the Th17 response.
(a) GARP immunostaining in synovial biopsies from RTX-treated RA patients at T0 and T12 (n=12). (b) qPCR of the expression of TNF and IFNγ. in inflamed synovial tissue from patients with RA before and after treatment with rituximab (n=4). (a) Results are the mean (+/- SEM) surface/Nuclei ratio and digital quantification was performed using ImageJ, with 6 digitalized pictures (400x magnification) analyzed for each sample at T0 and T12. (b) Data is given as relative mRNA expression (2-.CT x 1000). *P<0.05 using Wilcoxon matched-pairs signed-rank tests.

The effect of rituximab on Th17 responses is mediated through depletion of B cells

To further investigate the effects of rituximab on the Th17 response, we used an in-vitro system in which peripheral blood mononuclear cells (PBMCs) from healthy volunteers were stimulated with the potent Th17-inducing stimulus *C. albicans* (16) in the absence or presence of rituximab. Notably, antigen presenting cells (APCs) are needed together with memory T cells in order for *C. albicans* to induce a Th17 response in human PBMCs (17). When PBMCs were treated with rituximab, all CD20 positive cells were depleted (Fig. 3a). PBMCs exposed to *C. albicans* showed a clear Th17 response reflected by a significant production of IL-17A and IL-22 (Fig. 3b). Baseline production of cytokines was undetectable in all experiments performed (data not shown). In the presence of rituximab, the IL-17A production induced by *C. albicans* was reduced by 50% and IL-22 production by 20-30% (Fig. 3b), while the addition of 10 μg of human IgG as a control had no inhibitory effect (data not

shown). Flow cytometry showed that the reduction in IL-17A production by rituximab was associated with a decrease in the percentage of IL-17A producing T-cells (Fig. 3c). No IL-17A positive cells were observed in the CD4 negative fraction (Supplemental data, Fig S2). In line with the results obtained in vivo, the exposure to rituximab did not have any effect on the Th1 or TNFα response (Fig. 3b). To determine whether the effect of rituximab on the *C. albicans*-induced Th17 response was mediated by B cell depletion, the Th17 responses of PBMCs from four patients with X-linked agammaglobulinemia (XLA) were investigated. Patients with XLA do not have circulating functional B lymphocytes and are deficient in immunoglobulins. PBMCs from these patients that completely lack mature B cells exhibited a Th17 response upon stimulation with *C. albicans* (Fig 3d). When these PBMCs were stimulated with *C. albicans* in the absence or presence of rituximab, no difference was observed in the Th17 cytokine profile (Fig. 3d), and Th17 cells were not reduced in the presence of rituximab in contrast to healthy volunteers (Supplemental data, Fig. S2). These results suggest that rituximab exerts its effects through the depletion of B cells.

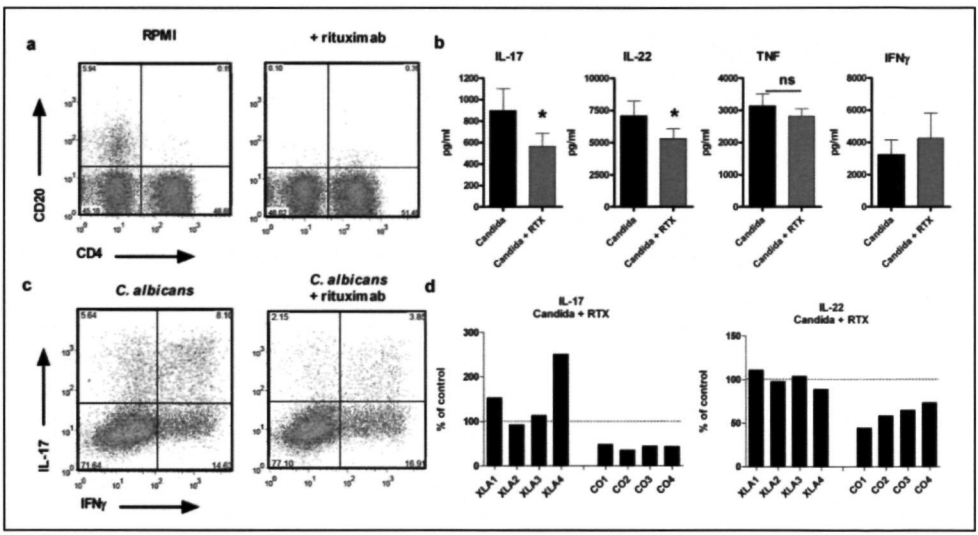


Figure 3. The effect of ritximab on Th17 responses is mediated through depletion of B cells.
(a) Flow cytometry analysis of CD4 and CD20 on human PBMCs cultured for 5 days with *C. albicans* in the presence or absence of rituximab, and then exposed for 4 h to PMA and ionomycin. (b) Human PBMCs were incubated for 5 days in the presence of human serum with either medium or *C. albicans* and in the presence or absence of rituximab 10 ug/ml. Cytokines in the supernatants were measured by ELISA. (c) Intracellular cytokine staining of IL-17A and IFNγ in human PBMCs (n=5) stimulated for 5 days with *C. albicans*, with or without rituximab. Cells were gated for CD4 and data is given as % of total gated CD4 positive cells. (d) PBMCs from four patients with X-linked agammaglobulinemia (XLA) and 4 healthy controls were treated as in (b). Values after exposure to *C. albicans* were set at 100% and data are shown for incubation with *C. albicans* in the presence of rituximab and is given as percentages compared to incubation with *C. albicans* alone. (a,c) Data from one healthy volunteer, that represents the pattern observed in two separate experiments with a total of n=5. Data are pooled and expressed as mean ± SEM. * $p < 0.05$ using Wilcoxon matched-pairs signed-ranks tests. RTX=rituximab.

Discussion

In the present study we demonstrate that rituximab inhibits the Th17 response in humans. Rituximab reduces the local Th17 response in patients with RA and this is associated with a decrease in inflammation and a better clinical outcome. Interestingly, the effect of rituximab is Th17 specific, since rituximab had no effects on Treg, Th1 or TNF responses. Inhibition of the Th17 response by rituximab is lost in the absence of B lymphocytes. These data strongly argue that B cells are able to modulate the Th17 response, and rituximab inhibits the Th17 function through B cell depletion.

Our data linking B lymphocytes to an IL-17A response shed light on the enigmatic observation that B-cell-targeted therapy with rituximab is beneficial in patients with autoimmune diseases such as RA and MS(2, 3). These findings would fit with the accumulating evidence that Th17 cells play an important role in the pathogenesis of RA(18), and MS (19). In this respect, our findings point to a so far undiscovered role for B cells in the pathology of human autoimmune diseases. The studies in patients with XLA (which lack functional B lymphocytes) showing that *Candida*-induced IL-17A production was not inhibited by rituximab, provide a strong argument for a key role of functional B cells in the IL-17A response. Nevertheless, we also entertained the possibility that cells other than B cells are directly affected by rituximab. It has been reported that a subset of T cells are CD20 positive (20), and these could be the target for rituximab. We found that only CD4 positive cells expressed IL-17A after stimulation with *C. albicans*, and CD4 positive cells did not show CD20 expression (data not shown), making it unlikely that rituximab acted directly on Th17 cells.

The studies performed in patients with RA who received treatment with rituximab support a relationship between B cell depletion, inhibition of the IL-17A response and clinical benefit. The depletion of CD20+ cells in the affected joints after 12 weeks of treatment with rituximab was associated with clear reduction of the Th17 cells as well as by a decrease of IL-17A, IL-21 and IL-22, and this in turn was accompanied by an improvement in clinical status, as measured by DAS. Most interestingly, these data suggest that B cell depleting therapies in RA work by virtue of a reduction of the Th17 response. An important question that had to be addressed was whether rituximab specifically inhibited the Th17 response or that it had additional effects which could explain the improvement in clinical outcome. It has been suggested that drugs for RA may work, in part, by promoting the function or increasing the numbers of Tregs (21). We found however no effects of B cell depletion on local Tregs in vivo. The proinflammatory Th1 response has been shown to contribute to the pathogenesis of RA (22), yet no effects on the Th1 effector cytokine IFNγ were observed when B cells were depleted by rituximab in vitro and in vivo. Importantly, targeting TNFα as a therapy has revolutionized the treatment of RA, demonstrating an important role for TNFα in RA. We therefore investigated the possibility that rituximab additionally inhibited TNFa production. We found no effects on TNFα in vitro and in vivo. Notably, this provides a rationale for investigating the combination of rituximab with anti-TNF therapy in patients with active RA. Altogether, these data advocate the concept that the Th17 response is specifically inhibited by B cell depletion.

In conclusion, the present study demonstrates an important role for B cells in the Th17 response during inflammation and underscore the concept that B cells are key players in regulating immune processes (23-25). It is tempting to speculate that the effect of B cell-depleting treatment in other Th17 related diseases, such as MS, Crohn's disease and psoriasis, is also based on the mechanism proposed in this paper. We feel that this insight may lead to novel therapeutic strategies for the large group of patients with Th17 related immune diseases.

Acknowledgements

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Authorship Contributions and Disclosure of Conflicts of Interest

F.L.V, B.L., R.J.M., P.D., M.G.N., W.B. B., J.W.M., L.A.B designed research, analyzed data and wrote the paper. F.D.P.contributed vital reagents and contributed to the design of research. F.L.V., R.J.M., K.T., M.I.K., I.G-R, performed the experiments and analyzed the data.

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Supplemental data

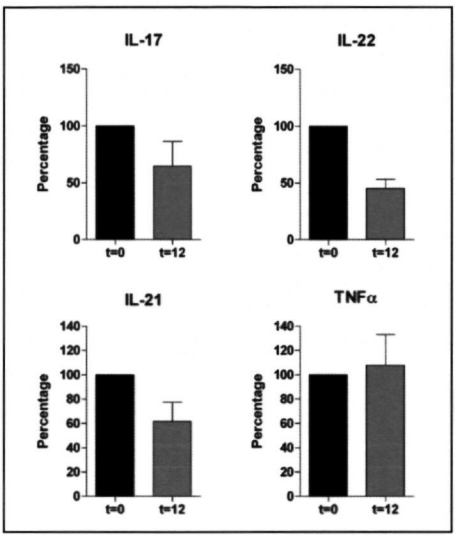


Figure S1. Microarray analysis of the synovial tissue before and after treatment with rituximab.
Microarray analysis of the expression of IL-17AA, IL-21, IL-22, TNFα in the affected synovial tissue from 12 patients with RA before (black bars) and after (grey bars) treatment with rituximab.

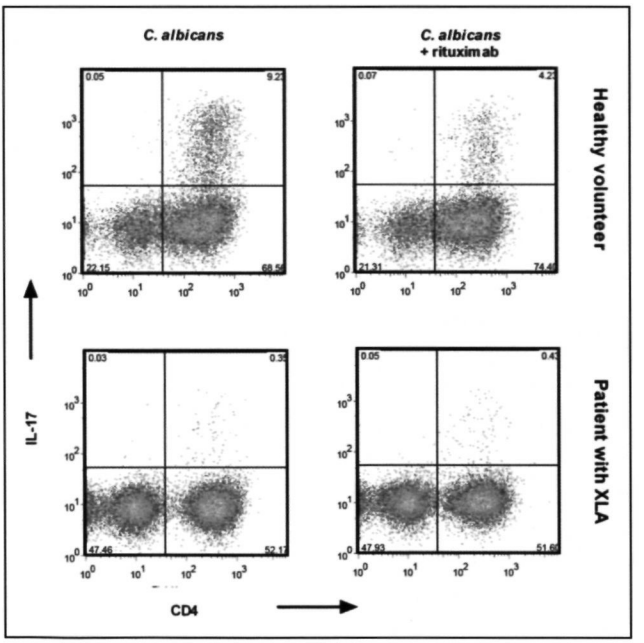
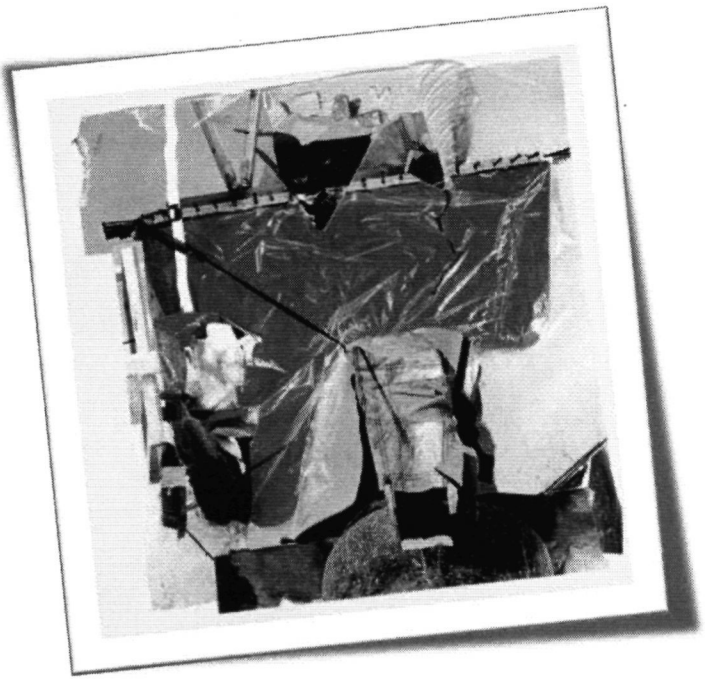


Figure S2. Rituximab reduces IL-17A producing cells in healthy controls but not in patients with agammaglobulinemia (XLA).
Intracellular cytokine staining of CD4 and IL-17A in PBMCs from a healthy volunteer and a patient with XLA cultured for 5 days with *C. albicans*, with or without rituximab.

Milder clinical hyperimmunoglobulin E syndrome phenotype is associated with partial interleukin-17 deficiency

Clin Exp Immunol. 2010 Jan;159(1):57-64



van de Veerdonk FL, Marijnissen R, Joosten LA, Kullberg BJ, Drenth JP, Netea MG, van der Meer JW.

Summary

Mutations in the signal transducer and activator of transcription 3 (STAT3) were reported to cause hyperimmunoglobulin E syndrome (HIES). The present study investigates T_H17 responses triggered by the relevant stimuli *S. aureus* and *C. albicans* in five "classical" HIES patients and a family with three patients that all had a milder HIES phenotype. We demonstrate that patients with various forms of HIES have different defects in their Th17 response to *S. aureus* and *C. albicans*, and this is in line with the clinical features of the disease. Interestingly, a partial deficiency of IL-17 production, even associated with STAT3 mutations, leads to a milder clinical phenotype. We also observed defective T_H17 responses in patients with the "classical" presentation of the disease but without STAT3 mutations. These data demonstrate that the defective IL-17 production in response to specific pathogens can differ between patients with HIES and that the extend of the defective T_H17 response determines their clinical phenotype.

Introduction

Hyperimmunoglobulin E syndrome (HIES) is a primary immunodeficiency disorder characterized by recurrent staphylococcal skin abscesses, pulmonary infections, mucocutaneous candidiasis, skeletal and dental abnormalities, and elevated serum imunoglobulin E concentrations (1, 2). Although most cases of HIES are sporadic, familial cases are encountered, mostly with an autosomal dominant mode of inheritance (3).

Recently, mutations in the evolutionary conserved SH2 and DNA-binding domains of the signal transducer and activator of transcription 3 (STAT 3) were found to be present in approximately 60% of the patients with HIES (4, 5). STAT3 is an important component of receptor signalling pathways for several cytokines, including interleukin-6 (IL-6) and IL-10, and patients with HIES have shown defective responses to these cytokines (4). The role of STAT3 for the normal signaling of IL-6 receptor has important consequences for the normal host defense. The IL-6/STAT3 pathway is crucial, together with other cytokines such as IL-1β and IL-23, for the normal development of $CD4^+$- T_H17 cells (6, 7). Because IL-17 has an important role for the activation of the neutrophil-dependent immunity (8), defective T_H17 generation as a result of STAT3 mutation may play an important role in the pathogenesis of HIES.

In a recent paper, Milner et al have demonstrated that T lymphocytes from patients with HIES are unable to differentiate into T_H17 after mitogenic stimulation (9). These data were supported by two reports that also showed defective generation of T_H17 when anti-CD3/anti-CD28/IL-2 or cytokine cocktails were used (10, 11). These studies reported the defective generation of T_H17 using mitogenic cocktails in patients with established mutations in the SH2 and DNA-binding domains of STAT3. In contrast, patients with atopic dermatitis and high IgE, but without skin and respiratory infections and without STAT3 mutations, had normal T_H17 responses (9, 12).

In the present paper, we aimed to extend these initial findings by investigating the generation of T_H17 cells and IL-17 production by relevant microbial stimuli for HIES. In addition, we assessed T_H17 profiles in three distinct groups of patients: "classical" HIES

patients with STAT3 mutations in the SH2/DNA-binding domains, "classical" HIES without STAT3 mutations, and a family with "variant" HIES that we described as having a milder clinical phenotype (13), and with a deletion of a triplet in the linker domain. The differences in the degree of IL-17 production defects after stimulations with *Staphylococcus aureus* or *Candida albicans* determined the severity of the clinical phenotype.

Materials and methods

Patients and controls

Eight patients with the clinical diagnosis of HIES at the outpatient clinic for infectious diseases and immunodeficiencies of the Department of General Internal Medicine of Radboud University Nijmegen Medical Centre were enrolled in the study. Three of these patients were family members. After informed consent, blood was collected from eight healthy, nonsmoking volunteers who were free of infectious or inflammatory disease and the enrolled HIES patients by venipuncture into 10 ml EDTA syringes (Monoject). STAT3 mutation analysis was kindly performed in the Laboratory of Human Molecular Biology and Genetics, Catholic University of the Sacred Heart, Milan, Italy, head Prof. Roberto Colombo.

Microorganisms

C. albicans ATCC MYA-3573 (UC820), a strain well described elsewhere (14) was used. *C. albicans* was grown overnight in Sabouraud broth at 37°C, cells were harvested by centrifugation, washed twice, and resuspended in culture medium (RPMI-1640 Dutch modification, ICN Biomedicals, Aurora, OH) (15). *C. albicans* was heat killed for 1 hour at 100°C and all experiments were performed with heat-killed *C. albicans.* The clinical isolate of *S. aureus* was heat-killed and used in a dosage of 10^7/ml.

In-vitro cytokine production

Separation and stimulation of PBMCs was performed as described previously (16). Briefly, the PBMCs fraction was obtained by density centrifugation of diluted blood (1 part blood to 1 part pyrogen-free saline) over Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden). PBMCs were washed twice in saline and suspended in culture medium supplemented with gentamicin 1%, L-glutamine 1%, and pyruvate 1%. The cells were counted in a Bürker counting chamber, and their number was adjusted to 5×10^6 cells/ml. 5×10^5 PBMCs in a volume of 100 µl per well were incubated at 37°C in round-bottom 96-wells plates (Greiner, Nuremberg, Germany), in the presence of 10% human pooled serum with stimuli or culture medium alone, and where indicated with the cytokines IL-6 and IL-10 (100 ng/ml). After 5 days of incubation, supernatants were collected and stored at –20°C until assayed.

Cytokine assays

IL1β and IL-17 concentrations were measured by commercial ELISA kits (R&D systems); IFNγ and IL-10 (Pelikine Compact, Sanquin, Amsterdam, The Netherlands); according to the manufacturer's instructions.

Intracellular cytokine staining

PBMC cells were stimulated as described above and restimulated for 4-6 hours with PMA (50 ng/ml; Sigma) and ionomycine (1 ug/ml; Sigma) in the presence of Golgiplug (BD biosciences) according to manufacturers protocol. Cells were first extracellularly stained using an anti-

CD4 APC antibody (BD biosciences). Subsequently the cells were fixed and permeabilized with Cytofix/ Cytoperm solution (BD biosciences) and then intracellularly stained with anti-IFNγ PE (eBiosciences) and anti-IL-17 FITC (eBiosciences). Samples were measured on a FACS Calibur and data were analyzed using the CellQuest-Pro software (BD biosciences).

Statistical analyses
The differences between groups were analyzed by the Mann-Whitney U test, and considered statistically significant when $p \leq 0.05$. Data is presented as the cumulative result of all experiments performed, unless otherwise indicated. Data are given as median or mean ± SEM unless otherwise indicated.

Results

Patients
In table 1, the clinical description of the patients with HIES are summarized. All patients were of Dutch ancestry. In Fig.1 the pedigrees of the HIES family is presented. Of note, the clinical data of the HIES family have been published elsewhere (13, 17). Blood sampling and TH17 profile were assessed in cells isolated from the three HIES patients in the third generation of the family, and the five patients with "classical" HIES. In the large HIES family with a milder HIES (variant HIES) the STAT3 gene had a deletion of a triplet, leading to the deletion of serine in position 560 of the linker domain. Three of the five "classical" HIES patients had known STAT3 mutations (R382W twice and V463del)(5) (Table 1). Two of the patients with "classical" HIES had no STAT3 mutation.

Defective IL-17 and IFNγ production in HIES patients in response to S. aureus and C. albicans
To investigate the immunological functional properties with respect to the TH17 responses in HIES patients with different mutations, PBMC from healthy volunteers, "classical" HIES patients and three members from a HIES family with a milder form of disease (variant HIES), were assessed for the capacity to mount IL-17 responses. We have developed a new methodology of T_H17 generation using human PBMC stimulated with whole microbial stimuli relevant for HIES: *S. aureus* and *C. albicans* (18). HIES patients had a defective response to *C. albicans*, although IL-17 was measurable in all patients (Fig. 2a). Interestingly, there was a completely absent IL-17 production in PBMC stimulated with *S. aureus* in all "classical" HIES patients (Fig. 2b). In contrast, PBMC isolated from the variant HIES patients, bearing the STAT3 mutations in the linker domain, were able to produce IL-17 in response to *S. aureus*, albeit at lower concentrations when compared to healthy volunteers (Fig. 2b,c). IFNγ production was distorted in HIES patients when compared to healthy controls, while IL-10 was found to be elevated in HIES patients when stimulated with both *S. aureus* and *C. albicans*.

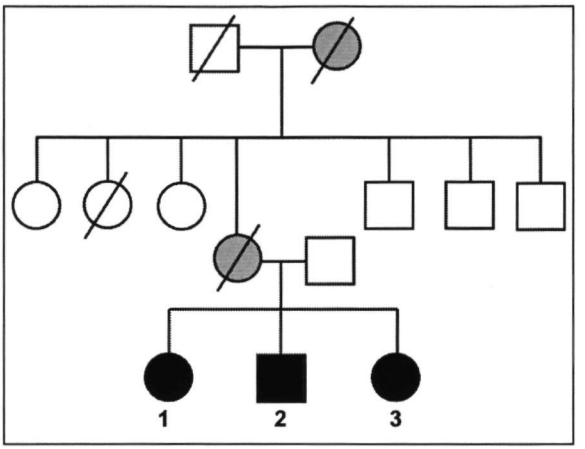


Figure 1. The pedigree of a family with HIES of Dutch ancestry.
Clinical symptoms characteristic for HIES were recorded in a large kindred, with patients affected (closed black symbols are family members affected STAT3 mutation) from several generations. Closed grey symbols represent suspected for the STAT3 mutation but not proven. *Line through symbol* represents deceased family member.

Table 1. Clinical characteristics of the patients with hyperimmunoglobuline E syndrome.

HIES Patients	Gender/ age	infections					Other abnormalities	IgE IU/l	STAT3 Mutation
		Localization			etiology				
		lung	skin	other	bacteria	fungi			
"Variant"									
1	F 45	No	Yes		*S. aureus*	*C. albicans*	Palatoschizis Fractures	900	S560del
2	M 46	No	Yes		*S. aureus*	*C. albicans*	Abnormal dentition	16700	S560del
3	F 38	No	Yes		*S. aureus*	*C. albicans*	Hypertension	7210	S560del
"Classical"									
1	F 27	Yes	Yes	Bartholinitis	*S. aureus*	*Aspergillus*		3600	V463del
2	M 40	Yes	Yes		*S. aureus*	*Aspergillus*	Giant chalazia	17200	R382W
3	M 39	Yes	Yes	Osteomyelitis	*S. aureus*			1762	R382W
4	M 43	Yes	Yes			*C. albicans*		2340	None
5	M 36	Yes	Yes	Spondylodiscitis	*S. aureus*			16000	None

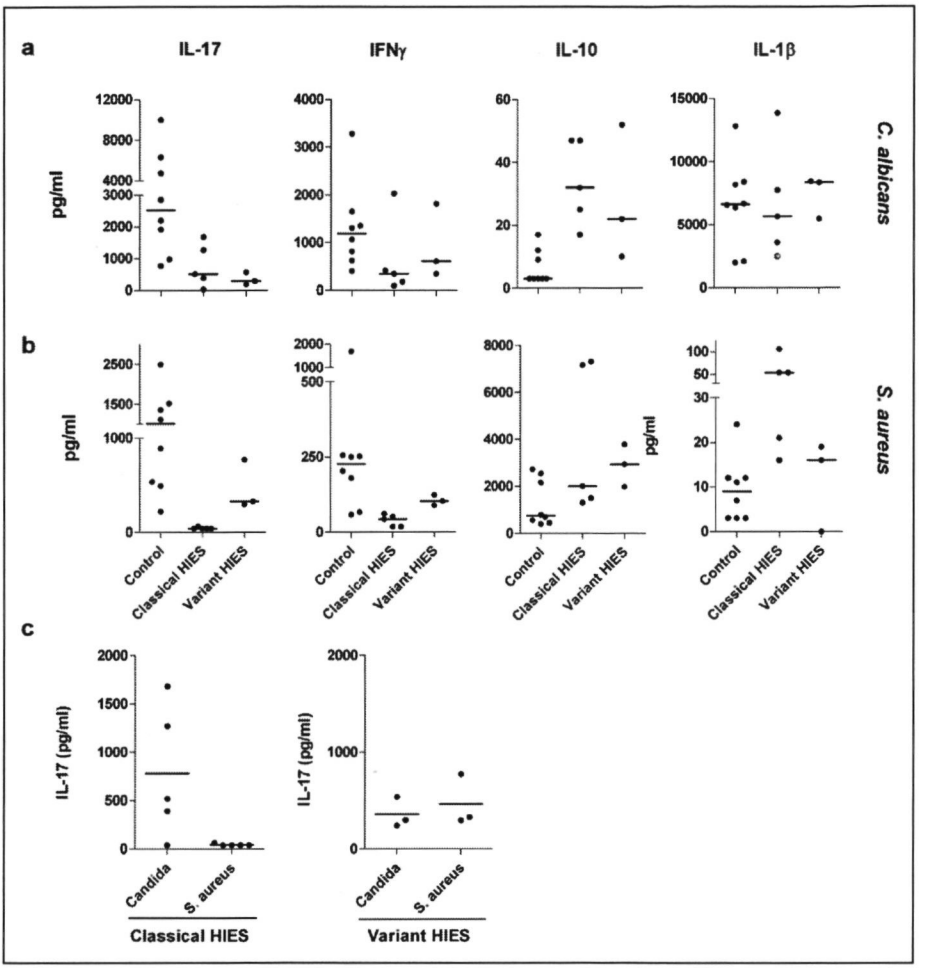


Figure 2. Defective IL-17 and IFNγ production in HIES patients in response to *S.aureus* and *C. albicans*.
Human PBMCs from healthy controls (n=8), "classical" HIES (n=5) and three members of the HIES family bearing the STAT3 linker domain mutation, were stimulated for 5 days with *C. albicans* (a) or *S. aureus* (b). Cytokines were measured by ELISA. (c) IL-17 production by PBMC stimulated as in (a) and (b) from "classical" HIES patients and familial HIES patients.

Normal induction of T_H17 cells in familial variant HIES patients

The in-vitro stimulations described above suggest that HIES patients have a significant defect in the generation of T_H17 cells. This was indeed the case for the patients with "classical" HIES, either bearing STAT3 mutations or not (Fig. 3). Surprisingly, when the familial variant HIES patients were challenged with disease related microorganisms, they showed a clear induction of single IL-17 positive and IL-17/INFγ positive CD4+ cells, comparable to normal controls (Fig 3).

HIES patients have a defect in IL-6, but not in IL-10, signaling

IL-6 augmented the IL-17 production induced by *C. albicans* and *S. aureus* in cells isolated from healthy controls (Fig. 4a). No effect was apparent in the HIES patients, independently

of the type of STA3 mutation. In contrast to IL-6, IL-10 reduced the amount of IL-17, and this effect was observed both in healthy controls and the HIES patients (Fig. 4b).

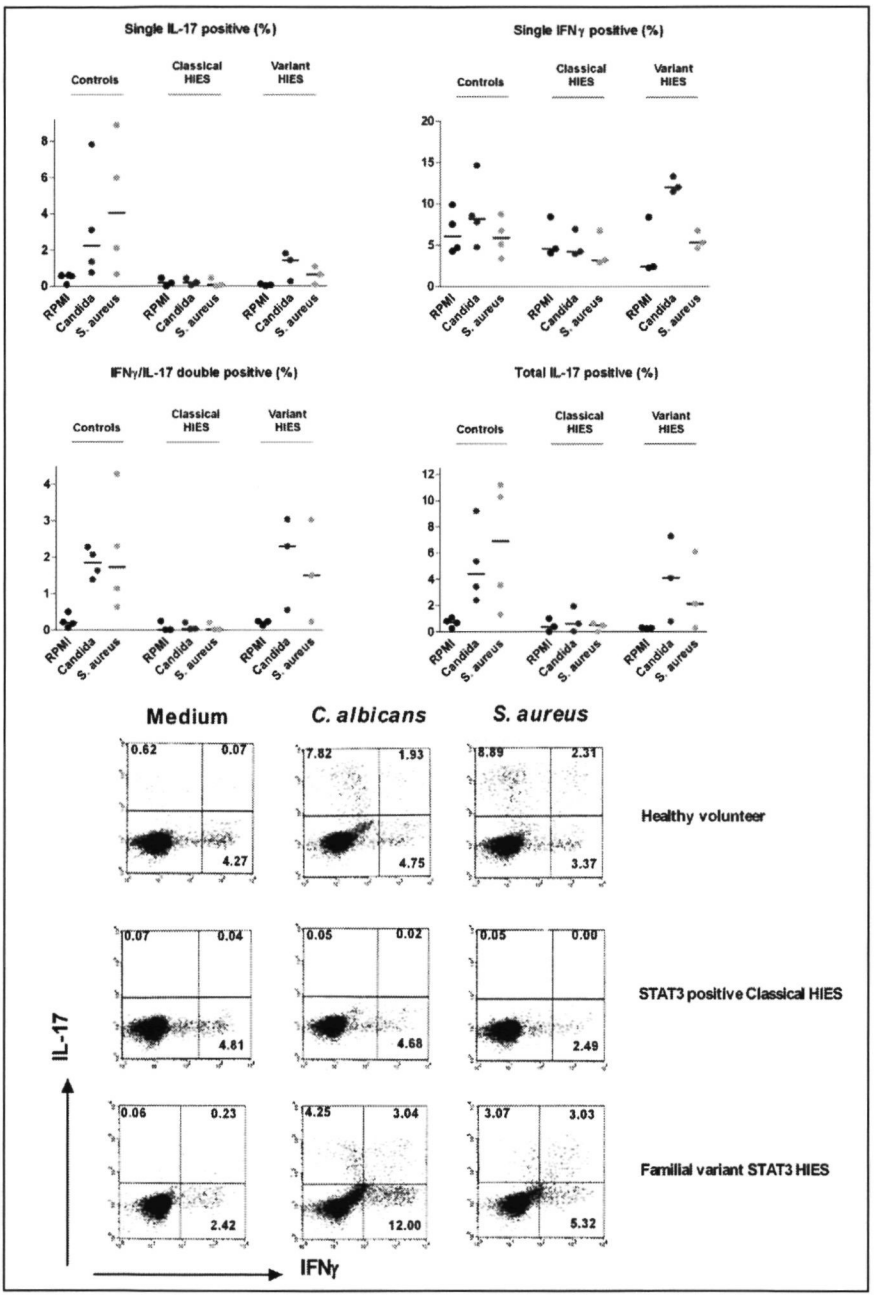


Figure 3. TH17 cell generation in HIES patients.
Human PBMCs from healthy controls (n=8), "classical" HIES (n=5) and three members of the HIES family bearing the STAT3 linker domain mutation, were stimulated for 5 days with *C. albicans* or *S. aureus*. The IL-17- or IFNβ- producing TH17 cells were assessed by intracellular staining (see description in the Methods).

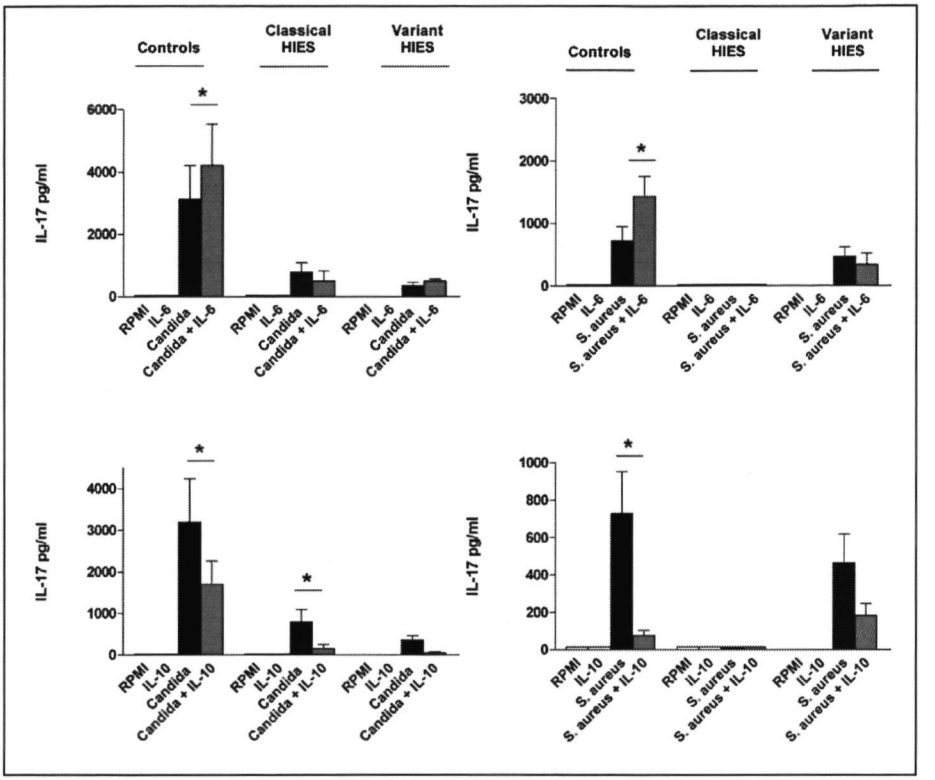


Figure 4. Defective IL-6, but not IL-10, pathway in HIES patients.
Human PBMCs from healthy controls (n=8), "classical" HIES (n=5) and three members of the HIES family bearing the STAT3 linker domain mutation, were stimulated for 5 days with *C. albicans* or *S. aureus*. The effect of either IL-6 or IL-10 on the induction of IL-17 was assessed by ELISA. *p<0.05

Discussion

Mutations in the SH2 and DNA-bindings domain of STAT3 have been reported to be the cause of disease in a large proportion of HIES patients (4). These mutations function as dominant-negative mutations (4) and result in a defective T_H17 response in these patients (9, 10), explaining many of the clinical features of HIES. In the present study we confirm on the one hand the relationship between HIES and defective T_H17 responses, but on the other hand we also refine this notion to include the relationships between the type of STAT3 mutation, the immunological response to relevant microbial stimulation, and the clinical phenotype of the patients.

All studies to date have used an artificial methodology to generate T_H17 cells and induce IL-17 production in cells from HIES patients, that used either mitogenic antibodies such as anti-CD3 and anti-CD28 (9, 10) or recombinant cytokine cocktails (11). We have recently developed a novel method to induce IL-17 production and generate T_H17 cells using exclusively microbial stimulation (18), method that mimics much closer the in-vivo conditions during infection. Although we can confirm the defective T_H17 generation and IL-

17 production by cells isolated from patients with HIES (9-11), several important aspects are now apparent when using this improved methodology. Firstly, the defective IL-17 induction differs between stimulation with *S. aureus* or *C. albicans*. When T_H17 responses were assessed, both these two microorganisms that are the most important in HIES patients were equally defective in generating $CD4^+$ $IL\text{-}17^+$ cells. Surprisingly however, *C. albicans* was still capable to stimulate approximately 20 to 30% of the normal IL-17 production, while *S. aureus* was completely defective as an IL-17 stimulus in HIES patients (Fig.1c). This finding is important as it may explain why it is mostly mucosal and nail bed infection which is the most common *Candida* complication in HIES patients (83% of them in one large study), while systemic candidiasis is relatively rare (3). Notably, patients with chronic mucocutaneous candidiasis, which have the same clinical spectrum of *Candida* infection [19], have also been reported to have a specific defect in *Candida* induced IL-17 production [20]. This supports the conclusion that IL-17 is important in mucosal anti-*Candida* host defense and that the lower IL-17 found in our patients is indeed clinically relevant.

Secondly, an important observation of our study is represented by the indistinguishable immunological responses in patients with the "classical" clinical form of HIES, independent on the presence or absence of STAT3 mutations. All these patients that had a strong phenotype of the disease displayed similar defects in IL-17 production and T_H17 generation. Our data are supported by the report of one HIES patient without STAT3 mutation and defective T_H17 responses (21), and strongly suggests that in patients with the "classical" presentation of HIES but in which no STAT3 mutation is found, defects in the same immunological pathways are most likely the cause of the disease. This may also imply that the defective T_H17 responses are a more sensitive diagnostic tool for HIES.

Thirdly, one of the most interesting finding of our study is the description of a clear association of a milder phenotype of the disease in a Dutch family with a less severe defect in IL-17 production, likely due to the linker domain triplet that did not lead to a frameshift (13). Patients from this family suffer from skin infections with *S. aureus*, candidiasis of the nail beds (but not of the mucosae), dermatitis, hyper IgE and eosinophilia, but they lack any respiratory infections (either with *S. aureus* or other pathogens). With regard to the non-infectious manifestations, persistent primary dentition, a rather common sign in HIES, was seen in one patient of this family, one patient displayed scoliosis, cheilognathopalatoschisis and pathological fractures, and one patient suffered from an aortic aneurysm. The defects in IL-17 responses to *S. aureus* in cells isolated from this family were milder compared to the "classical" HIES patients, as they were still able to release approximately 30% of the normal IL-17 production. In line with the presence of candidiasis as a clinical symptom in the family, the IL-17 production after *C. albicans* stimulation was equally defective compared to the other patients.

In addition to IL-17, other defects in the cytokine response of HIES patients have also been reported, such as a defective IFNγ production (17, 22), and increased GM-CSF (23). In line with these previous studies, IFNγ production was decreased in HIES patients in our study, while IL-10 release was significantly higher compared to controls. The production of IFNγ was defective in response to both *C. albicans* and *S. aureus*. IFNγ is the prototype of T_H1 cytokines and plays a crucial role in the activation of the innate and adaptive host response against these pathogens [24]. Therefore the defective IFNγ response could be at least as

relevant as the defect found in IL-17. Furthermore, it should be kept in mind that IFNγ therapy is a relatively safe therapeutic option [25] and it has been reported that recombinant IFNγ can enhance neutrophil chemotactic responses in patients with HIES [26]. Alltogether, these data strongly argue for a dysbalance of T_H subsets in patients with HIES, with defective responses of the proinflammatory subsets T_H1 and T_H17, and increased function of the antiinflammatory T_H2 subset.

In contrast to the T_H-derived cytokines, the release of IL-1βwas normal in HIES patients. As IL-1β is important for the generation of T_H17 cells (27), this result suggests that it is not a defective IL-1β/IL-1RI axis that is responsible for the defects of IL-17 production in HIES patients. This hypothesis is sustained by the normal generation of T_H17 responses in individuals with MyD88 or IRAK4 mutations, that are defective in the IL-1RI signalling (as well as TLR and IL-18R pathways) (11).

The defective generation of T_H17 responses in HIES must therefore be located at the level of another immunological pathway, and the most obvious is the IL-6/STAT3 axis (6). To test this hypothesis, we investigated the effect of IL-17 costimulation with microbial stimuli in combination with IL-6. While IL-6 potentiated the production of IL-17 induced by *C. albicans* or *S. aureus* in healthy individuals, no such effect was observed in either the "classical" HIES or the family with the variant HIES. The striking observation that the members of the HIES family were able to generate T_H17 cells upon contact with pathogens suggests that the linker domain of the STAT3 gene is involved in another unknown function of the STAT3 molecule, and highlights the observation that a mutation at a different location in the STAT3 gene leads to a different functional and clinical outcome. In contrast to the defective responses to IL-6, the inhibitory effects of IL-10 on IL-17 production were similar in the healthy volunteers or the HIES patients, suggesting that STAT3 is redundant for IL-10 signaling leading to reduced IL-17 production.

In conclusion, the present study demonstrates that patients with HIES have differential defects of the IL-17 responses to the two main pathogens associated with the disease, *S. aureus* and *C. albicans*, and this is comparable with the clinical features of this syndrome. In addition, the extent of the T_H17 defect is due to the location of the STAT3 mutation, and is associated with the clinical phenotype in the patients. Furthermore, the defective T_H17 responses are a more sensitive marker of the disease in HIES patients than STAT3 mutations.

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All authors declare no Conflict of Interest.

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Chapter 17

Mutations in the CC-domain of STAT1 in Autosomal Dominant Chronic Mucocutaneous Candidiasis

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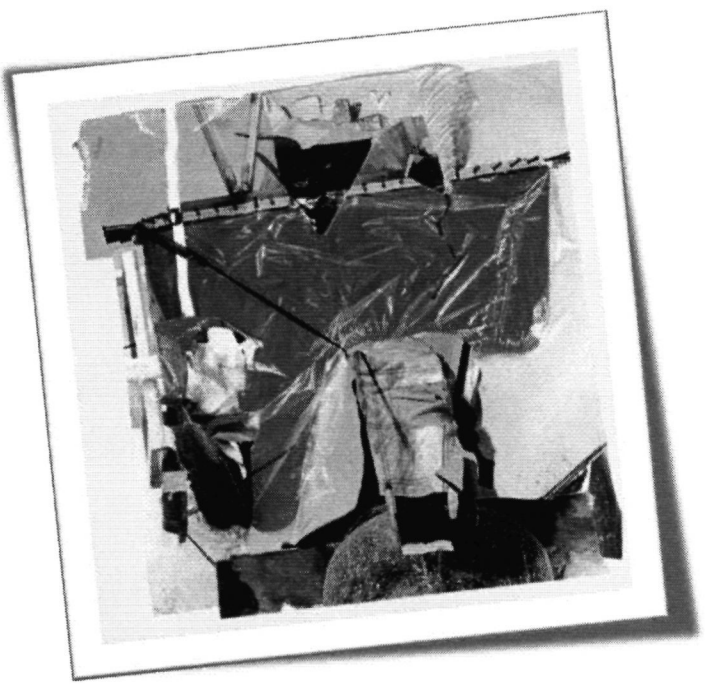

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Summary

Background: Chronic mucocutaneous candidiasis (CMC) is a primary immunodeficiency characterised by selective susceptibility to fungal infections of skin, nails and mucous membranes caused by *Candida* species. While patients with recessive CMC and autoimmunity have mutations in the *AIRE* gene (autoimmune regulator), the cause of autosomal dominant CMC is unknown.

Method: Twelve patients from five families with autosomal dominant CMC from the Netherlands and the United Kingdom were investigated. The immune pathways in these patients were investigated after incubation of peripheral blood mononuclear cells with different combinations of cytokines or microbial stimuli. Using the information obtained from these functional studies, we selected 100 genes most likely to contain the genetic defect responsible for autosomal dominant CMC. An array-based sequence capture assay, followed by next generation sequencing to simultaneously analyze these 100 genes, was used to find and identify relevant mutations.

Findings: Pronounced defects in the production of the T-helper 1 cytokine interferon-γ (IFNγ) and the T-helper 17 cytokines interleukin (IL)-17 and IL-22 were identified in CMC patients. The functional studies led to the conclusion that the defect would most likely be within the IL-12R and IL-23R signalling pathway. Using the array-based sequence capture assay, we found that all patients harboured heterozygous missense mutations in the CC domain of the STAT1 gene: patients from two families were heterozygous for the Arg274Trp mutation, while patients from three families were heterozygous for Ala267Val mutation. While the IL-12R and IL-23R pathways were defective, leading to absent Th1 and Th17 responses, the IFNγR pathway was intact in the CMC patients, thus explaining the absence of mycobacterial or viral infections.

Interpretation: Mutations in the CC domain of STAT1 underlie autosomal dominant CMC. These STAT1 mutations lead to defective Th1 and Th17 responses, and thereby to increased susceptibility to mucosal and skin fungal infections.

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Introduction

Chronic mucocutaneous candidiasis (CMC) is a primary immunodeficiency characterised by selective susceptibility to fungal infections of skin, nails and mucous membranes caused by *Candida* species and dermatophytes (1). There are several CMC subtypes: autosomal recessive CMC associated with autoimmune involvement of endocrine glands (autoimmune polyendocrinopathy candidiasis ectodermal dystrophy – APECED, OMIM 240300), autosomal dominant CMC with or without associated thyroid disease (OMIM 114580), and autosomal recessive, isolated CMC (OMIM 212050).

The genetic defect in APECED resides in the *AIRE* gene (autoimmune regulator), which has a key role in the induction of immunotolerance (2). Susceptibility to *Candida* infections in APECED is believed to be due to auto-antibodies to interleukin-17 (IL-17) and IL-22 (3), as the Th17 cytokines are crucial for mucosal antifungal immunity (4). In contrast, very little is known about the genetic defects underlying increased susceptibility to *Candida* in non-APECED patients with CMC. Previously we have demonstrated defective Th1/interferon-gamma (IFNγ) responses in CMC patients (5), and a recent study in two CMC patients suggested also defective Th17 responses in these patients (6). Defects in the recognition of β–glucans from *Candida* due to mutations in the dectin-1/CARD9 pathway have been shown to be responsible for an increased susceptibility to mucosal fungal infections (7, 8), but the clinical presentation in these patients is less severe than in the classic CMC.

In the present study, we assessed the immunological defects responsible for the increased susceptibility to skin and mucosal fungal infections in a Dutch family with autosomal dominant CMC without *AIRE* mutations. Based on the defective Th1 and Th17 responses in these patients, we selected a panel of 100 genes encoding for proteins involved in the induction and modulation of these responses. We identified mutations in the CC domain of the gene encoding Signal Transducer and Activator of Transcription 1 (*STAT1)* as the cause of the immunological defects. Genetic and functional studies in three unrelated CMC families from United Kingdom, as well as one patient from an unrelated Dutch family with CMC, confirmed this finding.

Methods

Patients and controls

Family #1: We investigated a Dutch Caucasian family in which the father (index patient), his daughter and son suffer from severe CMC since early youth. There is no known consanguinity. The clinical characteristics are summarized in Table 1, the pedigree is depicted in Fig 1A. The patients have severe chronic *Candida* infections of the oropharynx, as well as severe dermatophytosis and candidiasis of the feet (Fig 1B). The index patient was diagnosed with autoimmune hepatitis, while his daughter is known with autoimmune haemolytic anemia and pernicious anemia; she has anti-phospholipid antibodies, suffered from pulmonary embolism, and recently had *Pneumocystis jirovecii* pneumonia and symptomatic cytomegalovirus infection. The son had a very severe dermatophytosis (*Trichophyton rubrum*) of the feet, but no autoimmune phenomena. No *AIRE* mutations were present in the family (Van der Graaf, personal communication). Immunological analysis was performed on peripheral blood mononuclear cells (PBMCs) isolated from the three affected family members, the unaffected mother and two healthy controls. The index patient has eight siblings, all unaffected by CMC (Fig 1A). Genetic analysis was performed in all affected and unaffected family members.

Family #2, #3, and #4: To confirm our findings in family #1, eight patients classified as autosomal dominant CMC and thyroid disease, from three unrelated Caucasian families from the UK, were immunologically and genetically analysed. The characteristics of these patients are reported elsewhere (9) and summarised in Table 1 (supplementary text). These patients have manifest thyroid disease. All patients were screened for *AIRE* mutations to exclude APECED syndrome (10).

Family #5: Genetic analysis was also performed in one patient from a Dutch family with CMC. The mother of the patient and the patient himself suffered from oesophageal cancer (Table 1).

Ethical approval

The study protocol was approved by the Ethics Committee of Radboud University Nijmegen Medical Centre, and the Newcastle and North Tyneside Local Research Ethics Committee. Informed consent was obtained from all family members and healthy controls studied.

Immunological studies

PBMCs were obtained by density centrifugation and resuspended in RPMI medium supplemented with gentamicin 1%, L-glutamine 1%, and pyruvate 1%, as previously described (11). PBMCs (5×10^5 cells) were incubated at 37°C in 96-wells plates (Greiner, Nuremberg, Germany), with RPMI or with heat-killed *Candida albicans* $1x10^6$ microorganisms/ml (strain UC820), *E. coli* lipopolysaccharide (LPS, Sigma-Aldrich, 1 ng/ml) and/or IL-1β (10 ng/ml), IL-12 (10 ng/ml), IL-18 (50 ng/ml), IL-23 (10 ng/ml) (R&D systems, Minneapolis, MA), IFNγ (1 μg/ml Boehringer Ingelheim, Alkmaar, The Netherlands). After 24 hours, 48 hours (without serum) or 5 days of incubation (in the presence of 10% serum), supernatants were stored at –20°C. Cytokine concentrations were measured by commercial ELISA kits according to the manufacturer's instructions: IL1β, TNFα, IL-17, IL-22 (R&D Systems, Minneapolis, MA); IFNγ, IL-6 (Pelikine Compact, Sanquin, Amsterdam, The Netherlands). Supernatants from unstimulated PBMCs had no detectable cytokine concentrations (data not shown).

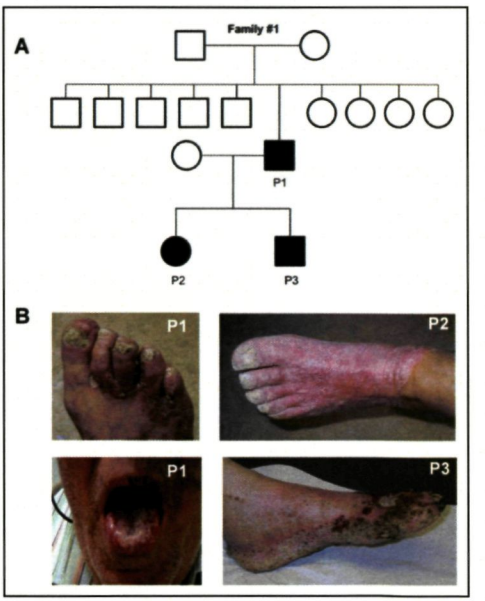


Figure 1. The pedigree of a family with autosomal dominant CMC of Dutch ancestry.
(A) Clinical symptoms characteristic for CMC were recorded in a Dutch family #1, with three patients affected (closed black symbols are family members affected) from two generations. (B) Pictures of clinical signs from affected family members with CMC.

Table 1. Clinical characteristics of 12 patients with autosomal dominant CMC from 5 families.

	Gender & age	Country of origin	Fungal infection	Hypo-thyroidism	Thyroid antibodies	other AID[1]
Family #1						
Patient 1	Male 67 y	NL	Oral Nails Skin	no	no	AI hepatitis
Patient 2	Female 38 y	NL	Oral Nails Skin	no	no	AI haemolytic anemia Pernicious anemia Anti –phospholipid antibodies
Patient 3	Male 37 y	NL	Oral Nails Skin	no	no	no
Family #2						
Patient 1	Female 59 y	UK	Oral Nails Skin	yes	yes	no
Patient 2	Male 36 y	UK	Oral Nails Skin	no	no	no
Patient 3	Male 29 y	UK	Oral Nails Skin	no	yes	no
Patient 4	Female 27 y	UK	Oral Nails Skin	no	no	no
Patient 5	Female 6 y	UK	Oral Skin	no	no	no
Family #3						
Patient 1	Female 37 y	UK	Oral Nails Skin Vaginal	yes	no	no
Patient 2	Male 32 y	UK	Oral Nails Skin	no	no	no
Family #4						
Patient 1	Female 40 y	UK	Oral Nails Skin Vaginal	yes	yes	no
Family #5						
Patient 1	Female 48 y	NL	Oral Nails Skin	no	no	no

[1] *AID = autoimmune disease*

Custom 12-plex sequence capture followed by Roche 454 sequencing

We applied array-based sequence capture followed by Roche 454 next generation sequencing to simultaneously analyze 100 genes (Table S1) from known immunologic pathways. We designed a custom-made 12-plex sequence capture array (Roche NimbleGen, Madison, WI, USA) including all known exons and untranslated regions (UTRs). In total, 1134 exons (of which 1059 were coding exons) from 100 RefSeq genes were selected, targeting 485,768 bases. After stringent probe selection by NimbleGen (Roche NimbleGen, Madison, WI, USA) (uniqueness tested by Sequence Search and Alignment by Hashing Algorithm-

SSAHA), a total of 443,488 bases were represented on an HD2 array, with 12-times 135000 oligonucleotide probes targeting the regions of interest. Sequence capture was performed in accordance with the manufacturer's instructions with a 'Titanium-optimized protocol' (Roche NimbleGen). 3µg of genomic DNA were used for sequence-capture hybridization. This included Titanium-optimized sequence primers with MID tags for barcoding (Table S2). After independent pre-hybridization ligation-mediated (LM-) PCR per DNA, a final amount of 1.125 µg DNA was hybridized for 72h to the customized array each one DNA to a individual sub-array, together with 37,5 µg Cot1 DNA and 0,24 µl of each 1 mM barcode complementary blocking oligos (Table S2). Subsequently, all DNAs were eluted together and amplified by ligation-mediated PCR. This pooled DNA library was used for massive parallel sequencing, with the use of a Roche 454 GS FLX sequencer with Titanium series reagents. All target and tiled regions of the array are available upon request.

Coverage statistics

The pooled sample was sequenced by using one full Roche-sequencing run, yielding on average 15.9 Mb of map-able sequence data per individual. Approximately 85.3% of the sequence data mapped back to unique regions of the human genome (hg18, NCBI Build 36.1), with the use of the Roche Newbler software (version 2.3). Of all mapped reads, 98.2% was located on or near the targeted regions (i.e. within 500 bp target proximity). This was sufficient to reach an average target coverage of 23.1-fold per individual (Table S3). For all regions of interest, only 5 regions sequences were not covered at all in any individual, all were GC-rich or repeat rich regions, therefore representing most likely an enrichment bias (Table S4). We observed a range of 5-29 regions without coverage per individual (on average 12 regions per individual). The Roche software detected on average 723 nucleotide variations per individual compared to the reference genome (Table S5). We used a custom-made data analysis pipeline to annotate detected variants with various kinds of information, including known single-nucleotide polymorphisms (SNPs), aminoacid consequences, genomic location, and evolutionary conservation.

Sequencing validation of STAT1 mutations

To validate the presence of *STAT1* mutations in the patients, conventional PCR assays and subsequently Sanger sequencing of the amplified DNA fragments were performed. All coding exons of the CC domain of *STAT1*, including exon 10, were amplified and analyzed. Primer sequences and PCR conditions are available upon request.

Results

Immunological defects in the Dutch family with CMC

The monocyte-derived cytokines IL-1β, IL-6 and TNFα in response to *Candida* were normal (Fig 2A, Fig S1A), as were the TLR4, TLR2 and dectin-1 receptor signalling pathways (Fig 2B) (12). In contrast, the production of the Th cytokines IFNγ, IL-17, and IL-22 in response to *Candida* was strongly reduced in the patients with CMC (Fig 2C).

We subsequently assessed the pathways leading to production of IFNγ, IL-17, and IL-22 by T-cells. The induction of IFNγ by the combination of IL-12 and IL-18 was partially impaired (Fig 2D). Stimulation with IL-12 alone resulted in undetectable IFNγ in the patients, in contrast to healthy controls (Fig 2D). Moreover, we detected no IL-17 and IL-22 production in the

patients (Fig 2E, Fig S1B). In contrast, mitogenic stimulation induced normal IFNγ production by cells of CMC patients (not shown). The IL-1RI-dependent pathway was intact, as shown by normal production of IL-6 in response to IL-1β (Fig 2E). We concluded that one of the molecules involved in both the IL-12 and IL-23 pathway is deficient in the Dutch family with CMC (Fig 2F).

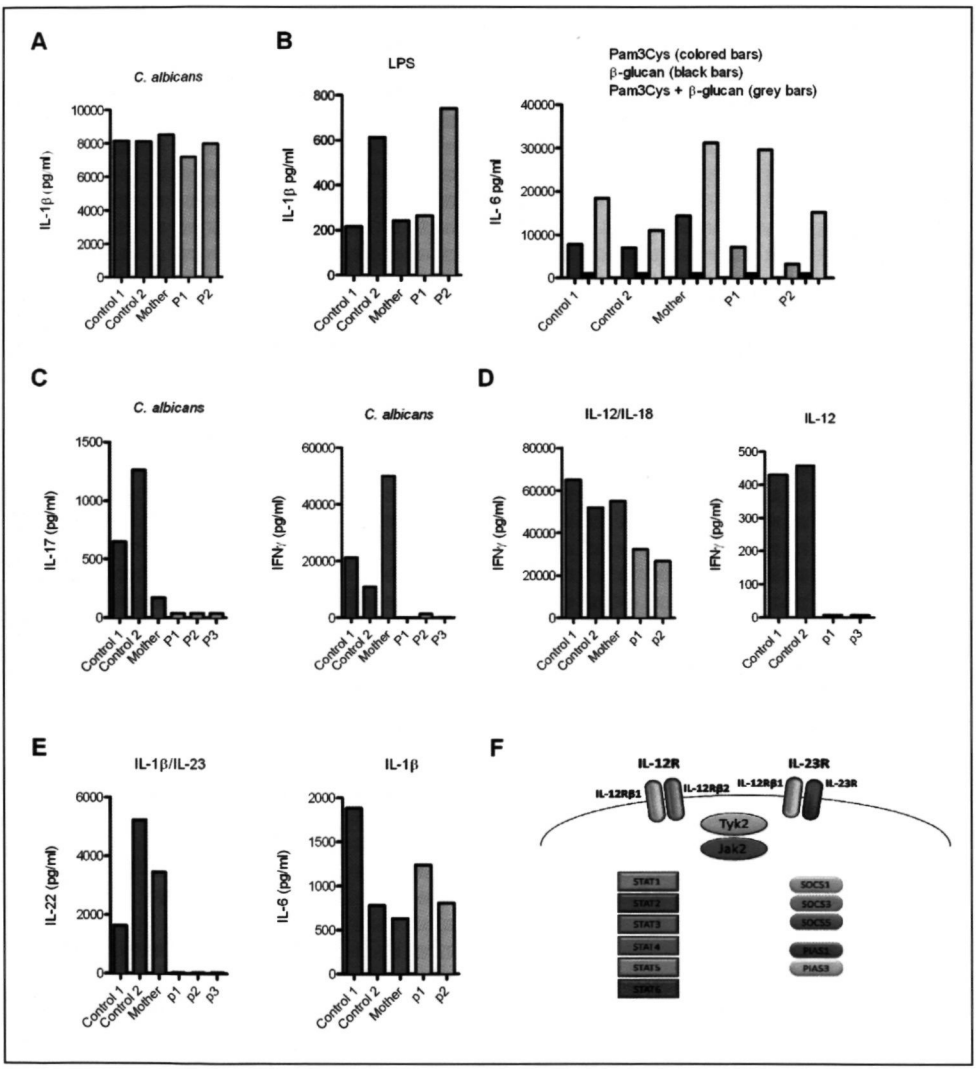


Figure 2. Immunological defects in the Dutch family with CMC.
Human PBMCs from healthy controls (n=2), the mother and three affected members of the Dutch family #1 with CMC (P1, P2, P3) were stimulated with (A) heat-killed *Candida albicans* (1x10e6 microorganisms/ml) for 24 hours, (B) LPS (1 ng/ml), Pam3Cys (10μg/ml), β-glucan (10μg/ml), or a combination of β-glucan for 24 hours, (C) heat-killed *C. albicans* (1x10e6 microorganisms/ml) for 5 days (IL-17) and 48 hours (IFNγ), (D) IL-12 (10 ng/ml)/IL-18 (50 ng/ml) for 48 hours, (E) IL-1β (10 ng/ml)/IL-23 (10 ng/ml) for 5 days and IL-6 (10 ng/ml) for 24 hours. Cytokines were measured by ELISA. (G) Scheme of molecules shared by the IL-12 and IL-23 pathway.

Missense mutation in the CC domain of STAT1 *in the Dutch family with CMC*

The *STAT3* genetic defect of hyper IgE syndrome (HIES) was not present in our three CMC patients. Neither did we find mutations in the gene for *STAT4*, another important molecule involved in both IL-23 and IL-12 pathways. Based on the cytokine defects found, we selected a panel of 100 genes encoding proteins known to induce or modulate Th1/Th17 responses (Table S1). Using array-based sequence capture followed by Roche 454 next generation sequencing, on average 723 variants were identified, of which 651 variants were found to correspond to known SNPs or to overlap with a known polymorphic region (dbSNP130). After we excluded variants from an in-house database, on average 38 novel variants remained (Table S5). Of these variants, only 4 were non-synonymous coding variants per individual, of which only between 0-3 variants were called in at least 20% of all reads, and therefore are considered as true heterozygous candidate variants. Of the total of 11 candidate variants, only 7 were observed in diseased individuals, of which 3 identical variants co-segregated in the three affected individuals from the family (Table S6). They all shared a heterozygous variant in exon 10 of the *STAT1* gene mapping to chromosome 2 (g.191,568,156G>A; c.820C>T; p.Arg274Trp).

The mutation leads to an aminoacid change (arginine to tryptophan) in the CC domain of STAT1 (Fig 3A). In all three affected individuals, *STAT1* was the only gene with a novel, heterozygous, non-synonymous coding variant, making it the most likely candidate gene for CMC. Three control individuals tested did not show any mutation in *STAT1*. When prioritising all novel, heterozygous, non-synonymous coding variants for base-pair conservation (phyloP values), the variants showing the highest conservation over all 44 species were the *STAT1* variants.

Confirmation of STAT1 *mutations in additional CMC patients and controls*

Next we investigated two families from the UK with affected family members with autosomal dominant CMC (Fig 4A, Table 1, and supplemental data). Using the same sequencing technique, an independent *STAT1* variant was found in a patient from one of the families (g.191,568,176G>A; c.800C>T; p.Ala267Val) also affecting exon 10. A second patient from another independent UK family showed very poor coverage for exon 10 of *STAT1*, however manual read inspection showed the same variant (g.191,568,176G>A; c.800C>T; p.Ala267Val) in 2 out of 5 reads. These mutations were confirmed by standard PCR amplification and sequencing of the CC domain (Fig 4B). *STAT1* sequencing in additional patients revealed Ala274Trp mutation in a member of a third independent UK family and an Ala267Val mutation in a patient from a second Dutch family with autosomal dominant CMC and oesophageal cancer (Fig 4A,B). Sequencing of *STAT1* did not identify any of these mutations in 162 unrelated healthy Caucasians.

Functional studies

Cytokine analysis in the CMC patients in the confirmation study revealed similar defects as those observed in family #1: defective IFNγ production in response to IL-12, and no IL-22 production in response to IL-1β and IL-23 (Fig 5A). In addition, we tested IFNγ signalling in the CMC patients from family #1. We observed that the addition of IFNγ to LPS resulted in an increased TNFα production by cells isolated from patients with autosomal dominant CMC and healthy controls, demonstrating that the IFNγ signalling is intact in the CMC patients (Fig 5B).

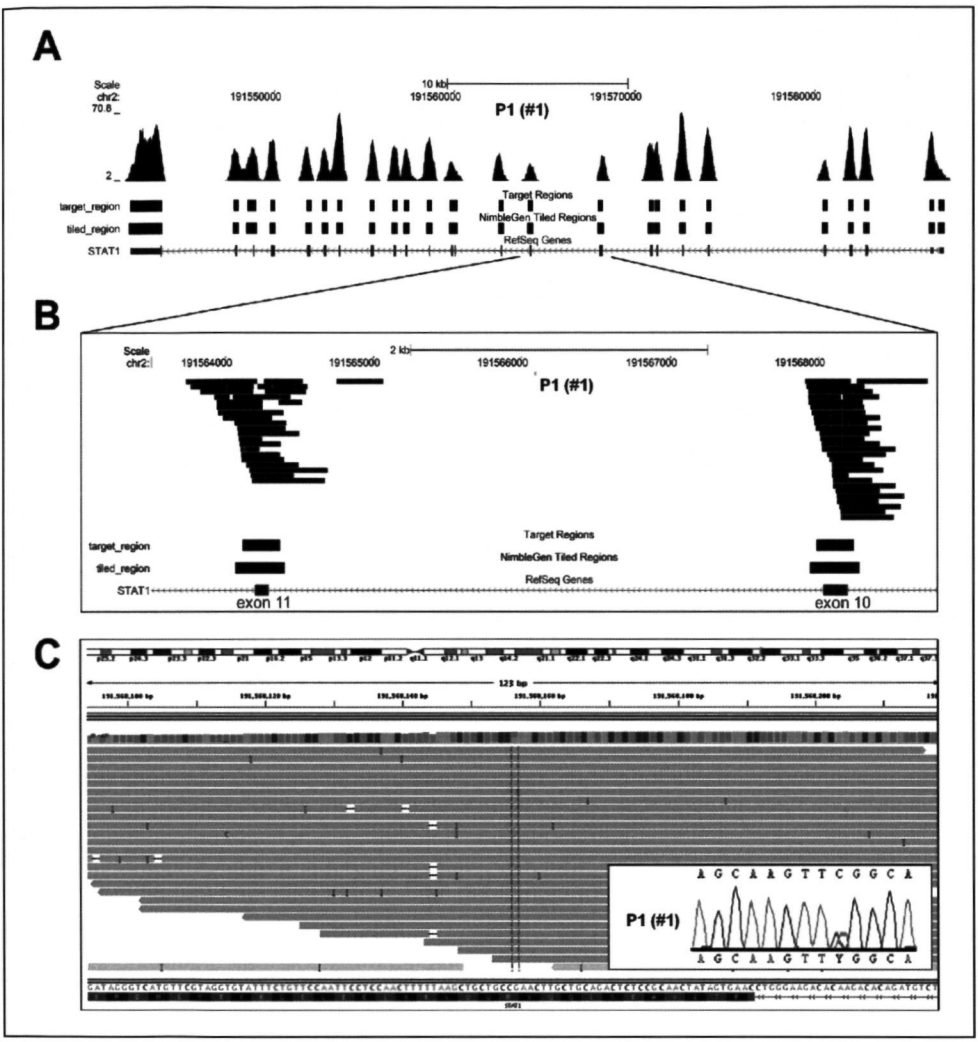
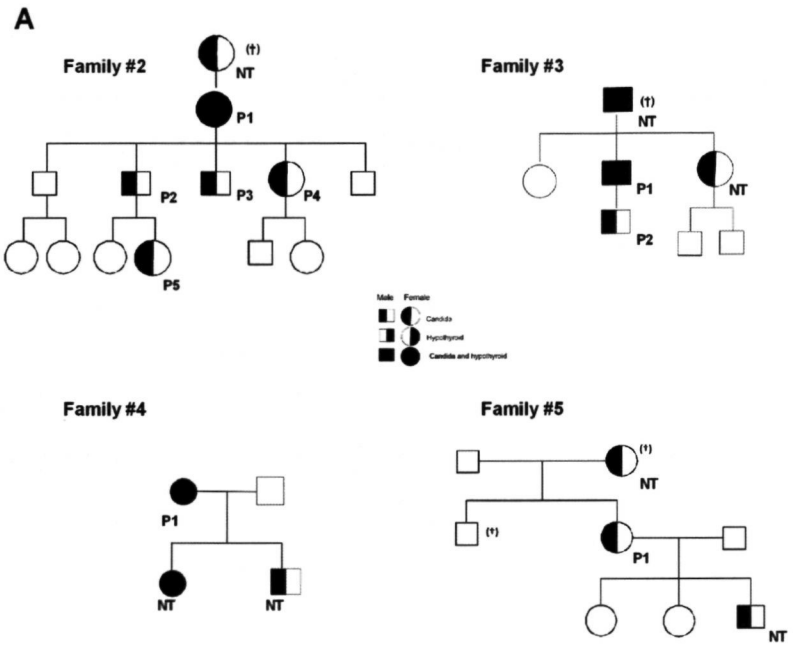


Figure 3. Next generation sequencing coverage and mutation detection for STAT1 in patient P1 - family #1. (A) Sequence read histogram uploaded to UCSC genome browser, displaying the sequencing depth of all exons of STAT1. Tracks displayed: Scale, chromosomal position, read depth histogram per bp (between 0 and 70-fold coverage), target regions, tiled regions (oligos represented on sequence capture array), refseq gene track. (B) Next generation sequencing read coverage of STAT1 exons 9 and 10. Tracks displayed: Scale, chromosomal position, individual 454 sequencing reads, target regions, tiled regions refseq gene track. (C) Mutation visualization in the IGV browser. Individual reads overlapping with the mutation are displayed, showing the heterozygous G>A mutation at genomic position chr2:191,568,156 (the reads are mapped and displayed on the + strand). The inserted caption displays the Sanger sequencing validation of the mutation (sequencing of the - strand shows heterozygous C>T mutation).



B

Family	Patient no.	gene	chromosome	genomic position (+ strand)	cDNA position	protein position
#1	Patient 1	STAT1	2q32.2	g.191568156G>A	c.820C>T	p.Arg274Trp
	Patient 2	STAT1	2q32.2	g.191568156G>A	c.820C>T	p.Arg274Trp
	Patient 3	STAT1	2q32.2	g.191568156G>A	c.820C>T	p.Arg274Trp
#2	Patient 1	STAT1	2q32.2	g.191568176G>A	c.800C>T	p.Ala267Val
	Patient 2	STAT1	2q32.2	g.191568176G>A	c.800C>T	p.Ala267Val
	Patient 3	STAT1	2q32.2	g.191568176G>A	c.800C>T	p.Ala267Val
	Patient 4	STAT1	2q32.2	g.191568176G>A	c.800C>T	p.Ala267Val
	Patient 5	STAT1	2q32.2	g.191568176G>A	c.800C>T	p.Ala267Val
#3	Patient 1	STAT1	2q32.2	g.191568176G>A	c.800C>T	p.Ala267Val
	Patient 2	STAT1	2q32.2	g.191568176G>A	c.800C>T	p.Ala267Val
#4	Patient 1	STAT1	2q32.2	g.191568156G>A	c.820C>T	p.Arg274Trp
#5	Patient 1	STAT1	2q32.2	g.191568176G>A	c.800C>T	p.Ala267Val

Figure 4 (A). Confirmation of *STAT1* mutations in patients with CMC.
Pedigrees of the additional four families with autosomal dominant CMC of which 9 patients were tested for *STAT1* mutations; three families from the United Kingdom with CMC and thyroid disease (family #2, #3, #4), and one Dutch family with CMC and oesophageal carcinoma (family #5). **(B)** Summary of all *STAT1* mutations identified.

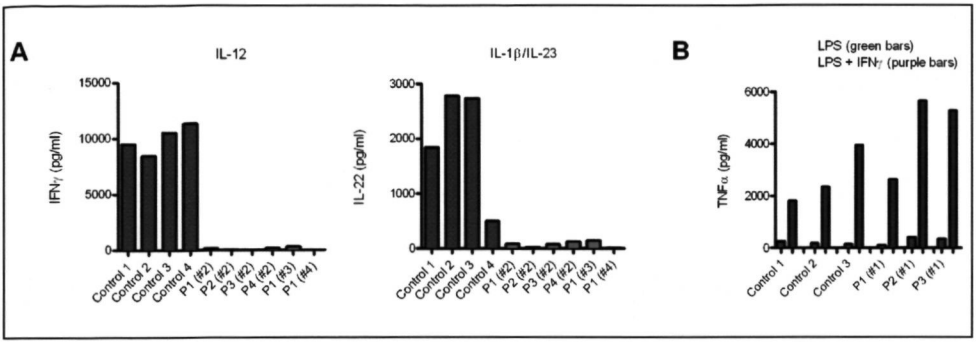


Figure 5. The IL-12 and IL-23 pathway is defect in patients with a mutation in the CC domain of *STAT1*, but the IFNγ pathway is intact.
(A) Human PBMCs from healthy controls (n=4), and CMC patients (n=6) were stimulated with IL-12 (10 ng/ml) for 48 hours or IL-1β (10 ng/ml)/IL-23 (10 ng/ml) for 5 days. (B) Human PBMCs from healthy controls (n=3), and CMC patients (n=3) were stimulated with LPS (1 ng/ml) with or without IFNγ (1 μg/ml) for 24 hours. Cytokines were measured by ELISA.

Discussion

In the present study, we demonstrate that mutations in the CC domain of *STAT1* are a major genetic defect in autosomal dominant CMC. These mutations lead to defective Th1 and Th17 responses characterized by low production of IFNγ, IL-17 and IL-22, crucial cytokines in the antifungal host defence of skin and mucosae.

We initiated the study by showing normal monocyte-dependent cytokine responses, but severely impaired Th1 and Th17 responses. These findings were in line with previous reports showing defective IFNγ (5), IL-17, and IL-22 production in CMC (6) and hyper IgE syndrome (HIES) (13), as well as with the increased susceptibility to oral candidiasis described in IL-17-defective mice (4). Since the clinical presentation somewhat resembles HIES with *STAT3* mutations (14, 15), we sequenced of the STAT3 gene. No *STAT3* mutations were identified in the family. The autosomal dominant mode of inheritance in the index family was not compatible with the recessive APECED syndrome; no mutations were found in the *AIRE* gene.

In subsequent functional studies, we obtained evidence that the defect was located in one of the molecules shared by the IL-12R and IL-23R pathway. Based on this knowledge, we selected a panel of 100 genes encoding proteins involved in the induction of Th1 and Th17 responses (Fig 2F, Table S1). The array-based sequence capture, followed by next generation sequencing to simultaneously analyze a large number of genes, yielded a mutation in the CC domain of STAT1 (R274W) present in all three affected members of the family, but in none of the unaffected individuals from the family or the healthy controls. As the index patient has nine unaffected siblings, none bearing a mutated *STAT1*gene, it is most likely that this is a *de novo* mutation in the index patient. When assessing basepair conservation (phyloP values), *STAT1* variants at this position showed a very high level of conservation over all 44 species. The variant co-segregation in the family showed very high Grantham scores, indicating a strong effect on the protein.

To strengthen the proof of the role of *STAT1* mutations as a cause of CMC, we performed a confirmation study in 9 CMC patients from 4 unrelated families from the UK and the Netherlands. All patients of family #2 and #3 from the United Kingdom were heterozygous for another mutation in the CC domain of *STAT1* (Ala267Val), as was the Dutch patient from family #5. The patient from family #4 from the UK had the same mutation as the Dutch patients from family #1. In 162 healthy controls no *STAT1* mutations were found, further supporting the notion that *STAT1* mutations in the CC domain are a major genetic cause of non-*AIRE* CMC.

It is of interest that *STAT1* mutations have been described before in patients with increased susceptibility to viral and/or mycobacterial infections (16-19). These mutations are located in the SH2 or DNA binding domains of STAT1 and result in defective signalling of IFNγ and/or type I IFN receptor pathways (16-19). On the other hand, a mutation in the CC domain of STAT1 (F172S) resulted in decreased STAT1 expression (20), while other mutations in the CC domain block dimerization of non-phosphorylated molecules (21). Our data show that mutations in the CC domain found in CMC patients are mainly involved in the regulation of Th1 and Th17 responses, possibly through the known interactions of STAT1 with STAT3 and STAT4 (22). In contrast, STAT1/STAT1 homodimers mediate signalling transmitted by the IFNγ receptor, that induce resistance to intracellular microorganisms (22). The biological function of STAT1 for mediating IFNγ-receptor signalling pathways was preserved in CMC patients, which may explain their apparently normal susceptibility to mycobacteria and viruses. It should be noted, however, that infections other than those caused by *Candida* and dermatophytes occurred in the Dutch and UK patients, ranging from bacterial chest infections to opportunistic infections caused by *Pneumocystis jiroveci* and cytomegalovirus.

Another important aspect is the co-morbidity found in some of the CMC patients. Two of the patients from family #1 displayed autoimmune phenomena (autoimmune hepatitis, autoimmune haemolytic anaemia, pernicious anaemia, and anti-phospholipid antibodies), despite a normal *AIRE* gene. STAT1 has been reported to be involved in the pathogenesis of autoimmune diseases (23). Since IL-17 is an inducer of autoimmunity, the defective Th17 responses cannot explain the observed autoimmune phenomena. Noteworthy, the daughter of patient 1 from family #4 also had autoimmune hepatitis, and shared the R274W mutation with family #1.

A substantial number of CMC patients have hypothyroidism (OMIM 114580). It has been shown before that thyroid stimulating hormone (TSH) induces Suppressor of Cytokine Signalling 1 (SOCS-1), which in turn can alter STAT1 phosphorylation (24). In this way, TSH might act as a negative regulator of cytokine action in thyroid tissue, which could be relevant to the pathogenesis of thyroid disease. Thus, mutated STAT1 may hamper the rescue of thyroid cells by TSH and hence lead to thyroid disease. An additional mechanism through which *STAT1* mutations can induce hypothyroidism is decreased iodine accumulation which occurs in *STAT1*-deficient mice (25). Finally, one of the Dutch CMC patients (and her deceased mother) and two members of the UK families suffered from oesophageal/oral carcinoma (9), and within this context it is relevant that the loss of function of STAT1 has been linked to oesophageal carcinoma (26, 27). It is striking that these families share the A267V mutation.

In conclusion, mutations in the CC domain of the *STAT1* gene are an important genetic cause of autosomal dominant CMC. The defective STAT1 affects mucosal host defence responses against *Candida* spp. through abnormal Th1 and Th17 responses. These findings will facilitate the diagnosis in patients with chronic *Candida* infections and may help to anticipate serious complications (such as oesophageal cancer). In addition, this study opens new insights into the pathogenesis of *Candida* infections that could be helpful for developing new immunomodulatory strategies against mucocutaneous candidiasis.

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Authors' contributions statement

F.L.vdV., J.W.M.vdM., D.L., J.A.V, and M.G.N. designed the study.
F.L.vdV., T.S.P., A.H., S.P.P., L.A.B.J., C.G., P.A., D.C.R., and C.A.A.vdG. performed the experiments.
T.S.P., A.H., C.G., P.A., and J.A.V. performed the statistics and the genetic analysis.
A.J.C., B.J.K., J.W.M. vdM., D.L., and M.G.N. recruited the patients and contributed to the clinical part of the study.
F.L. vdV., T.S.P., A.H., J.W.M.vdM., and M.G.N. wrote the first draft of the paper. All authors significantly contributed to the final version of the manuscript.

Conflict of interest

All authors disclose no financial and personal relationships with other people or organizations that could inappropriately influence their work.

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Supplemental data

UK Family #2

Patient 1: 59 yr old female; oral and nail candidiasis since adolescence; hypothyroidism (since young adulthood) with thyroid (TPO) antibodies, eczema, skin infections, blepharitis, enamel dysplasia (loss of all teeth), alopetia, vitiligo, squamous cell carcinoma of the mouth (5yrs ago), iron deficiency anemia. Patient 2: 36 yr old male; oral and nail candidiasis since early childhood, no hypothyroidism nor thyroid (TPO) antibodies, severe eczema, skin boils and abscesses, blepharitis, enamel dysplasia (loss of all teeth). Patient 3: 29yr old male; oral and nail candidiasis since adolescence, incipient hypothyroidism and thyroid (TPO) antibodies, severe eczema, severe blepharitis, mild enamel dysplasia, oesophageal dysmotility, gastro-oesophageal reflux disease (GORD). Patient 4: 27 yr old female; oral and nail candidiasis since 5yrs old; no hypothyroidism nor thyroid (TPO) antibodies, eczema, skin infections, blepharitis, corneal vascularisation, mild enamel dysplasia, severe iron deficiency anemia, oesophageal dysmotilyty, gastro-oesophaeal reflux disease (GORD). Patient 5: 6 yr old female; oral candidiasis, no other symptoms.

UK Family #3

Patient 1: 32yr oldmale; oral and nail candidasis since childhood, hypothyroidism (since young adulthood) no thyroid (TPO) antibodies, eczema, skin infections, blepharitis. Patient 2: 37yr female; oral, nail, vaginal candidiasis since early childhood, no hypothyroidism nor thyroid antibodies, iron deficiency anemia. Patient 3: 7 yr old male; oral candidiasis, no hypothyroidism, recurrent severe infections (sepsis, meningitis, pneumonia). Father of Patient 1: not included in this study, also had chronic candidiasis since childhood (oral and nail) and hypothyroidism; died of oesophageal squamous cell carcinoma.

UK Family #4

Patient 1: 40 yr old woman; oral, nail, vaginal candidiasis since adolescence, hypothyroidism (since young adulthood) with thyroid (TPO) antibodies, skin infections, iron deficiency anemia; has 2 affected children not included in this study (son, 13 yr old, oral candidiasis and auotimmune hepatitis and daughter, 11 yr old, oral candidiasis and hypothyroidism)

Table S1. Genes targeted by sequence capture This table contains the common gene name, the genomic position (hg18), the total number of exons, the total number of coding exons and the transcript ID of the largest isoform

AHR	chr7 17338276-17385775	12	11	NM_001621
AKT1	chr14 104306732-104333125	15	13	NM_001014432
FOS	chr14 74815284-74818665	4	4	NM_005252
ATF2	chr2 175647252-175741143	15	12	NM_001880
ATM	chr11 107598769-107745036	63	62	NM_000051
BATF	chr14 75058537-75083086	3	3	NM_006399
Bcl10	chr1 85504048-85516171	4	3	NM_003921
CARD9	chr9 138377262-138387939	13	12	NM_052813
CARD11	chr7 2912295-3050105	25	24	NM_032415
CD207	chr2 70910855-70916461	6	6	NM_015717
TYROBP	chr19 41087143-41091026	5	5	NM_003332
CLEC6A	chr12 8499858-8522193	6	6	NM_001007033
MAPK1	chr22 20443947-20551970	9	8	NM_002745
FCRLB	chr1 159959067-159964557	8	6	NM_001002901
FOXP3	chrX 48993841-49008232	12	11	NM_014009
GADD45b	chr19 2427135-2429257	4	4	NM_015675
GADD45G	chr9 91409748-91411289	4	4	NM_006705
IDO1	chr8 39890485-39905104	10	10	NM_002164
IFNA2	chr9 21374254-21375396	1	1	NM_000605
IFNAR1	chr21 33619084-33653998	11	11	NM_000629
IFNAR2	chr21 33524101-33558690	9	8	NM_207585
IFNGR2	chr21 33697072-33731698	7	7	NM_005534
CHUK	chr10 101938114-101979334	21	21	NM_001278
IKBKB	chr8 42247986-42309122	22	21	NM_001556
IKBKE	chr1 204710419-204736845	22	20	NM_014002
IL10RB	chr21 33,562,103-33,591,390	7	7	NM_000628
IL12A	chr3 161189323-161196500	7	7	NM_000882
IL12B	chr5 158674369-158690059	8	6	NM_002187
IL12RB1	chr19 18031371-18058742	17	17	NM_005535
IL12RB2	chr1 67545635-67635171	16	15	NM_001559
IL17A	chr6 52159144-52163395	3	3	NM_002190
IL17RA	chr22 15,945,849-15,971,389	13	13	NM_014339
IL18	chr11 111519186-111540050	6	5	NM_001562
IL18BP	chr11 71387606-71391498	7	5	NM_001039659
IL18RAP	chr2 102401686-102435456	12	10	NM_003853
IL21	chr4 123753233-123761661	5	5	NM_021803
IL22	chr12 66928292-66933548	5	5	NM_020525
IL23A	chr12 55018930-55020461	4	4	NM_016584
IL23R	chr1 67404757-67498238	11	10	NM_144701
IFNW1	chr9 21130631-21132144	1	1	NM_002177
IL21R	chr16 27321924-27369616	9	8	NM_181078
IL6R	chr1 152644293-152706812	10	10	NM_000565
IL22RA2	chr6 137506650-137536478	7	6	NM_052962
IL22RA1	chr1 24318848-24342198	7	7	NM_021258
IRF1	chr5 131846684-131854326	10	9	NM_002198
IRF2	chr4 185545870-185632720	9	8	NM_002199
IRF3	chr19 54854641-54860944	8	7	NM_001571
IRF4	chr6 336752-356443	9	8	NM_002460
IRF5	chr7 128365230-128377324	9	8	NM_001098630

IRF6	chr1:208027885-208046102	9	7	NM_006147
IRF7	chr11:602555-605999	11	10	NM_001572
IRF8	chr16:84490275-84513712	9	8	NM_002163
IRF9	chr14:23699569-23705614	9	8	NM_006084
JAK1	chr1:65071494-65204775	25	24	NM_002227
JAK2	chr9:4975245-5117995	25	23	NM_004972
JAK3	chr19:17796593-17819841	24	23	NM_000215
Malt1	chr18:54489598-54568350	17	17	NM_006785
MRC1	chr10:17891368-17993184	30	30	NM_002438
IL8	chr4.74825139-74828297	4	4	NM_000584
NFATc1	chr18:75261314-75390311	10	10	NM_006162
ST13	chr22:39550547-39582633	12	12	NM_003932
PIK3CG	chr7:106293160-106334828	11	10	NM_002649
PIAS1	chr15.66133626-66267458	14	14	NM_016166
PIAS3	chr1·144287345-144297903	14	14	NM_006099
PRKD3	chr2 37331150-37397726	18	18	NM_005813
PRKCE	chr2·45732547-46268633	15	15	NM_005400
SPI1	chr11:47332985-47356703	5	5	NM_001080547
GNB2L1	chr5·180596534-180603512	8	8	NM_006098
RASGRP3	chr2:33514920-33643162	19	16	NM_170672
RELB	chr19:50196552-50233292	12	12	NM_006509
RIPK1	chr6:3021997-3060420	10	10	NM_003804
RORA	chr15:58576755-58707024	12	12	NM_134260
RORC	chr1:150045171-150070972	11	11	NM_005060
RUNX1	chr21:35081968-35343465	9	8	NM_001754
Smad2	chr18:43613464-43711510	11	10	NM_001003652
Smad3	chr15:65145249-65274587	9	9	NM_005902
Smad4	chr18:46810581-46865409	13	11	NM_005359
SOCS1	chr16:11255775-11257540	2	1	NM_003745
SOCS3	chr17.73864457-73867753	2	1	NM_003955
SOCS5	chr2:46779603-46843431	2	1	NM_144949
SpiB	chr19:55614028-55624060	6	6	NM_003121
STAT1	chr2 191542007-191587221	25	23	NM_007315
STAT2	chr12:55021649-55040176	24	23	NM_005419
STAT5B	chr17:37604721-37681950	19	18	NM_012448
STAT6	chr12:55775460-55791428	22	22	NM_003153
SUMO1	chr2:202779148-202811567	5	5	NM_003352
Syk	chr9.92603891-92698304	14	13	NM_003177
MAP3K7IP2	chr6·149680756-149774440	7	6	NM_015093
TANK	chr2 161701712-161800928	8	7	NM_004180
TBK1	chr12:63132204-63182158	21	20	NM_013254
TGFBR1	chr9:100907233-100956294	9	9	NM_004612
TLR3	chr4:187227303-187243246	5	4	NM_003265
TLR4	chr9:119506431-119519587	4	2	NM_138554
TRAF3	chr14·102313569-102442381	12	10	NM_145725
TRAF6	chr11:36467299-36488398	8	6	NM_145803
TICAM2	chr5·114942247-114989610	4	1	NM_021649
TICAM1	chr19:4766992-4769451	1	1	NM_182919
TRIM21	chr11:4362703-4371502	7	6	NM_003141
TxK	chr4:47763167-47831030	15	15	NM_003328
TYK2	chr19:10322204-10352248	25	23	NM_003331

Table S2: Oligonucleotides used as bar-coded adaptors (Titanium optimized), and enhancing oligos for 12-plex sequence capture and subsequent 454 sequencing.

Ti-MID1-A	C*C*A*T*CTCATCCCTGCGTGTCTCCGACTCAGACGAGT*G*C*G*T
Ti-MID2-A	C*C*A*T*CTCATCCCTGCGTGTCTCCGACTCAGACGCTC*G*A*C*A
Ti-MID3-A	C*C*A*T*CTCATCCCTGCGTGTCTCCGACTCAGAGACGC*A*C*T*C
Ti-MID4-A	C*C*A*T*CTCATCCCTGCGTGTCTCCGACTCAGAGCACT*G*T*A*G
Ti-MID5-A	C*C*A*T*CTCATCCCTGCGTGTCTCCGACTCAGATCAGA*C*A*C*G
Ti-MID6-A	C*C*A*T*CTCATCCCTGCGTGTCTCCGACTCAGATATCG*C*G*A*G
Ti-MID7-A	C*C*A*T*CTCATCCCTGCGTGTCTCCGACTCAGCGTGTC*T*C*T*A
Ti-MID8-A	C*C*A*T*CTCATCCCTGCGTGTCTCCGACTCAGCTCGCG*T*G*T*C
Ti-MID10-A	C*C*A*T*CTCATCCCTGCGTGTCTCCGACTCAGTCTCTA*T*G*C*G
Ti-MID11-A	C*C*A*T*CTCATCCCTGCGTGTCTCCGACTCAGTGATAC*G*T*C*T
Ti-MID13-A	C*C*A*T*CTCATCCCTGCGTGTCTCCGACTCAGCATAGT*A*G*T*G
Ti-MID14-A	C*C*A*T*CTCATCCCTGCGTGTCTCCGACTCAGCGAGAG*A*T*A*C
Ti-MID1-Aprime	A*C*G*C*ACTCGTCTGAGTCG*G*A*G*A
Ti-MID2-Aprime	T*G*T*C*GAGCGTCTGAGTCG*G*A*G*A
Ti-MID3-Aprime	G*A*G*T*GCGTCTCTGAGTCG*G*A*G*A
Ti-MID4-Aprime	C*T*A*C*AGTGCTCTGAGTCG*G*A*G*A
Ti-MID5-Aprime	C*G*T*G*TCTGATCTGAGTCG*G*A*G*A
Ti-MID6-Aprime	C*T*C*G*CGATATCTGAGTCG*G*A*G*A
Ti-MID7-Aprime	T*A*G*A*GACACGCTGAGTCG*G*A*G*A
Ti-MID8-Aprime	G*A*C*A*CGCGAGCTGAGTCG*G*A*G*A
Ti-MID10-Aprime	C*G*C*A*TAGAGACTGAGTCG*G*A*G*A
Ti-MID11-Aprime	A*G*A*C*GTATCACTGAGTCG*G*A*G*A
Ti-MID13-Aprime	C*A*T*A*GTAGTGCTGAGTCG*G*A*G*A
Ti-MID14-Aprime	C*G*A*G*AGATACCTGAGTCG*G*A*G*A
Ti-MID-B	/5BioTEG/C*C*T*A*TCCCCTGTGTGCCTTGGCAGTC*T*C*A*G
Ti-MID-Bprime	C*T*G*A*GACT*G*C*C*A
Ti MID_1 Hyb enhancing oligo	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAG ACG AGT GCG T/3ddC/
Ti MID_2 Hyb enhancing oligo	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAG ACG CTC GAC A/3ddC/
Ti MID_3 Hyb enhancing oligo	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAG AGA CGC ACT C/3ddC/
Ti MID_4 Hyb enhancing oligo	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAG AGC ACT GTA G/3ddC/
Ti MID_5 Hyb enhancing oligo	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAG ATC AGA CAC G/3ddC/
Ti MID_6 Hyb enhancing oligo	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAG ATA TCG CGA G/3ddC/
Ti MID_7 Hyb enhancing oligo	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAG CGT GTC TCT A/3ddC/
Ti MID_8 Hyb enhancing oligo	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAG CTC GCG TGT C/3ddC/
Ti MID_10 Hyb enhancing oligo	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAG TCT CTA TGC G/3ddC/
Ti MID_11 Hyb enhancing oligo	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAG TGA TAC GTC T/3ddC/
Ti MID_13 Hyb enhancing oligo	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAG CAT AGT AGT G/3ddC/
Ti MID_14 Hyb enhancing oligo	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAG CGA GAG ATA C/3ddC/
Titanium Hyb enhancing B	CCT ATC CCC TGT GTG CCT TGG CAG TCT CAG /3ddc/

Each oligonucleotide should have phosphorothioate modifications in both the first four and the last four bases of the oligomers
The adapter B long oligonucleotide Ti-MID-B must be synthesized with a 5-prime biotin-TEG moiety
All oligonucleotides must be purified using HPLC
Oligos may be ordered lyophilized or in solution (TE, pH 8.0) at at fixed concentration (100µM)
100 nmole scale (adapter A) and 1 micromole scale (adapter B) is for 40 libraries

Table S3: Statistics on the target sequence coverage for 454 sequencing run

	Family #1, patient 1	Family #1, patient 2	Family #1, patient 3	Family #2, patient 5	Family #3, patient 1	Family #1, unaffected	Unrel. healthy control 1	Unrel. healthy control 2	Average
Total mapped bases [Mb]	16.502	14.188	16.285	6.913	27.974	17.672	12.416	15.067	**15.877**
On target*	73.4%	73.9%	73.8%	73.4%	75.8%	72.9%	71.6%	73.3%	**73.5%**
Near target** (500bp)	24.9%	24.4%	24.1%	25.1%	21.9%	25.3%	26.8%	24.9%	**24.7%**
Off target	1.7%	1.6%	2.2%	1.6%	2.2%	1.8%	1.6%	1.9%	**1.8%**
Average target coverage	23.8	20.7	23.7	10.3	41.5	25.3	17.6	21.7	**23.1**

* On target: mapping to bases included in the array design
** Near target: In 500 basepair (approximate fragment length) proximity of array targets
*** Off target: Mapping to other genomic positions in the genome

Table S4: Regions without coverage in all 8 CMC individuals

Chromosome	Start position	End position	Length	Target
chr14	102,313,490	102,313,823	334	*TRAF3* UTR
chr14	104,332,786	104,333,180	395	*AKT1* UTR
chr17	37,681,695	37,682,027	333	*STAT5B* UTR
chr9	100,907,145	100,907,367	223	*TGFBR1* UTR and partial exon1
chr9	100,907,373	100,907,472	100	*TGFBR1* partial exon1

Table S5: Statistics on variants called.

	Family #1, patient 1	Family #1, patient 2	Family #1, patient 3	Family #2, patient 5	Family #3, patient 1	Family #1, unaffected	Unrel. healthy control 1	Unrel. healthy control 2	Average
Total variants	740	730	707	443	918	847	673	723	723
of those SNVs	659	661	631	416	791	781	611	656	651
of those indels	81	69	76	27	127	66	62	67	72
Known SNPs (db SNP 130)	698	680	655	427	810	793	644	681	674
In house variants	9	14	16	8	14	15	7	7	11
Novel variants	33	36	36	8	94	39	22	35	38
of those coding (non-synonymous)	5	2	4	3	7	4	4	2	4
of those minimal 20% variant reads	2	1	1	2	1	0	3	1	1

Table S6: Summary of all novel non-synonymous variants identified in 8 individuals (5 CMC patients, 3 controls) (with *STAT1* variants in bold)

Sample ID	Chr.	Start	End	Ref.	Var.	No. reads	No. var. reads	% variation	Gene	Ref. AA	Var. AA	phyloP	Grantham score
Family #1, patient 3	**chr2**	**191,568,156**	**191,568,156**	**G**	**A**	**16**	**10**	**62**	**STAT1**	**R**	**W**	**6.78**	**177**
Family #1, patient 3	chr8	42,294,370	42,294,370	T	A	20	4	20	IKBKB	F	L	0.40	22
Family #1, patient 2	**chr2**	**191,568,156**	**191,568,156**	**G**	**A**	**17**	**9**	**53**	**STAT1**	**R**	**W**	**6.78**	**177**
Family #1, patient 1	**chr2**	**191,568,156**	**191,568,156**	**G**	**A**	**23**	**11**	**48**	**STAT1**	**R**	**W**	**6.78**	**177**
Family #2, patient 5	chr4	187,234,943	187,234,943	C	A	22	9	41	TLR3	T	N	5.05	65
Family #2, patient 5	chr19	4,769,178	4,769,178	C	T	14	6	43	TICAM1	R	Q	-2.04	68
Family #3, patient 1	**chr2**	**191,568,176**	**191,568,176**	**G**	**A**	**41**	**26**	**63**	**STAT1**	**A**	**V**	**6.78**	**64**
Unrel. healthy control 1	chr9	5,068,411	5,068,411	C	T	31	13	42	JAK2	P	S	5.40	103
Unrel. healthy control 1	chr6	137,517,930	137,517,930	T	-	25	10	40	IL22RA2	K	KX	0.56	*
Unrel. healthy control 1	chr9	119,516,114	119,516,114	G	T	26	9	35	TLR4	M	I	-0.42	10
Unrel. healthy control 2	chr11	4,363,464	4,363,464	T	C	25	8	32	TRIM21	E	G	0.92	98

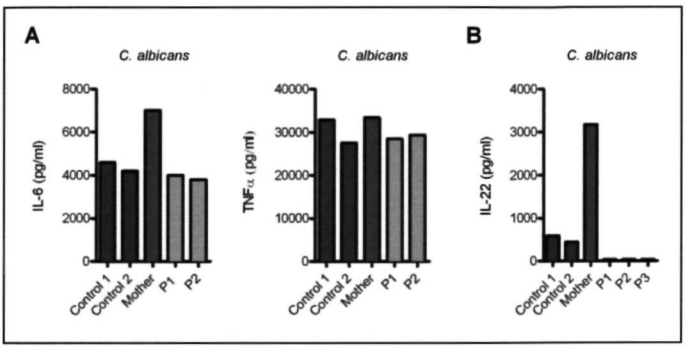

Figure S1. Human PBMCs from healthy controls (n=2), the mother and three affected members of the Dutch family #1 with CMC (P1, P2, P3) were stimulated with (A) heat-killed *Candida albicans* (1x10e6 microorganisms/ml) for 24 hours, (B) IL-1β (10 ng/ml)/IL-23 (10 ng/ml) for 5 days. Cytokines were measured by ELISA.

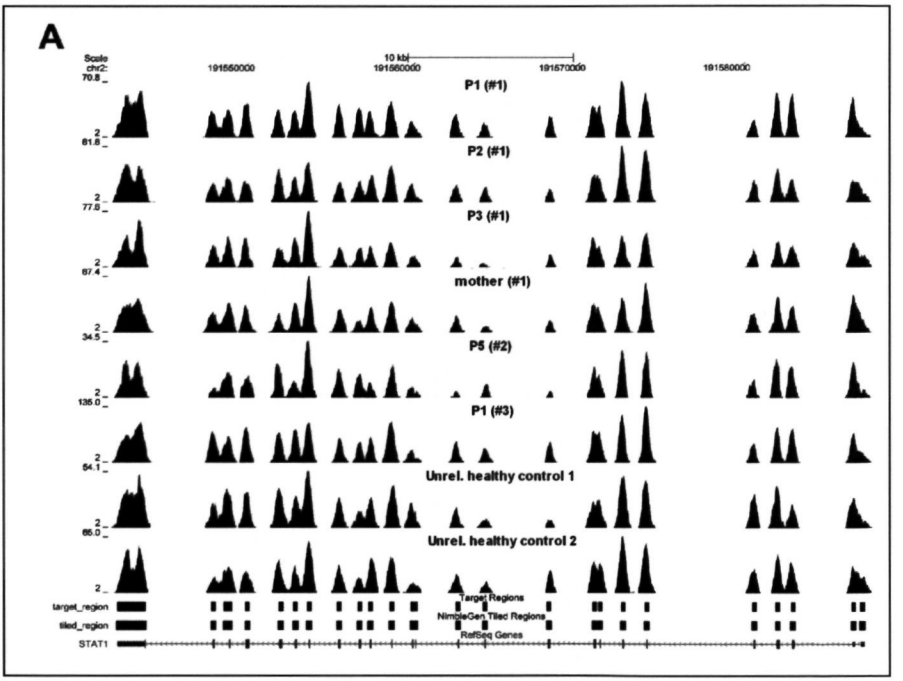

Figure S2. (A) Sequence read histograms uploaded to UCSC genome browser, displaying the sequencing depth of all exons of STAT1 for all patients included in the next generation sequencing assay. Tracks displayed: Scale, chromosomal position, read depth histogram per bp (between 0 and 70-fold coverage), target regions, tiled regions (oligos represented on sequence capture array), refseq gene track.

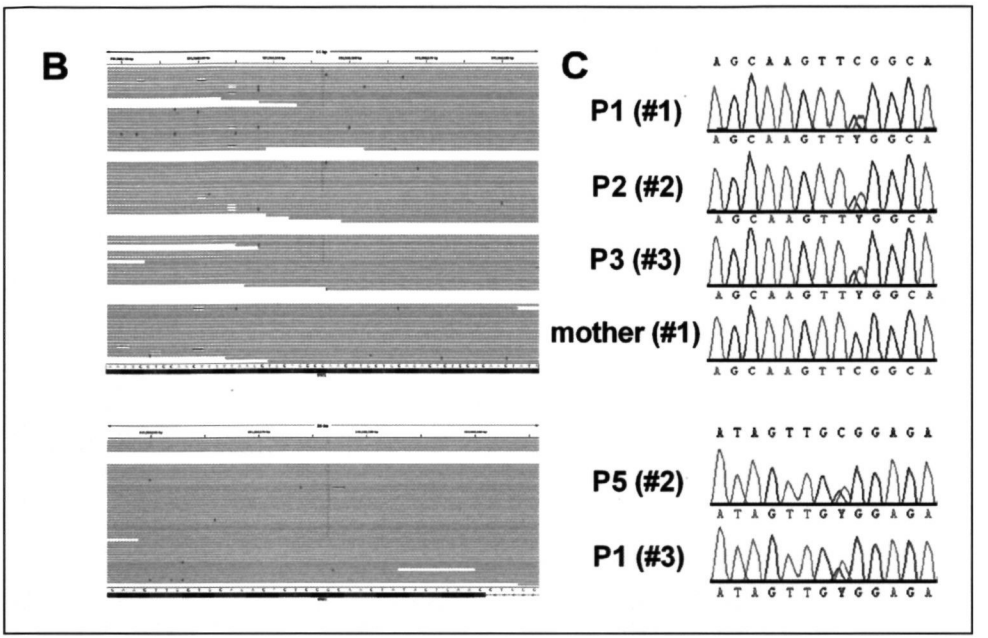

Figure S2. (B) Next generation sequencing reads coverage of STAT1 exons 9 and 10. Tracks displayed: Scale, chromosomal position, individual 454 sequencing reads, target regions, tiled regions refseq gene track. (C) Mutation visualization in the IGV browser. Individual reads overlapping with the mutation are displayed, showing the heterozygous G>A mutation at genomic position chr2:191,568,156 (the reads are mapped and displayed on the + strand). The inserted caption displays the Sanger sequencing validation of the mutation (sequencing of the - strand shows heterozygous C>T mutation) for patients 1, 2, 3, and the unaffected mother from family #1. Likewise, individual reads overlapping with the mutation are displayed, showing the heterozygous G>A mutation at genomic position chr2:191,568,176 (the reads are mapped and displayed on the + strand). The inserted caption displays the Sanger sequencing validation of the mutation (sequencing of the - strand shows heterozygous C>T mutation) for patient 5 from family #2 and patient 1 from family #3.

Chapter 18

Novel strategies for prevention and treatment of *Candida* infections: the potential of immunotherapy

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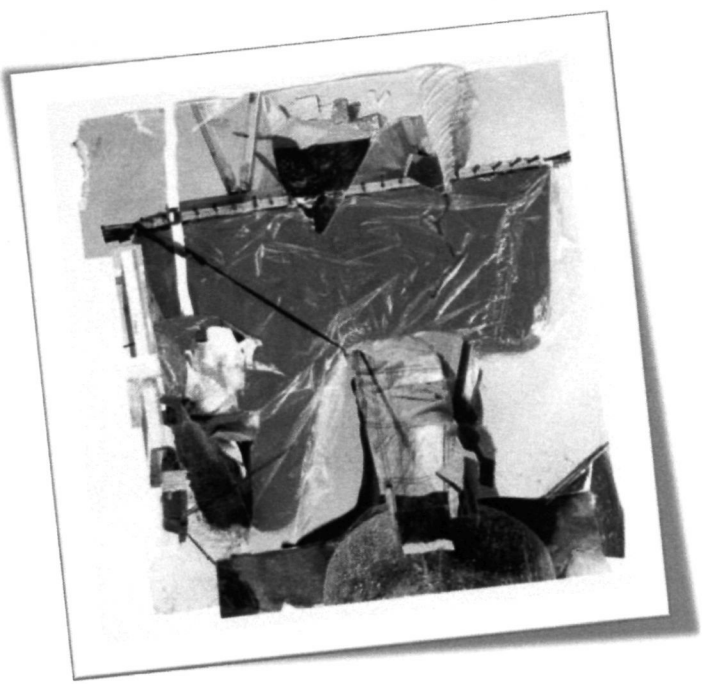

Veerdonk FL, Netea MG, Joosten LA, van der Meer JW, Kullberg BJ

Summary

Infections caused by *Candida* species continue to be a substantial cause of disease burden, especially in immunocompromised patients. New approaches are needed to improve the outcome of patients suffering from *Candida* infections, since it seems unlikely that the established standard treatment will drastically lower the morbidity of mucocutaneous *Candida* infections and the high mortality associated with invasive candidiasis. New insights into the mechanisms of the anti-*Candida* host response have contributed to the design of novel immunotherapeutic approaches that have been proposed as adjuvant therapy in *Candida* infections. This review presents an overview of novel strategies in the prevention and treatment of *Candida* infections, with a special focus on adjuvant immunotherapy.

Introduction

Candida infections can be divided into local infections and invasive candidiasis. The various forms of mucosal *Candida* infections induce a significant burden of morbidity: vulvovaginal candidiasis (VVC), oropharyngeal candidiasis (OPC) and chronic mucocutaneous candidiasis (CMC). VVC, the most common form of mucosal candidiasis, is widespread and may affect up to 75% of women of child-bearing age (1). It is characterized by pruritus, irritation and dyspareunia often accompanied by increased vaginal discharge. VVC can be divided into uncomplicated or complicated (2). Complicated VVC is defined as severe or recurrent disease, infection due to *Candida* species other than *C. albicans*, or VVC in an abnormal host (2). Recurrent forms of VVC (RVVC) are defined as four or more episodes of symptomatic VVC within one year (3). Notably, in patients with RVVC the associated costs of medical visits is high and their quality of life is significantly reduced (1).

OPC is a relatively common local infection occurring in high-risk groups of patients such as dental wearers, diabetic patients, individuals treated with broad spectrum antibiotics, infants and patients infected with HIV. It has been reported that 84% of HIV-infected individuals are asymptomatically colonized with *Candida* spp. in the oral cavity, with 55% developing at least one episode of OPC with clinical signs (4). If left untreated these lesions contribute considerably to the morbidity associated with HIV infection (5). Importantly, oropharyngeal candidiasis can be complicated by esophageal candidiasis. Esophageal candidiasis is accompanied by more serious complaints and predisposes to the development of systemic candidiasis (6, 7).

These mucosal manifestations of candidiasis may also be associated with primary immunodeficiencies. CMC represents a heterogeneous group of primary immunodeficiencies that are characterized by an inability to clear fungal infections. Consequently, persisting and recurrent infections of the skin and mucous membranes with *C. albicans* ensue (8). Some patients have autosomal recessive polyglandular autoimmune syndrome type I, also known as autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED) syndrome. This syndrome is caused by mutations in the autoimmune regulator (AIRE) gene and is characterized by CMC and endocrine disorders, such as hypoparathyroidism and Addison's disease (9). CMC also occurs without associated disorders and the genetic defect in these patients is not known (10). Another primary immunodeficiency accompanied by mucocutaneous candidiasis is the hyperimmunoglobulin E syndrome (HIES, Job's syndrome).

This disease is further characterized by recurrent staphylococcal skin abscesses, pulmonary infections, skeletal and dental abnormalities, and elevated serum immunoglobulin E concentrations (11, 12). Recently, it has been demonstrated that in large number of patients with HIES, a dominant negative mutation in STAT3 is the underlying cause of disease (13).

Disseminated candidiasis is a deep-seated organ infection with *Candida* spp. and/or candidemia. The clinical spectrum varies from minimal fever to severe sepsis with multiorgan failure (14). The microorganism gains access to the intravascular compartment either from the gastrointestinal tract or, less often, from the skin through the site of an indwelling intravascular catheter. Noteworthy, it has become apparent that health care workers' hands are commonly colonized with *Candida* spp. (15), and several studies have demonstrated that this is the main cause of the reported *Candida parapsilosis* outbreaks in pediatric ICU departments (16-18). *Candida* spp. has been reported as one of the most common pathogens that cause hospital acquired bloodstream infections in patients undergoing surgical or chemotherapeutic interventions and/or with underlying immunological deficiencies (19, 20). Importantly, nosocomial candidemia is associated with an unacceptable high mortality ranging between 30% and 50% (19, 21, 22).

In this review we will discuss novel approaches such as vaccination, antibodies, cytokine therapy, and adoptive transfer of primed immune cells that have the potential to improve the clinical outcome of patients with *Candida* infections.

Diagnosis and treatment of *Candida* infections

Patients with invasive candidiasis should receive effective treatment as soon as possible, since delays in starting antifungal therapy in candidemia are correlated with increased mortality (14, 23, 24). Therefore, it is of utmost importance to identify candidemia in critically ill patients as soon as possible. Several clinical scores have been developed to identify patients who are at high risk for developing candidemia (25, 26). The golden standard for the diagnosis of disseminated candidiasis remains microbiological identification of the yeast in the blood or the organs. Unfortunately, the microbiological methods have a low sensitivity, and additional methods have been developed to improve early fungal detection. Non-invasive techniques, such as measurement of mannan and anti-mannan antibodies and the detection of the fungal cell wall component (1-3)-β D-glucan (BDG) have the potential to be useful for early diagnosis of invasive candidiasis (27-29). Another promising technique is the use of polymerase chain reaction (PCR), which has been reported to have a high sensitivity and specificity and has good potential to provide species identification prior to blood culture positivity (30). Still, none of these techniques can definitively prove a *Candida* infection (31, 32). Since it remains difficult to establish a diagnosis of invasive candidiasis early in the course of infection, prophylactic therapy is often advocated. However, prophylactic treatment in high-risk groups, such as stem cell transplant recipients and high-risk populations in the ICU, is a controversial issue as outlined in the IDSA guidelines for the treatment of invasive candidiasis (24).

Diagnosis of VVC is usually made on the basis of clinical signs and symptoms (1), but since these are not specific it is wise to confirm it with a smear before starting antifungal therapy. Clinical signs and symptoms of oropharyngeal candidiasis in HIV patients are usually

suggestive of the diagnosis (33). However, in view of the differential diagnosis, frequent recurrence or persistence, microbiological culture to confirm the diagnosis of OPC, speciation and susceptibility testing are required.

The current standard treatment of *Candida* infections consists of antifungal agents such as azoles, echinocandins and amphotericin B compounds (Box 1). Despite these available antifungal agents the frequency of treatment failure is considerable, underscoring the necessity for new treatment strategies. In vitro experiments suggest that combinations of these antifungal agents might improve antifungal efficacy (34, 35). However, no clinical trial to date has demonstrated that combining antifungal agents results in additional efficacy for the treatment of invasive candidiasis. The lack of progress in terms of mortality due to disseminated *Candida* infections has led to the opinion among the experts that only a combination between standard antifungal treatment and the adjunctive immunotherapy will be able to decrease the mortality in *Candida* sepsis. A better understanding of the pathogenesis of host defense against *Candida* infections has made it possible to explore immunomodulatory interventions that might contribute to the standard clinical practice.

Pathogenesis of host defense against *Candida* infection

Host defense against *Candida* infection depends on intact mucosal and skin barriers, and adequate recognition of the fungus that subsequently triggers protective innate and adaptive antifungal defense mechanisms (36). The first line of defense is the skin and mucosa, which not only offers a mechanical barrier but also provides microbial antagonism with its associated normal flora. Once this first line of defense fails, protective innate and adaptive immune mechanisms will be activated that critically depend on appropriate pathogen recognition. Recognition of *Candida* is mediated by pattern recognition receptors (PRRs), which bind pathogen-associated molecular patterns (PAMPs). The most studied and well-known PAMPs of *Candida* are components of the fungal cell wall. The inner layer of the cell wall is composed of β-(1,3)-glucan covalently linked to β-(1,6)-glucan (37) and chitin, and an outer layer that consists of proteins that are heavily glycosylated by *N*-linked (38) and *O*-linked mannosylation (39). All these components have been reported as ligands for one or more PRRs, among which two classes collaborate in *Candida* recognition: toll-like receptors (TLRs) such as TLR2 and TLR4, and the C-type lectin receptors (CLRs) such as dectin-1, dectin-2, Mincle and mannose receptor (MR) (40-46). Some of these receptors exert a proinflammatory action (e.g., TLR4, dectin-1, MR), while others exert a more antiinflammatory effect (e.g., TLR2) (36). The type of response initiated by *Candida* depends on the complex interaction between the PRRs expressed by the different cell types present at the site of infection (47). Polymorphonuclear cells (PMNs), monocytes and macrophages are important for the main innate effector response: phagocytosis of *Candida* and the induction of reactive oxygen species (ROS) that can both damage and subsequently eliminate the fungus.

Proinflammatory cytokines such as tumor necrosis factor α (TNFα) and interleukin (IL)-1β are crucial for proper activation of PMNs. TNFα is essential for anti-*Candida* host defense through recruitment of neutrophils and phagocytosis, and deficiency results in higher mortality during experimental disseminated candidiasis (48). In addition, IFNγ produced by CD4 T lymphocytes is also important for stimulation of antifungal activity of PMNs. IFNγ

induces NO production by macrophages and *Candida*-specific immunoglobulin production (49). The central role of endogenous IFNγ in the resistance against systemic candidiasis has been underscored by the observation that knock-out mice deficient in IFNγ are highly susceptible to *C. albicans* infection (50, 51). Mice deficient in the cytokine IL-18, which plays a crucial role in the induction of IFNγ, are also more susceptible to disseminated candidiasis (52). Thus, the pathogenesis of invasive candidiasis seems to be linked to defects in phagocytosis and killing of *Candida* and defects, leading to IFNγ production.

Another source for the production of pro-inflammatory cytokines in the mucosa are epithelial cells, and they have been advocated to play a central role in the protection against fungal pathogens (53, 54). Epithelial cells can produce IL-8 and GM-CSF in response to *Candida* spp. (55-57). Furthermore, oral epithelial cells can upregulate the anti-fungal activity of neutrophils in vitro, and this effect was partially dependent on IL-1α (58). In addition, neutrophils can upregulate TLR4 expression on *C. albicans* infected human oral epithelium, and this was directly associated with protection against fungal invasion of the epithelium (59).

The adaptive immune responses that are crucial for antifungal protection are elicited by CD4+ T helper (Th)1 (IFNγ-producing) cells and Th17 (IL-17/IL-22-producing) cells. The *Candida*-specific Th1 response is induced by antigen presentation in the presence of the cytokine IL-12 (60), while Th17 responses are induced and maintained in the presence of IL-1 and IL-23 (61). In patients with HIV who have low CD4 counts, the incidence of oropharyngeal candidiasis is high (62). This underscores the importance of the T helper cell in mucosal anti-*Candida* host defense. Notably, in the absence of CD4+ T cells, CD8+ T cells also appear to play an important role in anti-*Candida* host defense (63, 64).

The recently discovered T helper subset Th17 cells have provided important novel insights into the pathogenesis of mucosal *Candida* infections. Th17 cells have been demonstrated to play a crucial role in host defense in experimental oropharyngeal *Candida* infection and disseminated candidiasis (65, 66). It has been shown that patients with HIES have a defect in the *Candida*- induced Th17 response (67, 68). Furthermore, patients with CMC have been linked to a defect in their Th17 response against *Candida* (69). A recent study shows that IL-17A deficient mice were equally susceptible to disseminated candidiasis, but in the same study the Th17 response induced by vaccination was associated with protection against disseminated candidiasis (70). However, Th17 responses have also been suggested to be detrimental for the host during fungal infection. In an experimental fungal infection model, both inflammation and infection were exacerbated by the Th17 response against *Candida albicans* and *Aspergillus fumigatus* (71). This is an important controversy that needs further investigation, since these observations will provide the rationale for choosing the correct adjuvant in fungal vaccine strategies.

There has been quite some speculations over the years whether patients with vulvovaginal candidiasis (VVC) have an underlying host defense defect (1). It has been reported that the increase of vaginal mannose binding lectin (MBL) levels in patients with VVC may be an effective immune response against *Candida albicans* infection, and that women with recurrent VVC have lower vaginal levels of MBL compared to controls (72). In addition, MBL polymorphisms have been associated with recurrent vulvovaginal candidiasis (73, 74).

Another polymorphism in the IL-4 (T-589) gene was found to correlate with a high prevalence of RVVC (75). One study has reported a role for neutrophils in the inflammatory response during VVC. Neutropenia in an experimental model of vaginal *Candida* infection had no effect on the fungal load during infection, but was significantly associated with decreased vaginal inflammation (76). Another study reported that symptoms of VVC appear to be due to an aggressive innate response by PMN (77).

Recently, new insights in the pathogenesis of RVVC have emerged. A family in which four women were affected by either recurrent vulvovaginal candidiasis or onychomycosis were found to be dectin-1 deficient (78). It was demonstrated that dectin-1 deficiency resulted in lower IL-17 production in response to *Candida albicans* and this most probably accounts for the clinical picture seen in these patients. These observations were further strengthened by another report which describes a family that was deficient in CARD9, a downstream molecule in the signaling cascade of dectin-1 (79). Patients with CARD9 deficiency had an impaired *Candida*-specific Th17 response, and female patients had a long history or early onset of vaginal candidiasis. These data suggest that Th17 responses are important for vaginal mucosal host defense.

Novel immunotherapeutic strategies

Insights into the anti-*Candida* host defense mechanisms have contributed to the development of immune interventions that have the potential to lower the morbidity and mortality associated with *Candida* infections. Immunomodulatory strategies under investigation range from vaccination, to therapeutic antibodies and recombinant cytokines, and adoptive transfer of primed immune cells.

Vaccination

Among those with invasive *Candida* infection, immunocompromised patients have the highest morbidity and mortality. Since vaccination depends on an appropriate host defense mechanism to provide protection, active and passive immunization in immunocompromised patients remains a challenge. Currently, there are no *Candida* vaccines clinically available. Nevertheless, several active and passive fungal vaccine approaches look promising and could prove to be an effective and safe strategy. A preliminary phase II trial with the oral vaccine D.561 performed already two decades ago in 22 patients with frequent recurrences of vulvovaginal candidiasis (VVC) showed promising results, but the true efficacy of the vaccine still has to be confirmed in a larger placebo-controlled trial (80). Several vaccine strategies have been tested in animal models with success. Diphtheria toxoid CRM197 conjugated with the algal antigen laminarin (Lam) was protective against both mucosal and systemic candidiasis in mice (81). Mannan protein conjugates induced protective antibody responses against experimental disseminated candidiasis and *Candida* vaginal infection (82). Vaccines based on the adhesins Als1p and Als3p were shown to cause a marked improvement in the fungal burden and survival of immunocompetent and immunocompromised mice with invasive and mucosal *Candida* infection (83-86). Furthermore, vaccination with live-attenuated *Candida* or the low virulent CA2 strain have been reported to provide protection against hematogenous *Candida* re-infection in animal models (87). Although these different vaccination strategies provide promising results in animal models with experimental

infections, their clinical safety and efficacy remains to be assessed in humans with *Candida* infections.

Important new insights have been gained in recent years regarding adjuvanticity. There are potent adjuvants that trigger the pattern recognition receptors (PRRs) of dendritic cells (DCs) which results in DC maturation. Different adjuvants induce different cytokine profiles, and dependent on this profile the immune response is shifted towards a Th1, Th2 or Th17 response. This enables to shape the kind of adaptive immune response elicited by the vaccine (Figure 1). Several adjuvants available act through PRRs or their pathways, such as the TLR9 agonist CpG DNA (88), the TLR4 agonist monophosphoryl lipid A (lipid A) (89), and alum which exerts its effects through cryopyrin (NALP3) (90). To improve the efficacy of current vaccines these insights should be exploited to specifically induce optimal defense against a pathogen. In candidiasis, this would mean that the adjuvant ideally facilitates a strong Th1 response in case of disseminated candidiasis and a predominant Th17 response during mucosal candidiasis. The dectin-1 ligand β-glucan that has the potential to induce both a Th1 and Th17 response, would be a good candidate for a *Candida* vaccine adjuvant (91). Mannans from yeasts induce a Th17 response through mannose receptor and/or dectin-2 are of potential interest for vaccination against mucosal *Candida* infections (40, 46). A recent study has demonstrated that mice immunized with als3p vaccine plus alum as an adjuvant were protected against disseminated candidiasis (70). Vaccination primed Th1 and Th17 lymphocytes which resulted in neutrophil recruitment and activation at the site of infection and more effective clearance of *Candida albicans* from the tissues.

Antibodies

Anti-*Candida* antibodies artificially induced and administered to patients can be protective and might have the potential to be used as immunotherapy. This is evident from the literature that has investigated the use of antibodies directed against *Candida* in **experim**ental models and patients with candidiasis. Since production of antibodies against the pathogen-specific heat shock protein 90 (Hsp90) is associated with recovery from invasive candidiasis in mice and also in patients, (92, 93), efungumab, a human recombinant shock protein 90 (Hsp90) is associated with recovery from invasive candidiasis in mice and also in patients, (92, 93), efungumab, a human recombinant antibody directed against the fungal Hsp90, has been developed. Efungumab was investigated in a double-blinded randomized multicentre study of 139 patients with invasive candidiasis (94). Treatment with liposomal amphotericin B was compared with liposomal amphotericin B in combination with efungumab. In patients with invasive candidiasis the combined therapy produced significantly better clinical and culture-confirmed outcome. However, questions were raised regarding the methodology (95) and additional studies are needed to establish its potential. Recently, pre-clinical data supporting synergy between efungumab and caspofungin in the treatment of invasive candidiasis has been reported (96). Other approaches include the use of monoclonal antibodies (mAb) and immune serum from mice that were vaccinated with *Candida*-mannan containing liposomes, these provided protection in mice with disseminated candidiasis (97, 98). Mice treated with recombinant anti-mannan human antibody were more resistant to disseminated candidiasis (99). The synthetic glycopeptide vaccines can induce protective antibodies in experimental systemic candidiasis in mice (100). In addition, it is reported that β-glucan-conjugate vaccination results in anti-β-glucan antibodies, which are effective against experimental murine vaginal candidiasis (101).

Another approach is the use of idiotypic antibodies. *Candida albicans* is highly susceptible to the so called yeast killer toxin (YKT). The monoclonal KT4 antibody neutralizes the effects of YKT. An idiotypic antibody that is directed against KT4 mAb appears to mimic the biological function of YKT (102), and exerts anti-candidal effects and protects against mucosal and systemic experimental candidiasis (102, 103). Treatment with such antibodies that are directly effective against *Candida* may become a therapy for the immunocompromised host.

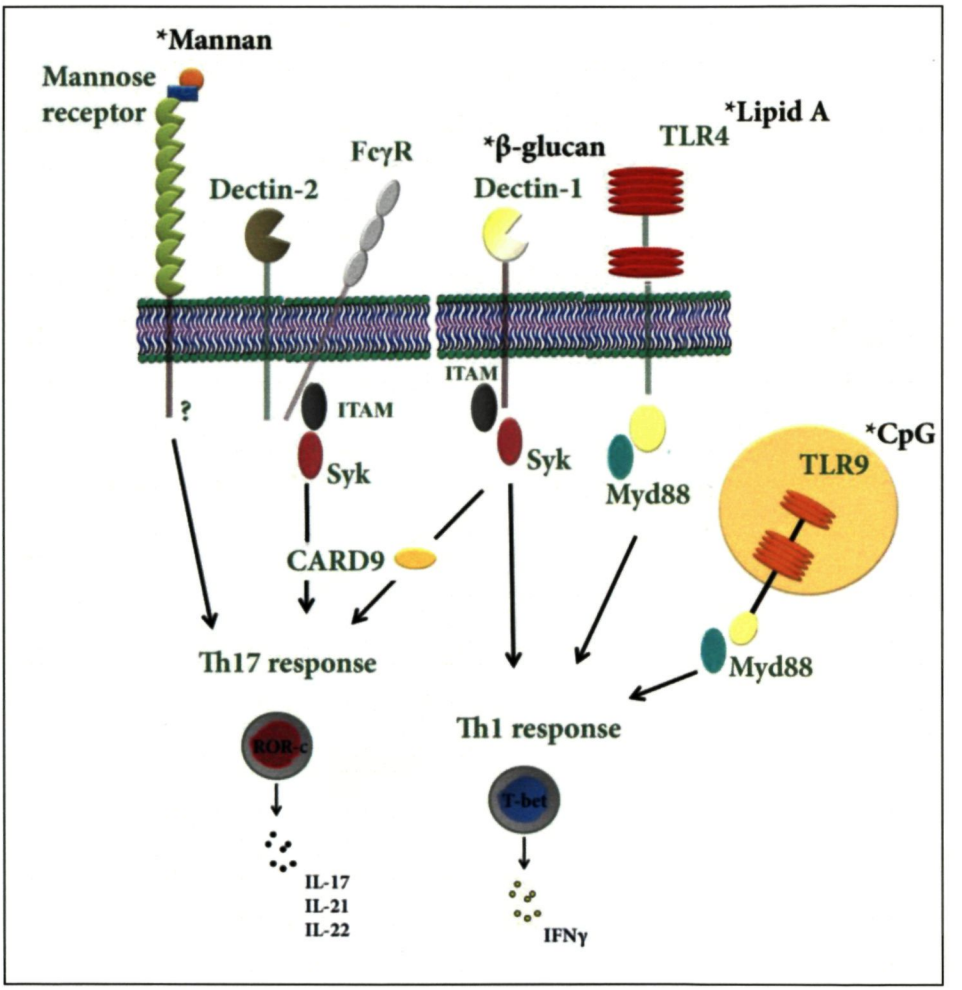

Figure 1. Adjuvants and induction of specific Th1 and or Th17 responses.
Overview of possible ligands (*) and their pathways that will result in a polarized T helper (Th) cell response. TLR=Toll like receptor; IL=interleukin; IFN=interferon; ROR-c=RAR-related orphan receptor C; T-bet= T-box expressed in T cells; Syk= Spleen tyrosine kinase; Myd88= Myeloid differentiation primary response gene (88), FcγR= Fcγ receptor.

Cytokine therapy

Cytokines are able to enforce host defense and therefore may be useful for immunomodulation during infections. GM-CSF accelerates hemopoiesis of myeloid cells, resulting in production of monocytes and neutrophils (104), and in pharmacological doses it leads to monocytosis and neutrophilia. It enhances phagocytosis and the release of ROS by PMNs (105), and prolongs the survival of neutrophils by inhibition of programmed cell death (106). It also upregulates dectin-1 expression on macrophages (107) and promotes fungicidal activity via upregulation of chititriosidase, which cleaves chitin present in the inner cell wall of *C. albicans* (108). In neutropenic mice with disseminated candidiasis, GM-CSF was shown to reduce lung damage and mortality (109). In two small studies in patients, Vazquez et al. investigated the use of granulocyte macrophage-colony stimulating factor (GM-CSF) as an adjunctive drug for clinically refractory mucosal candidiasis in patients with advanced AIDS. GM-CSF in combination with antifungal therapy appeared to lead to clinical and mycological improvement without adverse events (110, 111). Anecdotal reports in the literature of patients with disseminated candidiasis showed a favorable response to addition of GM-CSF (112, 113). A case report of a patient with a 17-year history of severe chronic mucocutaneous candidiasis who was treated with GM-CSF had a favorable response (114). Unfortunately, the patient had a severe anaphylactic reaction, which is an uncommon side effect of GM-CSF, and GM-CSF was stopped (114). Although these studies indicate that GM-CSF could be beneficial in the treatment of *Candida* infections, the experience is limited and controlled trials are lacking.

G-CSF is a hematopoietic growth factor that selectively promotes the proliferation and differentiation of neutrophils. Incubation of PMNs from healthy volunteers in vitro with G-CSF showed enhanced antifungal activity in damaging *Candida* pseudohyphae (115). G-CSF administered in humans also significantly enhanced PMN-mediated damage of *Candida* pseudohyphae (116). Furthermore, mice with disseminated candidiasis treated with recombinant G-CSF show significantly reduced mortality and lower fungal outgrowth (117-119); this benefit was less obvious during subacute or chronic candidiasis (117). In chronic gastrointestinal candidiasis in mice, G-CSF in combination with fluconazole did not show additional benefit over fluconazole alone in reducing the fungal burden (120). The first randomized placebo controlled trial addressing adjunctive immunotherapy in non-neutropenic patients with disseminated candidiasis, compared fluconazole alone with fluconazole and G-CSF. This phase 2 study indicated that administration of G-CSF is safe and showed a trend towards faster resolution of infection (121).

IFNγ is produced by T cells and NK cells and augments the cytotoxic function of macrophages and the killing of intracellular pathogens (122). IFNγ is also known to have activity on other important non-immune cells important in host defense, such as endothelial cells, epithelial cells and fibroblasts (123). Clinical experience with IFNγ therapy is greatest in patients with chronic granulomatous disease. In these patients it reduces the incidence of infections, including infections with *Aspergillus* (124). The effector mechanisms that are triggered by IFNγ are elusive. Several in-vitro and in-vivo studies support that IFNγ treatment is beneficial in the treatment of *Candida* infections. Various studies have shown that IFNγ increases anti-candidal function of macrophages (125-130). Other studies however were not able to show that IFNγ enhanced the capacity of murine macrophages (Marcil, *et al.*, 2002) or murine

pulmonary macrophages to kill *Candida* (131). Peritoneal and peripheral blood PMNs from IFNγ-treated mice showed enhanced killing of *Candida* (132). Incubation of human PMNs with IFNγ augments the capacity of PMNs to kill *Candida* (133). The administration of IFNγ reduces the fungal burden in mice with disseminated candidiasis (132). However, IFNγ failed to improve the efficacy of fluconazole in a murine model of experimental oral mucosal candidiasis (120). Although the literature is controversial regarding the role of IFNγ in anti-*Candida* host defense, a small study of three patients with disseminated candidiasis reported that additional IFNγ therapy was beneficial (112). Furthermore, administration of IFNγ in a HIV-infected patient with azole-resistent oropharyngeal candidiasis (OPC) resulted in a dramatic improvement (134). These case reports suggest that IFNγ could be beneficial as adjuvant antifungal therapy, but clinical trials are urgently needed to establish whether IFNγ is valuable in the treatment of *Candida* infections.

Adoptive transfer of primed immune cells

Another approach for antifungal immunotherapy would be the use of antigen primed dendritic cells (DCs) that are able to skew the adaptive immune response towards anti-*Candida* effector functions (135). Dendritic cells could be primed ex-vivo with antigens that induce specific cytokine profiles, and thereafter infused in the patient with *Candida* infection (136). It has been shown that Th1 dependent antifungal protection could be induced by DC vaccination in mice that received allogeneic bone marrow transplants (137). Furthermore, adoptive transfer of anti-*Candida* T cells has been proposed as potential immunotherapy in patients with *Candida* infection after hematopoietic stem cell transplantation (138). The generated human T cells were able to damage hyphal forms of *Candida* and significantly enhanced hyphal damage induced by human neutrophils. As the generated T cells do not seem to be affected by cryopreservation (138), there is the opportunity to generate anti-*Candida* T cells before the patients reach an immunocompromised status, and adoptively transfer these cells during infection when patients are immunocomprimised. Such new approaches to modulate the immune response offer elegant opportunities to enforce the immune system at the core of its failing anti-*Candida* defense mechanisms. However, the efficacy and potential adverse effects still have to be assessed in both animal models with experimental *Candida* infections, and ultimately in the patients.

Concluding remarks

Candida infections account for a high burden of morbidity and mortality. New therapeutic approaches are urgently needed to improve the outcome of the patients, as the currently available treatment options have not reduced the mortality and morbidity associated with *Candida* infections over the recent years. One solution would be the use of immunotherapy, which aims at improving host defense against *Candida*. The increase in understanding the mechanisms that underlie the pathogenesis of *Candida* infection brings the development of efficient and feasible immunotherapeutic strategies closer.

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Box 1. Standard treatment of *Candida* infections

Invasive candidiasis

- For neutropenic patients, patients with moderate to severe illness or hemodynamic instable patients, those with previous azole exposure, and/or patients that are at risk of infection by *C glabrata or C krusei*, echinocandins are recommended as primary treatment (24) Other patients may receive fluconazole treatment
- Once the patient is clinically stable, the *Candida* species is identified and its susceptibility is known, echinocandins may be switched to fluconazole or voriconazole and conversion to oral therapy should be considered
- Treatment should be continued for 2 weeks after the last positive blood culture If metastatic foci have occurred, treatment duration should be prolonged

Vulvovaginal candidiasis

- Successful treatment may be achieved with an oral or topical agent (2, 139), as these are equally effective (140) However, most often treatment does not prevent recurrences (1)
- Repeated treatment might select and induce drug resistance and a shift towards more resistant *Candida* species (141, 142)

Oropharyngeal candidiasis

- For mild disease topical therapy is recommended, for moderate to severe disease oral fluconazole is recommended (24)
- In HIV-infected patients, chronic suppressive therapy is usually unnecessary The use of highly active anti-retroviral therapy (HAART) is recommended to reduce recurrent infections (24)

Mucocutaneous candidiasis

- Fluconazole should be used as first line treatment in patients with chronic mucocutaneous candidiasis or hyper-IgE syndrome and most patients require chronic suppressive antifungal therapy (24) This is due to the extensive skin or nail involvement and the many relapses often seen in these patients

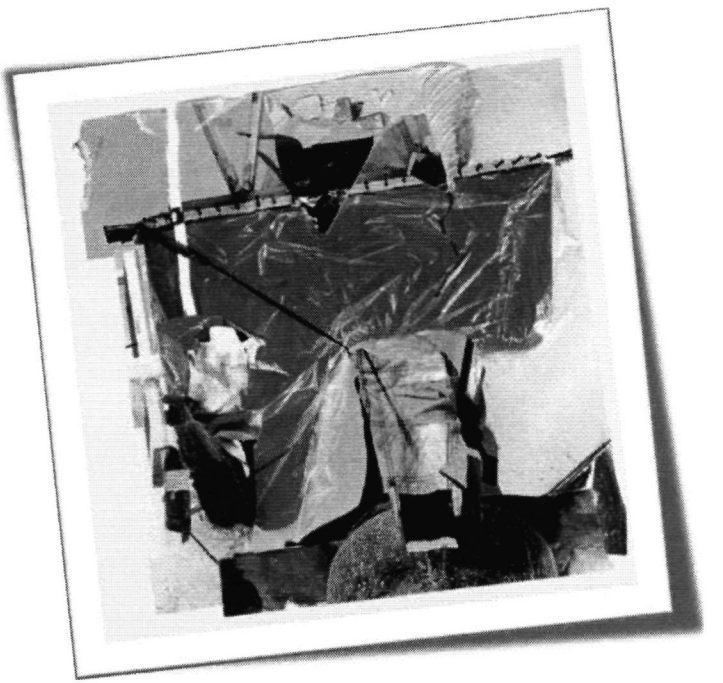
Table 1. Advantages and limitations of immunotherapeutic approaches.

Vaccination	- Broad spectrum activity - Low risk for the development of resistance - Long term effects	- Dependent on competence of host immune status; most patients with disseminated candidiasis are immune compromised - Might also induce disease-enhancing Ab - Relatively slow effects	Ribosomal cell fraction (D.561)	VVC (80)
			Diphtheria toxoid CRM 197 conjugated with Lam	VVC; disseminated candidiasis (81)
			Mannan protein conjugates	VVC; disseminated candidiasis (82)
			Adhesins Als1p and Als3p	Oropharyngeal candidiasis (84) ; disseminated candidiasis (85)
			Als3p conjugated with alum	Disseminated candidiasis (Lin, *et al.*, 2009)
			Live attenuated *Candida* strain CA2	Disseminated candidiasis (87)
Antibodies (Ab)	- Can be directly fungicidal independent of host immune status - Very specific (e.g. strain specific) - Rapid effects - Could be used as monotherapy	- May induce the development of anti-antibodies - Potentially toxic	Recombinant Ab against heat shock protein 90 (Efungumab)	Invasive candidiasis (94, 96)
			Anti-mannan Ab	VVC (82); Disseminated candidiasis (82, 97, 143)
			Anti-β-glucan Ab	VVC (101); disseminated candidiasis (81)
			Idiotypic antibodies (YKT neutralizing Ab KT4)	VVC (103); disseminated candidiasis (102)
Cytokines	- Experience with efficacy and safety in patients - Already on the market - Rapid effects	- Mainly effective as adjunctive therapy - Might influence the pharmacokinetics and efficacy of the combined antifungal drug - Inflammation as a potential consequence	GM-CSF	Refractory mucosal candidiasis (110, 111, 114); disseminated candidiasis (112, 113, 144)
			G-CSF	Disseminated candidiasis (121, 145, 146)
			IFNγ	Oropharyngeal (in HIV) candidiasis (134); disseminated candidiasis (112, 132)
Adoptive transfer of primed immune cells	- Opportunity to develop immune cells before immunocompromised status	- Risk for developing graft-versus-host disease after transplantation	Adoptive transfer of anti-*Candida* T cells or pulsed dendritic cells	Disseminated candidiasis (136, 138)

Ab=antibody; VVC=vulvovaginal candidiasis; G(M)-CSF=granulocyte (macrophage)-colony stimulating factor; IFN=interferon;

Chapter 19

Summary and conclusions



Innate pattern recognition of *Candida albicans*

The elementary function of the innate immune system is to recognize and eliminate pathogenic microorganisms. In **Chapter 2,** we gave an overview of the recognition of fungal pathogens by the innate immune system and proposed a model in which convergence and specificity shape the innate immune response. Fungal cell wall components are recognized by Toll like receptors (TLRs) and C-type lectin receptors (CLRs). Despite the fact that the signaling pathways of these receptors converge into a limited set of adaptor molecules and transcription factors, the innate immune response maintains its specificity by different mosaics of receptors that are stimulated by certain fungi, and by the complex interactions between the various pathways. In the end, this will lead to a tailored immune response to the invading microorganism.

In **Chapter 3** we have focused on the role of TLR9 in the host defense against *Candida*. TLR9 recognizes unmethylated CpG dinucleotides that are common in microorganisms but not in vertebrate DNA (1). Several reports suggested that TLR9 can recognize fungal DNA (2, 3). When mice deficient in TLR9 were infected intravenously with *Candida albicans*, we did not observe differences in mortality and fungal growth in the organs compared to control mice. Interestingly, the cytokine response of peritoneal macrophages of TLR9 deficient mice exposed to *Candida albicans* differed from that of wild-type macrophages. Human peripheral blood mononuclear cells also demonstrated an altered cytokine profile in response to *Candida albicans* when TLR9 was blocked. Most strikingly, we observed that IL-10 production was reduced in TLR9 deficient cells and TLR9 blocking, indicating that IL-10 production induced by *Candida albicans* is at least in part dependent on TLR9.
The fact that we found no significant difference susceptibility between TLR9 deficient mice and control mice does not necessarily imply that TLR9 is not important in anti-*Candida* host defense. This may be explanained by the presence of parallel signaling routes, as has been shown in studies with experimental *M. tuberculosis* and *Tryposoma cruzii* infections (4, 5). In these studies, TLR2 or TLR9 deficient mice were much less susceptible to experimental infection than mice deficient in both TLR2 and TLR9. This could also be the case for *Candida* infection, meaning that TLR9 deficiency may lead to a significantly increased susceptibility to *Candida* infection when there are additional immune defects. Since TLR9 in *Candida* infection is important for the anti-inflammatory cytokine IL-10, TLR9 deficiency combined with other defects might result in a strong pro-inflammatory condition during *Candida* infection that could be detrimental for the host. More research is needed to address these issues.

TLR1 and TLR6 can form heterodimers with TLR2 and recognize specific molecular patterns. TLR2/1 heterodimers recognize bacterial triacyl lipopeptides (6) and TLR2/6 heterodimers recognize diacyl lipopeptides and lipoteichoic acid (7). Although the role of TLR2 for the recognition of *Candida* has been studied earlier, nothing is known about TLR1 and/or TLR6 in anti-*Candida* host defense. In **Chapter 4** we investigated the role of TLR1 and TLR6 in anti-*Candida* host defense. Splenocytes isolated from TLR6 deficient mice with disseminated candidiasis showed an increased IFNγ production and a decreased IL-10 production in response to *Candida albicans*. In contrast, TLR1 deficiency did not result in a different cytokine profile. Although we observed a role for TLR6 in controlling the balance between Th1 and Th2 balance during fungal infection, it was redundant during disseminated

candidiasis in mice. Furthermore, TLR1 deficient mice were equally susceptible to disseminated candidiasis. However, because differences between murine and human TLRs are known to be present (8), our findings do not yet completely exclude a role of TLR1 or TLR6 in innate immunity to *Candida* infections in humans. Notably, a recent study suggested an association between the susceptibility to invasive aspergillosis and TLR6 and TLR1 polymorphisms (9). The potential role of TLR1 and TLR6 is currently being investigated in a large cohort of patients with invasive candidiasis.

The role of the inflammasome for the host defense against *Candida albicans*

Stimulation and release of pro-inflammatory cytokines is an essential step for the activation of an effective innate host defense, and subsequently for the modulation of adaptive immune responses. Interleukin-1β (IL-1β) and IL-18 are important pro-inflammatory cytokines that play a critical role in fungal infection (10, 11). On the one hand they activate monocytes, macrophages, and neutrophils, and on the other hand they induce adaptive cellular responses. IL-1β and IL-18 are secreted as inactive precursors, and the processing of pro-IL-1β and pro-IL-18 depends on cleavage by proteases. One of the most important enzymes that cleaves these cytokines is the serine protease caspase-1, which in turn is activated by several protein platforms called the inflammasomes (12).

In **Chapter 5** we demonstrate the dichotomy in the capacity of cells of the mononuclear phagocyte lineage to release active IL-1β (Figure 1). Monocytes possess a constitutively activated caspase-1. Therefore, a single stimulatory event with a TLR ligand will lead to the release of active IL-1β. By contrast, macrophages need two distinct signals: one signal that induces transcription and translation, and a second signal that activates caspase-1. These two signals will finally result in IL-1β processing and secretion. The demonstration that monocytes have a constitutive activation of caspase-1, uncouples the necessity for human primary monocytes to activate caspase-1 by pathogen-associated molecular pattern recognition. This likely represents an adaptation of the monocyte and the macrophage to their respective environment. Circulating monocytes function in the surveillance of an essentially pathogen-free environment, i.e., the bloodstream, so they must respond promptly to any danger signal. Macrophages are confined to an environment (e.g. alveolar space, mucosal surfaces) in which they are constantly exposed to microbial stimuli and danger signals. A sensitive response to release IL-1β for each encounter with such stimuli would result in chronic deleterious inflammatory reactions. This can be prevented by the requirement of a second signal for the activation of the inflammasome and release of active IL-1β. Such a second signal would be available at sites of infection, trauma or necrosis, sites where ATP levels are elevated and trigger the P2X7 receptor.

The pathways that are important for IL-1β production induced by *Candida albicans* were investigated in **Chapter 6**. We demonstrate that the mannose receptor pathway and the TLR2/dectin-1 pathway, but not TLR4 or TLR9 are necessary for the induction of IL-1β production in response to *Candida albicans*. In our investigations into the role of the inflammasome, we confirmed that caspase-1 was crucial for IL-1β production in monocytes. But surprisingly, the enzyme was constitutively activated in monocytes. Next, we explored whether NLRs (Ipaf and Nlrp3) are important for IL-1β production induced by *Candida albicans*. We found no difference in IL-1β production between macrophages deficient in

either Nlrp3 or Ipaf when they were exposed to *Candida albicans*. Thus these data cast doubt on the role of the pathogen-mediated inflammasome activation in *Candida* infection.

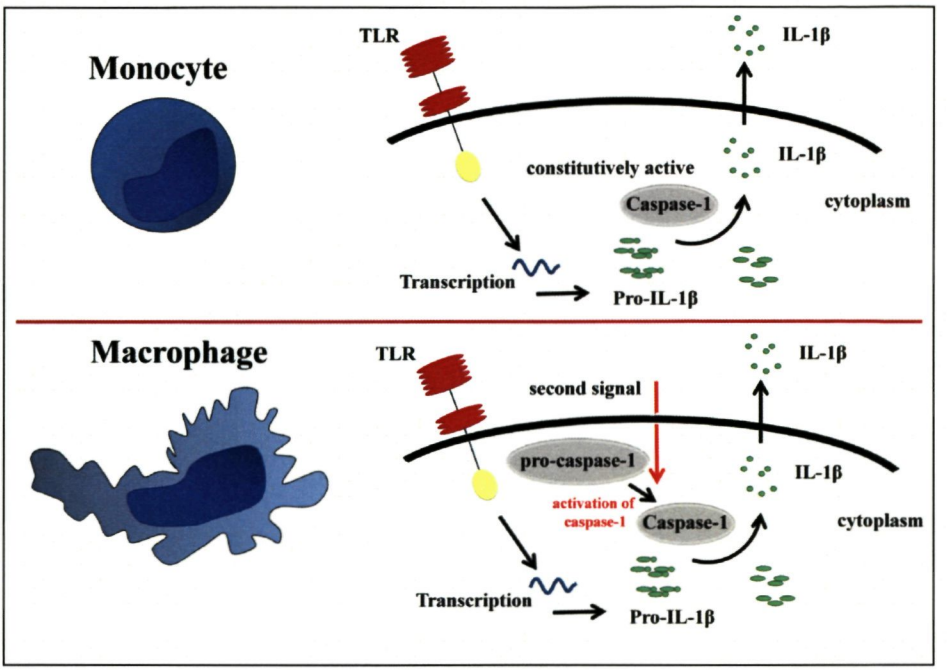


Figure 1. Differential IL-1β secretion pathways in monocytes and macrophages.
Caspase-1 is constitutively active in monocytes, and these cells release mature IL-1β after single stimulation with a TLR ligand. In contrast, macrophages need two signals: one signal such as a TLR-ligand that induces transcription, and a second signal that induces inflammasome activation, IL-1β processing and secretion.

Shortly after the data in Chapter 6 were published, additional studies appeared in the literature that suggested a protective role of the Nlrp3 inflammasome in *Candida* infection (13-16). These studies reported that Nlrp3 was essential for host defense against Candida. In **Chapter 7** we addressed the role of Nlrp3 in a murine model of disseminated candidiasis. In contrast to the studies mentioned (13-16), we found no role for Nlrp3 in disseminated candidiasis. Notably, our observation that Nlrp3 is not essential during disseminated candidiasis underscores the argument that IL-1β processing during disseminated *Candida* infection does not require pathogen-mediated inflammasome activation. It is of course possible that the use of Nlrp3 deficient mice generated in different institutes, and infection with different *Candida* strains could explain the contradictory observations found in our study and that of other studies. The discrepancy between these studies is not well understood and merits further investigation.

In **Chapter 8** we studied the inflammasome components ASC, caspase-1 and P2X7 (the ATP receptor) in mice with disseminated candidiasis. Caspase-1 and ASC, but not P2X7, were essential for anti-*Candida* host defense. Mice with disseminated candidiasis that were deficient in caspase-1 or ASC showed a reduced survival rate and higher fungal loads in the kidneys than infected control mice. Interestingly, we observed that caspase-1 and ASC were

essential for protective Th1 and Th17 responses, which implies a novel role for the inflammasome. These results indicate that the innate immune system controls an important aspect of adaptive immunity by controlling the pro-inflammatory cytokines IL-1β and IL-18. The vaccine adjuvant alum activates the inflammasome and thereby caspase-1. Interestingly it was recently shown that vaccination with Als3p (a *Candida* antigen) together with alum as a vaccine adjuvant, induced protective adaptive immunity in mice with disseminated candidiasis through the induction of Th1 and Th17 responses (17). It is tempting to speculate that alum induces or boosts such protective Th1 and Th17 responses through the activation of caspase-1. The way of how alum is able to shape the antigen- induced adaptive immune response is currently under investigation.
We observed an unexpected role for ASC in disseminated candidiasis. The kidneys of ASC-deficient mice infected with *Candida albicans* displayed large numbers of infiltrating neutrophils, and this was neither observed in the other knockout mice nor in control mice. In addition, ASC-deficient splenocytes produced large amounts of TNF in response to *Candida albicans*. These findings suggest an important role for ASC in the control of TNF-mediated inflammation; apparently ASC also acts in an inflammasome-independent manner during infection, and this definitely needs further investigation.

Th17 and antifungal host defense

The Th17 lymphocyte subset is characterized by the production of IL-17 and thereby leads to the recruitment of neutrophils (18). The discovery of this subset has provided new insights into the pathogenesis of autoimmune diseases and host defense against infections. In **Chapter 9** we describe the delicate balance between the protective and potentially harmful effects of Th17 responses in infection. On the one hand, Th17 cells provide protective host defense against bacterial and fungal infections by inducing production of defensins and recruitment of neutrophils to the site of infection. On the other hand, Th17 responses seem to contribute to viral persistence in viral infection and may be detrimental for the host in the chronic inflammation of parasitic infection. These insights need to be taken into account if one considers to exploit the Th17 responses in immunomodulatory therapy.

Chapter 10 describes the main pathways for the Th17 response induced by *Candida*. We discovered that *Candida albicans* evokes a robust IL-17 response in human peripheral blood mononuclear cells and could demonstrate that interaction between the mannoproteins on the surface of *Candida albicans* and the mannose receptor on monocytes/macrophages is essential for the induction of IL-17. Stimulation of the TLR2/dectin-1 pathway by itself does not lead to induction of IL-17. However, the TLR2 and dectin-1 pathways do have the capacity to amplify IL-17 production induced by the mannose receptor. These findings are in line with the results presented in Chapter 6, where we we demonstrate that the mannose receptor and TLR2/dectin-1 pathways are needed for IL-1β production induced by *Candida albicans*. IL-1β in turn is required for the Th17 response in humans. This study underscores the dominant role of saccharides (such as mannoproteins and glucans) and C-type lectins in initiating Th17-mediated host defense and inflammation. To pull this further, we tend to hypothesize that the protein parts of the mannoproteins of *Candida albicans* are processed and presented at the surface of the antigen-presenting cells and subsequently trigger the T cell receptor and activate the T cell, while the mannan parts are responsible for inducing a

cytokine profile that polarizes the immune response towards a Th17 response. We are currently investigating this hypothesis.

Prostaglandin E2 (PGE2) contributes to Th17 responses in experimental models of autoimmune disease (19). Since we found a robust IL-17 production in human cells exposed to *Candida albicans,* we explored the contribution of PGE2 to the Th17 response induced by *Candida* (**Chapter 11**). The addition of a non-steroidal anti-inflammatory drug (NSAID) completely blocked PGE2 production and resulted in decreased IL-17 production when cells were stimulated with *Candida albicans*. Mannoproteins of *Candida albicans* and the mannose receptor appeared to have a prominent role in the production of PGE2. In addition, TLR2 synergizes with dectin-1 in the production of PGE2. Notably, IL-1β is not affected by blocking PGE2 and is probably responsible for the observed residual IL-17 production in the absence of PGE2. It is tempting to speculate that the use of an NSAID can reduce Th17 responses in patients, and in this way may ameliorate detrimental inflammation. This may be one explanation for the beneficial effects of NSAIDs in a chronic inflammatory disease such as rheumatoid arthritis.

It has been suggested that the IL-17 response may be deleterious in fungal infection as it leads to an overwhelming neutrophil-mediated inflammation (20). Evidence for this was derived from experimental candidiasis and aspergillosis in animals (21). In other animal studies of mucosal and disseminated candidiasis a clear protective role of IL-17 responses was seen(22, 23). In **Chapter 12,** we addressed this controversy, and investigated the role of IL-17 in two different mouse models, namely one of invasive candidiasis and another of zymosan-mediated multi-organ failure. We observed that IL-17 receptor knockout animals were more susceptible to disseminated candidiasis than control mice and had equal survival rates to control mice in zymosan-induced multi-organ failure. These findings demonstrate that IL-17 does not contribute greatly to the inflammatory process that leads to organ failure in fungal sepsis, and support the concept that the IL-17 pathway is protective in antifungal host defense.

In **Chapter 13** we assessed the role of Th17 responses in host defense against *Aspergillus*. In contrast to the high IL-17 production seen with human peripheral blood mononuclear cells stimulated with *Candida albicans*, we did not observe any IL-17 production when human cells were exposed to *Aspergillus fumigatus*. Furthermore, we found very low IL-17 concentrations in the bronchoalveolar lavage fluid and serum of patients with invasive aspergillosis. This is in sharp contrast with the data obtained from the experimental *Aspergillus* infection models (20, 21). Interestingly, we observed that *Aspergillus* is a potent inducer of IFNγ production, which is the prototypical cytokine of the Th1 response. Since IFNγ is known to suppress Th17 responses (24), we explored whether the strong Th1 responses induced by *Aspergillus* were responsible for the observed low IL-17 production. We found no difference in the capacity of *Aspergillus* to induce IL-17 when we blocked IFNγ, making it unlikely that the low IL-17 is due to a strong Th1 response. It has been suggested that the tryptophan metabolite L-kynurenine is able to dampen fungal *Aspergillus*-induced IL-17 production (20). Interestingly, we found that *Aspergillus* itself is able to convert tryptophan into L-kynurenine, and that the supernatant of live *Aspergillus* has the capacity to decrease IL-17 production by mitogen-activated T cells. Altogether, it is likely that Th17 responses are not a major factor in host defense against *Aspergillus* infection. This is also in

line with the clinical picture of patients with hyper IgE syndrome who completely lack Th17 responses, yet they rarely display invasive infections with *Aspergillus*.

The inflammasome/Th17 axis in clinical syndromes

Patients with chronic granulomatous disease (CGD) have a defect in the NADPH oxidase system and as a consequence cannot generate NADPH-dependent reactive oxygen species (ROS) (25). Interestingly, NADPH-dependent ROS are reported to be necessary for inflammasome activation (26). Therefore, the loss of ROS would theoretically result in defective inflammasome activation, decreased caspase-1 activation, less IL-1β processing and ultimately decreased IL-1β production. This would be in line with the generally assumed pro-inflammatory role of ROS. However, patients with CGD (as well as CGD mice) have a pro-inflammatory status, which to date is still poorly understood (20, 27). To address the role of NADPH-dependent ROS in the activation of the inflammasome, we studied peripheral blood mononuclear cells isolated from patients with CGD in **Chapter 14**. Strikingly, we observed that cells isolated from CGD patients produced greater amounts of IL-1β in response to stimuli. When the caspase-1 status was assessed, it was found to be more activated in CGD cells than in control cells. In contrast to existing idea, these data show that the absence of NADPH-dependent ROS results in increased inflammasome activation and would explain why patients with CGD have an inflammatory phenotype.

B lymphocyte-depleting therapy with rituximab has been unexpectedly beneficial in T cell-mediated autoimmune diseases such as multiple sclerosis (28). The efficacy of rituximab in rheumatoid arthritis also raised a lot of questions, since there was no clear effect on the presence of autoantibodies and the clinical beneficial effects were observed faster than one would expect from the rate of disappearance of antibodies (29). We realized that Th17 cells have been implicated in the pathogenesis of multiple sclerosis and rheumatoid arthritis (30) and hypothesized that B cells are important for the Th17 response. An answer to this hypothesis is provided in **Chapter 15.** Twelve patients with rheumatoid arthritis were treated with rituximab. The treatment resulted in efficient B cell depletion in vivo and led to a statistically clinical improvement in these patients. Before and 12 weeks after treatment we obtained synovial biopsies from affected knee joints. We were able to demonstrate that the Th17 response was reduced after treatment with rituximab. Staining of IL-17 in the inflamed synovium was significantly decreased after treatment. We next addressed the question whether rituximab specifically inhibited the Th17 response or inhibited all T cell responses. No effects on Th1 or Treg responses were observed. In addition, we found no effect of rituximab on TNF responses indicating that the clinical improvement was not due to effects on TNF production. To obtain a better understanding of how rituximab inhibits the Th17 response we used the in-vitro model of *Candida albicans* stimulation. Cells stimulated with *Candida albicans* produced significantly less IL-17 and IL-22 when rituximab was added to the culture. We entertained the possibility that rituximab could directly deplete T cells, since it has been reported that a subset of T cells expresses CD20 (31). We observed no CD20 expression on T cells that also expressed IL-17. Moreover, in cells isolated from patients with agammaglobulinemia, who do not have functional B cells, the inhitory effect of rituximab on the Th17 response was lost, supporting a direct effect of rituximab on B cells. Thus we demonstrated an unexpected role for B cells in the specific regulation of the Th17 response, and this may explain why rituximab works in multiple sclerosis and rheumatoid

arthritis. These findings may also have important implications for the development, research, and treatment of Th17 mediated diseases.

Patients with hyper IgE syndrome (HIES) are particularly prone to infections with *Candida albicans* and *Staphylococcus aureus* (32). It is reported that some 60% of patients with HIES have mutations in STAT3 and as a consequence have a defective Th17 response (33). In **Chapter 16** we describe three patients with HIES from the same family with a STAT3 mutation in the linker domain; interestingly these patients are phenotypically different from sporadic HIES patients. The most striking feature is that they are not highly susceptible to *S. aureus* pneumonia, which is a common feature in patients with HIES (34). We found that cells isolated from the patients of the HIES family had residual IL-17 production upon stimulation with *S. aureus*, which is in contrast with the absolute defect of IL-17 production in the sporadic HIES patients that were reported previously (33). These data suggest that the IL-17 response is particularly critical for host defense against *S. aureus* at the level of the lung epithelium. In addition, all HIES patients with mucocutaneous candidiasis had defective IL-17 production in response to *Candida albicans*, underscoring the important role for Th17 cells in host defense against mucosal candidiasis. These HIES patients also suggest that different STAT3 mutations result in clinically variant phenotypes of HIES. Moreover, the mutation in the linker domain of STAT3, which has not been described before, resulted in normal intracellular levels of IL-17 after mitogenic stimulation demonstrated by FACS analysis, but ultimately lower IL-17 concentrations in the supernatants when cells were exposed to relevant microbial stimuli. These data underscore a direct relationship between the degree of the defect in the Th17 response and the severity of the clinical picture in patients with hyper IgE syndrome.

In **Chapter 17**, we demonstrate that autosomal dominant chronic mucocutaneous candidiasis (AD-CMC) is due to mutations in the CC domain of the *STAT1* gene. We had found that CMC patients have a deficient production of Th1 and Th17 cytokines in response to *Candida*, and the IL-12 and IL-23 pathway are both functionally compromised in cells isolated from patients with CMC. With next-generation sequencing we analyzed genes that coded for the molecules shared by the IL-12 and IL-23 pathway and genes involved in the differentiation of the Th17 response. Interestingly, all patients from 5 AD-CMC families from the Netherlands and the United Kingdom were positive for a mutation in the CC domain of *STAT1*, whereas no mutations have been found in unaffected siblings and 162 healthy controls. Three families carried the A267V mutation (one Dutch and two UK families), and two families carried the R274W mutation (one Dutch and one from the UK). Interestingly, in the three families with A267V mutation, several patients with CMC also suffered from oral/oesophagal carcinoma indicating a role for STAT1 in the pathogenesis of oral/oesophagal carcinoma. This is in line with previous reports linking STAT1 deficiency to oesophageal carcinoma. Furthermore, the two families with the R274W mutation included CMC patients that also had autoimmune phenomena, such as autoimmune hepatitis and autoimmune haemolytic anemia. The role of STAT1 in the pathogenesis of oral and oesophageal cancer and autoimmune diseases merits further investigation. This study shows the power of next generation sequencing technologies combined with well-designed and robust functional assays to elucidate the pathogenesis of hereditary diseases.

It is unlikely that the current treatment options will significantly reduce the morbidity of mucosal *Candida* infection and the high mortality associated with disseminated candidiasis. This underscores the need to develop new treatment strategies in the fight against *Candida* infection. **Chapter 18** discusses the present knowledge that has contributed to approaching alternatives such as vaccination, antibodies, cytokine therapy or adoptive transfer of primed immune cells, which all are promising, but yet have to be demonstrated to be safe and effective. It also points out that it is necessary to address controversies such as the role of the Th17 response in antifungal host defense, since it is essential to know whether augmentation or inhibition of the Th17 response would be more likely to be beneficial in patients with fungal infection.

General conclusions

By understanding the failing defense mechanisms that underlie the occurrence of a specific *Candida* infection, we are on the edge of developing immunotherapeutic strategies that will support standard treatment regiments in patients with candidiasis. The experiments performed in this thesis aim to contribute to the large and dynamic field of research in immunology that brought us where we are today. Many challenges lie ahead: understanding the molecular mechanisms by which mutations in the CC domain of STAT1 lead to Th1 and Th17 deficiency in autosomal dominant chronic mucocutaneous candidiasis or the reasons why patients with chronic granulomatous disease are especially susceptible to *Aspergillus* infection. These are but a few examples that will finally contribute to the understanding of antifungal host defense. Moreover, the role of the immune system stretches out far beyond host defense against infection into virtually every area of the medical sciences. By combining the knowledge of the different areas of research, we will move forward in our appreciation and understanding of the full magnitude of the importance of the immune system and its unknown functions. This will help us in the development of strategies that can modulate the immune response during health and disease in such a way that it will be valuable for a better prevention and treatment of infections in general, and fungal infections in particular.

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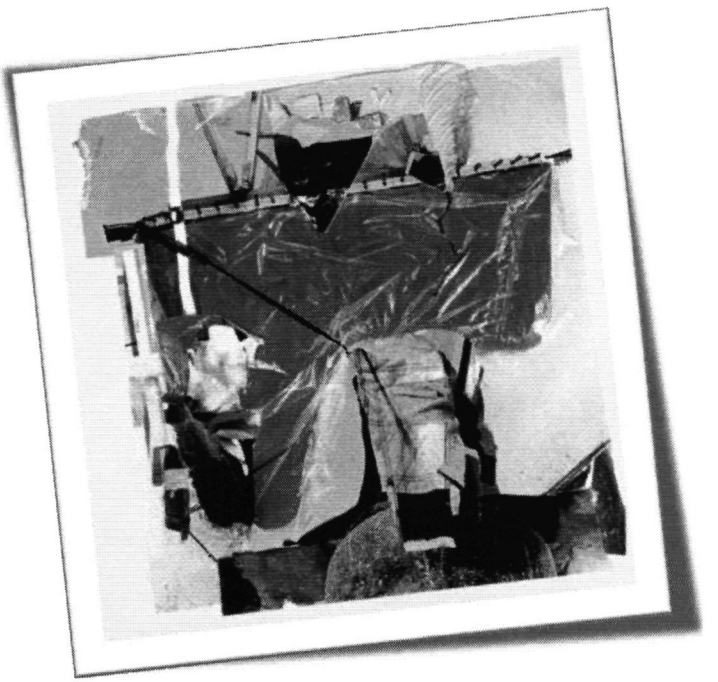


Nederlandse samenvatting

Aangeboren afweer tegen *Candida albicans*

Een elementaire functie van het aangeboren afweersysteem is het herkennen van ziekteverwekkende micro-organismen opdat ze vervolgens geëlimineerd kunnen worden. In **hoofdstuk 2** geven we daarom een overzicht over hoe schimmels worden herkend door het aangeboren afweersysteem en beschrijven we een model dat verklaart hoe het aangeboren afweersysteem specifiek op een ziekteverwekker kan reageren. De celwand componenten van schimmels worden herkend door Toll-like receptoren (TLR's) en C-type lectine receptoren (CLR's). Ondanks het feit dat de signaalwegen van deze receptoren convergeren tot een beperkt aantal adapter moleculen en transcriptiefactoren, kan het aangeboren afweersysteem zijn specificiteit behouden doordat meerdere signaalwegen tegelijkertijd kunnen worden gestimuleerd en er complexe interacties zijn tussen deze verschillende signaalroutes. Uiteindelijk zal dit leiden tot een op maat gesneden afweerreactie gericht tegen het binnendringende micro-organisme.

In **hoofdstuk 3** hebben we de rol van TLR9 bij de herkenning van en de afweer tegen de schimmel *Candida* onderzocht. TLR9 herkent ongemethyleerde CpG dinucleotiden die voorkomen in DNA van micro-organismen, maar niet in de mens (1). Verschillende studies hebben gesuggereerd dat TLR9 het DNA van schimmels kan herkennen (2, 3). Om de rol van TLR9 in systemische *Candida* infecties te bestuderen hebben we TLR9 deficiënte muizen intraveneus geïnfecteerd met *C. albicans*. We zagen geen verschil in de vatbaarheid voor *Candida* infectie in TLR9 deficiënte muizen ten opzichte van de controle groep. Echter, de cytokine reactie van TLR9 deficiënte macrofagen die werden blootgesteld aan *C. albicans* verschilde wel van die van controle macrofagen. Humane perifere bloed mononucleaire cellen vertoonde ook een afwijkend cytokine profiel als ze gestimuleerd werden met *C. albicans* in de aanwezigheid van een TLR9 blokker. Het meest opvallende was dat de interleukine (IL)-10 productie was verminderd in TLR9 deficiënte cellen en in de humane cellen waarbij TLR9 was geblokkeerd. Dit geeft aan dat de IL-10 productie geïnduceerd door *C. albicans* ten minste gedeeltelijk afhankelijk is van TLR9.

Het feit dat we geen significant verschil vonden in de gevoeligheid tussen TLR9 deficiënte muizen en controle muizen betekent niet noodzakelijkerwijs dat TLR9 niet belangrijk is voor een optimale afweerreactie tegen *C. albicans*. Dit kan worden verklaard door observaties die zijn beschreven in studies met experimentele *Mycobacterium tuberculosis* en *Tryposoma cruzii* infecties (4, 5). In deze studies waren TLR2 of TLR9 deficiënte muizen veel minder gevoelig voor een experimentele infectie dan muizen die deficiënt waren voor zowel TLR2 en TLR9. Dit zou ook kunnen gelden voor systemische *Candida* infecties, wat betekent dat een TLR9 tekort kan leiden tot een verhoogde gevoeligheid voor systemische *Candida* infecties mits er ook nog andere immunologische defecten bestaan. Omdat TLR9 belangrijk is voor de productie van het anti-inflammatoire cytokine IL-10, kan TLR9 deficiëntie, in combinatie met andere afwijkingen, leiden tot een sterke pro-inflammatoire reactie tijdens een systemische *Candida* infectie die uiteindelijk nadelig kan zijn voor de gastheer. Meer onderzoek is nodig om deze observaties verder te bestuderen.

TLR1 en TLR6 kunnen heterodimeren vormen met TLR2. Deze heterodimeren herkennen

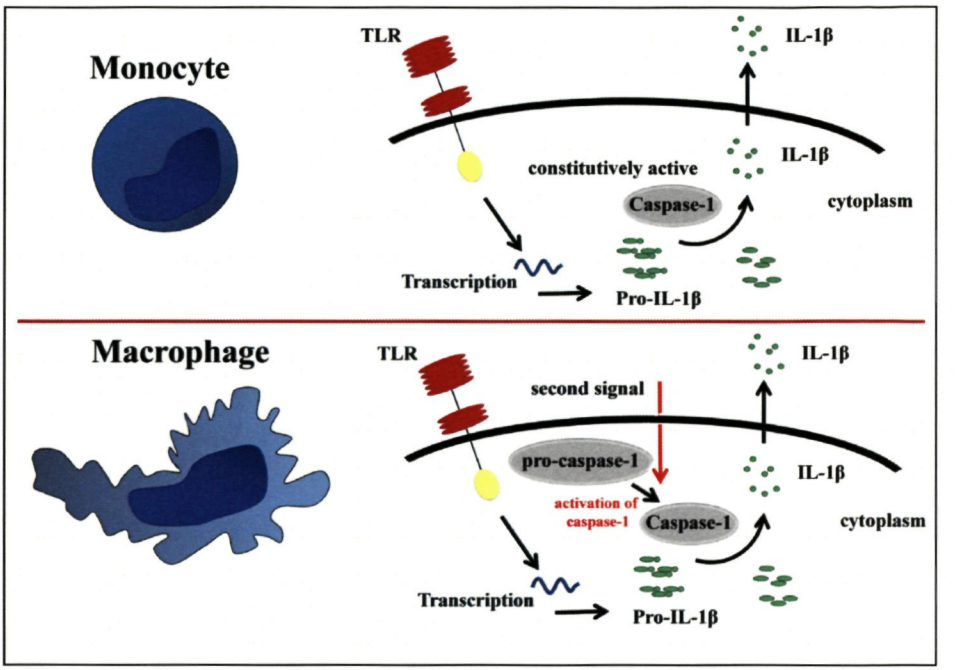
specifieke moleculaire patronen. TLR2/1 heterodimeren herkennen bacteriële triacyl lipopeptiden (6) en TLR2/6 heterodimeren herkennen diacyl lipopeptiden en lipoteichoïnezuur (7). De rol van TLR2 in de herkenning van *Candida* is al eerder onderzocht, echter er is niets bekend over de functies van TLR1 en/of TLR6 in de afweerreactie tegen *Candida*. In **hoofdstuk 4** beschrijven we de rol van TLR1 en TLR6 in invasieve candidiasis. Miltcellen van TLR6 deficiënte muizen met een systemische *Candida* infectie vertoonden een toegenomen IFNγ productie en een verminderde productie van IL-10 wanneer ze gestimuleerd werden met *C. albicans*. Miltcellen met een TLR1 deficiëntie vertoonden geen veranderd cytokine profiel na stimulatie met *C. albicans*. Alhoewel we vonden dat TLR6 een rol speelde in de beheersing van het evenwicht tussen Th1 en Th2 reacties, bleek TLR6 overbodig te zijn voor het herstel van muizen met een systemische *Candida* infectie. TLR1 deficiënte muizen waren ook even vatbaar voor systemische candidiasis als controle muizen. Omdat er verschillen tussen muis en mens beschreven zijn met betrekking tot TLR functie in vivo (8), sluiten onze bevindingen niet uit dat TLR1 en TLR6 toch een belangrijke rol kunnen spelen in de aangeboren immuniteit gericht tegen *Candida* bij de mens. Een recente studie suggereerde een verband tussen de gevoeligheid voor invasieve aspergillose en TLR6 en TLR1 polymorfismen (9). De potentiële rol van TLR1 en TLR6 polymorfismen wordt momenteel onderzocht in een groot cohort van patiënten met invasieve candidiasis.

De rol van het inflammasoom in de afweer tegen *Candida albicans*

Pro-inflammatoire cytokinen spelen een belangrijke rol in de activering van de aangeboren afweerreactie, en zorgen tevens voor de modulatie van de verworven immuniteit. Interleukine-1β (IL-1β) en IL-18 zijn belangrijke pro-inflammatoire cytokinen voor de afweer tegen schimmelinfecties (10, 11). Enerzijds activeren ze monocyten, macrofagen, en neutrofielen, en anderzijds induceren ze een beschermende verworven cellulaire respons. IL-1β en IL-18 worden in eerste instantie geproduceerd als inactieve precursor pro-IL-1β en pro-IL-18. Om actief te kunnen worden, moeten deze percursor moleculen eerst "geknipt" worden. Deze splitsing van pro-IL-1β en pro-IL-18 is afhankelijk van proteasen. Een van de belangrijkste enzymen die deze percursors splitst is het serine protease caspase-1, dat op zijn beurt weer geactiveerd wordt door een platvorm van eiwitten die tezamen het inflammasoom worden genoemd (12).

In **hoofdstuk 5** beschrijven we het verschil tussen monocyten en macrofagen met betrekking tot de activering van het inflammasoom en IL-1β productie (Figuur 1). Monocyten hebben een constitutief geactiveerd caspase-1. Daardoor kan een stimulatie met een enkelvoudig TLR ligand direct leiden tot het vrijkomen van actief IL-1β. Macrofagen hebben twee verschillende signalen nodig om actief IL-1β te produceren: een signaal dat de transcriptie van IL-1β induceert, en een tweede signaal (exogeen) dat leidt tot caspase-1 activering. Omdat monocyten een constitutief geactiveerd caspase-1 hebben, is in deze cellen activering van caspase-1 door exogene gevaar signalen niet nodig. Dit verschil tussen monocyten en macrofagen is te beschouwen als een vorm van aanpassing van deze cellen aan hun omgeving. Circulerende monocyten bevinden zich in de bloedbaan en bewaken zo een omgeving die in wezen vrij is van ziekteverwekkers. Monocyten moeten dus snel kunnen reageren op elk signaal van gevaar. Macrofagen komen in weefsel voor (bijvoorbeeld alveoli in de long, slijmvlies van het gastro-intestinale systeem) waar ze voortdurend worden blootgesteld aan microbiële stimuli en gevaar signalen. Als een

enkelvoudige prikkel in deze situatie direct zou leiden tot de productie van actief IL-1β zou dit kunnen resulteren in een schadelijke chronische ontstekingsreactie. De noodzaak van een tweede signaal om uiteindelijk actief IL-1β te kunnen produceren, is aldus een logisch veiligheidsmechanisme om chronische inflammatie te voorkomen.



Figuur 1. Inflammasoom activatie in monocyten en macrofagen.
Caspase-1 is constitutief actief in monocyten, en actief IL-1β kan direct na een enkelvoudige stimulatie met een TLR ligand geproduceerd worden. Macrofagen hebben echter twee signalen nodig: een eerste signaal, zoals een TLR ligand dat de transcriptie van IL-1β induceert, en een tweede signaal dat leidt tot de activering van het inflammasoom en vervolgens IL-1β productie.

Het onderzoek in hoofdstuk 6 beschrijft de routes die betrokken zijn bij de IL-1β productie die door *C. albicans* geïnduceerd wordt. We hebben aangetoond dat de mannose-receptor en de TLR2/dectin-1 route, maar niet de TLR4 of TLR9 route, nodig zijn voor de inductie van IL-1β productie. Caspase-1 was belangrijk voor de IL-1β productie door monocyten en we vonden tevens dat caspase-1 constitutief geactiveerd was in monocyten. Vervolgens hebben we onderzocht of de Nod like receptoren (NLR's) (IPAF en NLRP3) van belang waren voor de IL-1β productie die wordt veroorzaakt door *C. albicans*. We vonden geen verschil in IL-1β productie in macrofagen die deficiënt waren voor NLRP3 of IPAF als ze werden blootgesteld aan *C. albicans*. Deze gegevens werpen een nieuw licht op de rol van het inflammasoom tijdens infecties.

Kort nadat de gegevens in hoofdstuk 6 werden gepubliceerd, verschenen er in de literatuur aanvullende studies die een beschermende rol van het NLRP3 inflammasoom in de afweer tegen *C. albicans* rapporteerden (13-16). Deze studies vonden dat NLRP3 essentieel was voor de afweer tegen experimentele *Candida* infectie in muizen. In **hoofdstuk 7** hebben wij de rol

van NLRP3 daarom nader bestudeerd in een muismodel van systemische candidiasis. In tegenstelling tot de andere studies (13-16), vonden wij dat NLRP3 géén belangrijke rol had bij candidiasis. Dit ondersteunt het argument dat IL-1β tijdens systemische infecties niet geheel afhankelijk is van pathogeen geïnduceerde inflammasoom activering. Het is natuurlijk mogelijk dat deze tegenstrijdige waarnemingen verklaard kunnen worden door gebruik van NLRP3 deficiente muizen afkomstig van verschillende instituten, of door infecties met verschillende *Candida* stammen. Echter, de discrepantie tussen onze waarnemingen en de andere studies is niet goed opgehelderd en behoeft nader onderzoek.

In **hoofdstuk 8** onderzochten we hoe belangrijk de inflammasoom componenten ASC, caspase-1 en P2X7 (de ATP-receptor) zijn in de afweer tegen systemische *Candida* infecties. Caspase-1 en ASC, maar niet P2X7, waren essentieel voor een goede afweer tegen systemische candidiasis. Muizen die deficient waren in caspase-1 of ASC vertoonden een verhoogde vatbaarheid voor een systemische *Candida* infectie. We vonden dat caspase-1 en ASC belangrijk waren voor een beschermende reactie van het verworven afweersysteem door Th1 en Th17 cellen. Deze resultaten laten zien dat het aangeboren afweersysteem ook een belangrijke rol speelt in het moduleren van het verworven afweersysteem door middel van de regulatie van IL-1β en IL-18.

Het vaccin adjuvant alum activeert het inflammasoom en dus caspase-1. Recentelijk is aangetoond dat vaccinatie met Als3p (een *Candida*-antigeen) samen met alum, beschermend werkt in muizen met een systemische *Candida* infectie. Deze beschermende afweerreactie was afhankelijk van een vaccin geïnduceerde Th1 en Th17 reactie (17). Het is verleidelijk om te speculeren dat alum deze beschermende Th1 en Th17 reacties induceert door caspase-1 te activeren. Het mechanisme hoe alum een verworven afweerreactie kan beïnvloeden wordt momenteel onderzocht.

We vonden een onverwachte functie van ASC in muizen met een systemische candidiasis. De nieren van ASC deficiente muizen die geïnfecteerd waren met *Candida* vertoonden grote infiltraten die bestonden uit neutrofielen. Dit werd niet waargenomen in de NALP3 en caspase-1 deficiënte muizen, noch in controle muizen. ASC deficiënte miltcellen produceerden bovendien grote hoeveelheden TNFα na stimulatie met *C. albicans*. Deze bevindingen suggereren dat ASC een belangrijke rol speelt in in de regulatie van TNFα afhankelijke ontstekingsreacties. Blijkbaar heeft ASC ook een inflammasoom onafhankelijke functie tijdens infectie en dit verdient zeker verder onderzoek.

Th17 en anti-schimmel afweer

Th17 lymfocyten worden gekenmerkt door de productie van IL-17 (18). De ontdekking van deze subgroep van T helper cellen heeft nieuwe inzichten gegeven in de pathogenese van vele auto-immuunziekten en heeft bijgedragen aan een beter begrip van de afweer tegen bacteriele infecties en schimmelinfecties. In **hoofdstuk 9** beschrijven we het delicate evenwicht tussen de beschermende en potentieel schadelijke effecten van Th17 reacties tijdens een infectie. Aan de ene kant hebben Th17 cellen een belangrijke rol in de afweer tegen bacteriële en schimmelinfecties door het induceren van defensines en de werving van neutrofielen naar de plaats van infectie. Aan de andere kant kunnen Th17 cellen bijdragen aan het persisteren van virus infecties en kunnen ze schadelijk zijn voor de gastheer door het

in stand houden van chronische ontstekingen bij parasitaire infecties. Met deze inzichten moet rekening worden gehouden als men immunomodulatoire behandelingen wil ontwikkelen.

Hoofdstuk 10 beschrijft de belangrijkste signaalroutes die betrokken zijn bij de Th17 reactie die optreedt wanneer humane mononucleaire cellen gestimuleerd worden met *C. albicans*. We hebben ontdekt dat *C. albicans* een zeer uitgesproken IL-17 productie induceert. Dit blijkt te berusten op een interactie tussen de mannoproteïnen die zich bevinden op het celoppervlak van *Candida albicans* en de mannose receptor die zich bevindt op de celmembraan van de monocyt/macrofaag. Stimulatie van de TLR2/dectin-1 route leidt op zichzelf niet tot de inductie van IL-17, echter de TLR2 en dectin-1 signaalroutes hebben wel het vermogen om de IL-17 productie door stimulatie van de mannose receptor te versterken. Deze bevindingen zijn in overeenstemming met de resultaten die zijn gepresenteerd in hoofdstuk 6, waar we aantonen we dat de mannose receptor en TLR2/dectin-1 nodig zijn voor IL-1β productie; IL-1β is namelijk erg belangrijk voor het induceren van een Th17 reactie. Deze studie onderstreept de dominante rol die suikers (zoals mannose structuren en glucanen) en de Calcium-afhankelijke suikerherkennende moleculen, de C-type lectines, spelen in het initiëren van Th17 afweerreacties. We hebben een model voorgesteld waarin de eiwitstructuren van de mannoproteïnen van *C. albicans* belangrijk zijn voor T helper cel activering en de mannose structuren verantwoordelijk zijn voor het induceren van een cytokine profiel dat de T helper cel polariseert richting Th17. We zijn deze hypothese momenteel aan het onderzoeken.

Prostaglandine E2 (PGE2) speelt een belangrijke rol in de initiatie van Th17 reacties in experimentele modellen van auto-immuunziekten (19). Omdat we een robuuste IL-17 productie vonden in menselijke cellen die werden blootgesteld aan *C. albicans*, onderzochten we de rol van PGE2 in de Th17 reactie die geïnduceerd wordt door *Candida* (**hoofdstuk 11**). De toevoeging van een niet-steroïdale anti-inflammatoire geneesmiddel (NSAID) blokkeerde de PGE2 productie volledig en resulteerde in een afname van IL-17 productie door cellen die gestimuleerd werden met *C. albicans*. Mannoproteïnen van *C. albicans* en de mannose-receptor bleken een prominente rol te spelen in de productie van PGE2. Daarnaast versterkten de TLR2 en dectin-1 signaalroutes de productie van PGE2. Interessant genoeg werd IL-1β niet beïnvloed door het blokkeren van PGE2. Dit is waarschijnlijk de verklaring voor de resterende IL-17 productie na blokkade van PGE2. Deze observaties geven aan dat het gebruik van een NSAID Th17 reacties zou kunnen verminderen in patiënten. Dit zou de reden kunnen zijn waarom NSAID's gunstige effecten hebben in chronische ontstekingsziekten, zoals reumatoïde arthritis.

Er is gesuggereerd dat de IL-17 respons onwenselijk is tijdens schimmelinfecties, omdat het bijdraagt aan een ontstekingreactie die schadelijk is voor de gastheer (20). Deze suggestie is afgeleid van onderzoek dat is verricht in muizen met experimentele candidiasis en invasieve aspergillose (21). In andere dierstudies die experimentele *Candida* infecties hebben bestudeerd, heeft de Th17 reactie duidelijk een beschermende functie (22, 23). In **hoofdstuk 12** hebben we geprobeerd deze controverse te onderzoeken door Th17 reacties te bestuderen in twee verschillende muismodellen, namelijk invasieve candidiasis en zymosan-gemedieerd multi-orgaanfalen. We zagen dat IL-17 receptor deficiënte (IL-17R-/-) dieren meer vatbaar waren voor systemische candidiasis dan controle muizen. Echter, IL-17R-/-

muizen hadden gelijke overlevingskansen in het model van zymosan-geinduceerd multi-orgaanfalen vergeleken met controle muizen Deze bevindingen tonen aan dat IL-17 niet in grote mate bijdraagt aan schadelijke ontstekingsprocessen die leiden tot orgaanfalen tijdens invasieve schimmelinfecties, maar ondersteunen juist het concept dat de IL-17 signaalroute beschermend is tijdens schimmelinfecties

In **hoofdstuk 13** hebben we de rol van de Th17 reactie in de afweer tegen *Aspergillus fumigatus* onderzocht In tegenstelling tot de hoge IL-17 productie die wordt geinduceerd door *C albicans*, vonden we een relatief lage IL-17 productie door humane mononucleaire cellen die werden blootgesteld aan *Aspergillus fumigatus* Bovendien vonden we zeer lage IL-17 concentraties in de bronchoalveolaire lavage vloeistof en serum van patienten met invasieve aspergillose. Dit staat in schril contrast met de gegevens die zijn verkregen uit experimentele *Aspergillus* infectie modellen (20, 21) Verder vonden wij dat *Aspergillus* een zeer krachtige interferon (IFN)γ respons teweegbrengt in humane cellen IFNγ is het prototypische cytokine van een Th1 respons Omdat het bekend is dat IFNγ Th17 reacties onderdrukt (24), hebben we onderzocht of de sterke Th1 reactie verantwoordelijk was voor de lage IL-17 productie We vonden geen verschil in het vermogen van *Aspergillus* om IL-17 te induceren wanneer we IFNγ blokkeerden Deze observatie maakt het onwaarschijnlijk dat de lage IL-17 productie door *Aspergillus* te wijten is aan een sterke Th1 respons Er is gesuggereerd dat L-kynurenine (een tryptofaan metaboliet) in staat is om *Aspergillus* geinduceerde IL-17 productie te dempen (20) We hebben gevonden dat *Aspergillus* zelf in staat is om tryptofaan om te zetten in L-kynurenine, en dat het supernatant van levende *Aspergillus* de capaciteit heeft om de IL-17 productie van mitogeen geactiveerde T-cellen te verminderen Concluderend is het zeer waarschijnlijk dat Th17 reacties niet cruciaal zijn voor een optimale afweer tegen *Aspergillus* infecties Dit is ook in overeenstemming met het klinisch beeld van patienten met het hyper-IgE syndroom Deze patienten kunnen helemaal geen Th17 reacties induceren, maar krijgen zelden invasieve infecties met *Aspergillus*

Het inflammasoom en Th17 reacties in klinische syndromen

Patienten met chronische granulomateuze ziekte (CGD) hebben een defect in het NADPH oxidase systeem en als gevolg daarvan kunnen ze geen zuurstof radicalen (reactive oxygen species, ROS) produceren (25) Het is beschreven dat NADPH afhankelijke ROS essentieel zijn voor de activering van het inflammasoom (26) Dit zou in overeenstemming zijn met de algemeen geaccepteerde gedachte dat ROS met name pro-inflammatoir zijn Echter, patienten met CGD (evenals CGD muizen) hebben een pro-inflammatoire status, wat tot op heden nog steeds niet goed is verklaard (20, 27) In **hoofdstuk 14** hebben we de rol van NADPH afhankelijke ROS in de activering van het inflammasoom bestudeerd door cellen te isoleren van patienten met CGD We vonden dat de CGD cellen grotere hoeveelheden IL-1β produceerden na stimulatie Tevens bleek caspase-1 meer geactiveerd te zijn in CGD cellen dan in controle cellen In tegenstelling tot het dogma dat ROS pro-inflammatoir zijn en essentieel zijn voor de activering van het inflammasoom, blijkt uit onze gegevens dat ROS deficientie uiteindelijk resulteert in een actiever inflammasoom en meer IL-1β productie Dit zou kunnen verklaren waarom patienten met CGD een pro-inflammatoir fenotype hebben

CD20 is een molecuul dat voorkomt op alle B-cellen Rituximab is een geneesmidel dat bindt aan CD20 waardoor B-cellen worden geelimineerd Ziektes die worden veroorzaakt door B-

cellen kunnen dan ook behandeld worden met rituximab. Echter, merkwaardigerwijs heeft rituximab ook een gunstig effect op sommige T-cel gemedieerde auto-immuunziekten zoals multiple sclerose (28). Ook de werkzaamheid van rituximab bij patienten met reumatoïde artritis roept vragen op, immers er is geen effect van rituximab op de ziektegeassocieerde autoantilichamen en het gunstige effect treedt veel sneller op dan wat men zou verwachten bij het verdwijnen van autoantilichamen (29). Gezien het feit dat Th17 cellen een prominente rol spelen in de pathogenese van zowel multiple sclerose als reumatoïde artritis (30), formuleerden we de hypothese dat B-cellen een rol spelen bij de regulatie van de Th17 reactie. Deze hypothese is uitgewerkt en bestudeerd in **hoofdstuk 15**. Twaalf patiënten met reumatoïde artritis werden behandeld met rituximab. De behandeling resulteerde in een efficiënte B-cel depletie en leidde tot een klinische verbetering bij deze patiënten. Vóór en 12 weken na de behandeling werden synoviale biopten genomen uit aangedane kniegewrichten. De Th17 reactie in de aangedane gewrichten in deze patiënten was na behandeling met rituximab minder. Vervolgens stelden we ons de vraag of rituximab de Th17 reactie specifiek remt of dat het meerdere T-cel reacties beïnvloed. We konden geen effect vinden van rituximab op Th1 of Treg reacties. Bovendien vonden we geen effect op TNFα, wat aangeeft dat de klinische verbetering niet te wijten was aan een verminderde TNFα productie. Om een beter begrip te krijgen hoe rituximab de Th17 reactie remt hebben we een in-vitro model gebruikt met *C. albicans* stimulaties. Cellen die gestimuleerd werden met *C. albicans* produceerden aanzienlijk minder IL-17 en IL-22 als rituximab werd toegevoegd aan het kweekmedium. Wij hebben vervolgens de mogelijkheid overwogen dat rituximab rechtstreeks de Th17 cellen zou kunnen depleteren, omdat er is gerapporteerd dat sommige T-cel subsets CD20 tot expressie kunnen brengen (31). Wij konden echter geen CD20 expressie op T-cellen aantonen die ook IL-17 produceerden. Bovendien vonden we dat rituximab geen effect had op de Th17 reactie in cellen die geïsoleerd werden van patiënten met een X-linked agammaglobulinemie (deze patienten hebben geen naiëve en geen mature B-cellen). Dit ondersteunt de hypothese dat het effect van rituximab op TH17 ectivatie toch verloopt via een direct effect op B-cellen. Concluderend beschrijft deze studie een rol voor B-cellen in de regulatie van de Th17 reactie. Hiermee kan worden verklaard waarom rituximab effectief is in patiënten met multiple sclerose en reumatoïde artritis. Onze bevindingen kunnen belangrijke gevolgen hebben voor de ontwikkeling, het onderzoek en de behandeling van Th17 gemedieerde ziekten.

Patiënten met het hyper IgE syndroom (HIES; Job syndroom) zijn bijzonder gevoelig voor infecties met *Candida albicans* en *Staphylococcus aureus* (32). Het is beschreven dat circa 60% van de HIES patiënten mutaties hebben in het *STAT3* gen met als gevolg daarvan een deficiënte Th17 reactie (33). In **hoofdstuk 16** beschrijven we drie HIES patiënten afkomstig uit één gezin. Zij hebben allemaal dezelfde *STAT3* mutatie in het linker-domein en hebben een fenotype dat interessant genoeg verschilt van dat bij patiënten met een sporadisch HIES. Het meest opvallende verschil is dat, in tegenstelling tot vrijwel alle andere HIES patiënten, onze patiënten niet extra vatbaar zijn voor *S. aureus* longontstekingen (34). We vonden dat de cellen van HIES patiënten met een linker-domein mutatie weldegelijk IL-17 produceerden als ze gestimuleerd werden met *S. aureus*, terwijl de cellen van patiënten met een sporadisch HIES absoluut geen IL-17 produceerden (33). Deze gegevens suggereren dat de IL-17 respons bijzonder belangrijk is voor de afweer tegen *S. aureus* op het niveau van longepitheel. De cellen van alle HIES patiënten die ook een mucocutane candidiasis hadden, vertoonden ook een defecte IL-17 productie na stimulatie met *C. albicans*. Dit benadrukt de

belangrijke beschermende rol van de Th17 reactie in de afweer tegen mucosale *Candida* infecties. Deze studies suggereren verder dat verschillende *STAT3* mutaties kunnen resulteren in verschillende klinische karakteristieken. Een interessante bevinding was dat de cellen met een mutatie in het linker-domein van *STAT3* een normale intracellulaire productie van IL-17 lieten zien na mitogene stimulatie (aangetoond door FACS analyse), hoewel in de supernatanten duidelijk lagere concentraties IL-17 werden gevonden na mitogene stimulatie. Concluderend suggereert deze studie een directe relatie tussen de mate van Th17 deficiëntie en de ernst van het klinische beeld bij patiënten met het hyper IgE syndroom en geeft ze aan dat IL-17 essentieel is voor de afweer tegen een *S. aureus* pneumonie.

In **hoofdstuk 17** hebben we aangetoond dat autosomaal dominant chronische mucocutane candidiasis (AD-CMC) veroorzaakt wordt door mutaties in het CC domein van het *STAT1* gen. We hebben gevonden dat T-cellen van patiënten met CMC nauwelijks in staat zijn om karakteristieke Th1 en Th17 cytokinen te produceren als ze gestimuleerd worden met *Candida*. In deze cellen bleek er tevens een gestoorde respons te bestaan op de cytokinen IL-12 en IL-23. Met next-generation sequencing analyseerden we de genen van de moleculen die gedeeld worden door de IL-12 en IL-23 signaalroutes en de genen die betrokken zijn bij de differentiatie van de Th17 reactie. Alle patiënten van de 5 AD-CMC gezinnen afkomstig uit Nederland en Engeland hadden een mutatie in het CC domein van STAT1, terwijl er geen mutaties werden gevonden in de niet aangedane broers en zussen en in 162 gezonde controles. Drie families droegen de A267V mutatie (een Nederlandse en twee Engelse families), en twee gezinnen droegen de R274W mutatie (een Nederlandse en een Engelse). De patiënten met een A267V mutatie bleken ook orale en oesofagale tumoren te hebben, wat wijst op een rol voor STAT1 in de pathogenese van orale en oesofagale carcinomen. Dit is in overeenstemming met eerdere studies die hebben laten zien dat STAT1 gebreken geassocieerd zijn met slokdarmcarcinoom. Patiënten met een R274W mutatie hadden daarentegen geen carcinomen, maar een auto-immuun hepatitis of een auto-immuun hemolytische anemie. De rol van STAT1 in de pathogenese van orale en slokdarmcarcinomen en auto-immuunziekten verdient nader onderzoek. Dit onderzoek toont aan dat next generation sequencing in combinatie met goed ontworpen functionele studies de potentie heeft om de pathogenese van erfelijke ziekten op te helderen.

Het is niet waarschijnlijk dat de huidige behandelmogelijkheden de morbiditeit van mucosale *Candida* infecties en de hoge sterftecijfers van systemische candidiasis nog verder zal doen afnemen. Het is dus nodig om nieuwe therapeutische strategieën te ontwikkelen om *Candida* infecties te bestrijden. In **hoofdstuk 18** beschrijven we de huidige studies die hebben bijgedragen aan de ontwikkeling van vaccinatie, therapeutische antistoffen, cytokine therapieën als behandelingsopties voor *Candida* infecties. Deze benaderingen zijn allen veelbelovend, maar de effectiviteit en veiligheid van deze behandelmethoden moeten nog worden aangetoond. Ook beschrijven we dat als we immunomodulatoire therapieën willen ontwikkelen het noodzakelijk is om controverses aan te pakken, zoals bijvoorbeeld de rol van de Th17 reactie tijdens schimmelinfecties.

Algemene conclusies

Door de mechanismen te ontrafelen die ten grondslag liggen aan de vatbaarheid voor *Candida* infecties kunnen we nieuwe strategieën ontwikkelen die kunnen leiden tot een vermindering van de morbiditeit en mortaliteit van *Candida* infecties. De experimenten uitgevoerd in dit proefschrift hebben een bescheiden bijdrage kunnen leveren aan het omvangrijke en dynamische veld van immunologisch onderzoek. Vele uitdagingen liggen nog voor ons, waaronder bijvoorbeeld het begrijpen van de moleculaire mechanismen waardoor mutaties in het CC domein van STAT1 leiden tot Th1 en Th17 deficiëntie in autosomaal dominante chronische mucocutane candidiasis, of de reden waarom een patiënt met chronische granulomateuze ziekte vooral gevoelig is voor een *Aspergillus* infectie.

De rol van het afweersysteem strekt zich uit tot vrijwel elk gebied van de medische wetenschap. Door de kennis van de verschillende gebieden van onderzoek te combineren, kunnen we een beter begrip krijgen van het afweersysteem en haar onbekende functies. Dit zal ons helpen bij de ontwikkeling van nieuwe strategieën die afweerreacties kunnen moduleren, zodat we in de toekomst beschikken over betere behandelingopties voor infecties in het algemeen en schimmelinfecties in het bijzonder.

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1. **F.L. van de Veerdonk**, MG Netea, CA Dinarello, JWM van der Meer. Anakinra for the inflammatory complications of chronic granulomatous disease. The Netherlands Journal of Medicine. **2011** (accepted)

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3. **Van de Veerdonk F.L.**, Blauw, G., Schneeberger P.M., Festen H P.M., *Q-koorts blijft diagnostische en therapeutische vragen oproepen*. Infectieziekten Bulletin, **2003** 14(5): p. 177-180.

Books, or contributions to books

1. Co-author of book entitled: "Acute Boekje"; Chapter: "Severe bacterial infections" ISBN: 978-90-8523-197-4

Curriculum Vitae

Frank Leo van de Veerdonk werd geboren op 15 juli 1975 te Oss. In 1992 haalde hij zijn HAVO diploma en in 1994 zijn VWO diploma aan het Mondriaan College in Oss. Vanaf 1994 studeerde hij geneeskunde aan de Vrije Universiteit van Amsterdam. Tijdens zijn studie heeft hij gewerkt als studentassistent celbiologie en liep hij een wetenschappelijke stage bij de afdeling immunologie aan de VU onder supervisie van prof. dr. C.D. Dijkstra. In 2001 haalde hij zijn arts examen.

Na zijn studie aan de VU werkte Frank tot 2002 als assistent cardiologie in het Jeroen Bosch Ziekenhuis in 's Hertogenbosch. Van 2002 tot 2005 werkte hij in hetzelfde ziekenhuis als assistent interne geneeskunde. In maart 2003 begon hij aan zijn opleiding tot internist in het Jeroen Bosch ziekenhuis (opleider dr. P.M. Netten), welke hij in 2006 voortzette in het Universitair Medisch Centrum St Radboud (opleiders prof. dr. J.W.M. van der Meer, dr. J. De Graaf, prof. dr. P.M.J. Stuyt en dr. C. Bleeker). In mei 2010 startte hij de opleiding infectieziekten (opleider prof. dr. B.J. Kullberg). In maart 2010 en februari 2012 verwacht hij respectievelijk zijn opleiding tot internist en infectioloog af te ronden.

Tijdens zijn opleiding interne geneeskunde en infectiologie is hij in mei 2007 gestart met zijn promotieonderzoek bij prof. dr. M. Netea, dr. L.A.B. Joosten, prof. dr. J.W.M. van der Meer en prof. dr. B.J. Kullberg. Hij heeft periodiek in 2009 en in 2010 gewerkt op de afdeling immunologie van St Jude Children's Research Hospital in Memphis, USA onder supervisie van dr. T.D. Kanneganti. Voor zijn ontdekkingen ontving hij recent een aantal internationale prijzen, onder andere van de European Society for Clinical Microbiology and Infection en International Cytokine Society. Dit jaar werd hij uitgenodigd om een sabbatical periode te doen in het laboratorium van Professor Charles Dinarello in Denver, Colorado, USA. Dinarello is de wereldautoriteit op het gebied van cytokinen en ontving recent de Crawfoord prize, de Albany prize en de Paul Ehrlich prijs.

In 2003 trouwde hij met Inge van den Akker. Hun zoon Rijf werd geboren in 2006 en hun dochter Juno in 2010.

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Veel mensen hebben aan dit proefschrift bijgedragen, enkele personen wil ik graag in het bijzonder bedanken.

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Prof. dr. Jos W.M. van der Meer, beste Jos. Al tijdens mijn opleiding in de kliniek wist je mij te enthousiasmeren met je volhardheid om een diagnose te stellen achter de meest complexe casus. Die aanstekelijke gedrevenheid deed mij altijd met extra zin en motivatie terugkeren naar de patiënten op de afdeling. Ook voor de wetenschap werk je met een zelfde bezieling. Een mooi voorbeeld vind ik nog steeds jouw idee om cellen van agammaglobulinemie patiënten te gebruiken om te laten zien dat het effect van rituximab via B cellen loopt. Zo heb je nog vele andere, slimme experimenten bedacht die dit proefschrift vorm gaven. Het is een groot voorrecht om met je samen te werken.

Dr. Leo A.B. Joosten. Leo, jouw gevoel voor humor en je enorme enthousiasme voor de wetenschap gaan geweldig samen. In Memphis, waar het hard werken was, hebben we ook heel veel lol gehad. (Bedankt voor de iPet en de -big- Mac.) Op de juiste momenten weet jij met humor stress te reduceren of tegenslagen te relativeren. Vele experimenten die bedacht zijn in dit proefschrift zijn mede door jouw creatieve geest ontworpen. Ik kan altijd bij je binnenlopen en je bent altijd even hartelijk en gastvrij.

Prof. dr. Bart-Jan Kullberg, beste Bart-Jan. Je bezit een indrukwekkende hoeveelheid kennis en ik waardeer de energie waarmee je die deelt en overdraagt bijzonder. Je hebt me vanaf het begin gestimuleerd om onderzoek te doen en je stond altijd open voor nieuwe ideeën. Daar ben ik je zeer dankbaar voor. Dank ook dat je de mogelijkheid creëert om een periode in Denver te kunnen werken.

Prof. dr. Charles Dinarello, dear Charles, you are a wonderful person. Open, straight and one of the greatest scientists I have ever met. Thank you for inviting me to Frankfurt where you got the Paul Ehrlich prize and introduced me to some very interesting people, such as Stanley Sheldon. I look forward to work in your lab this summer.

Dear Tirumala, I would like to thank you for the opportunity to work in St Jude Children's hospital. This experience I will never forget. I would also like to thank Robby, Patrick, Maggy, Tall Chris, Texas Chris, Sirish, Parash, Zaki and Subbarao for the good discussions and fun we had.

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Ook mijn paranimfen Quirijn en Theo wil ik bedanken. Theo, Ik heb heel veel leuke discussies met je gehad en voor de meest lastige problemen vond jij altijd wel een creatieve oplossing. Q, je bent een goede arts, een goede onderzoeker en een goede vriend. Dat maakt het fantastisch samenwerken met jou.

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Stellingen behorend bij het Proefschrift:

The interplay between innate immunity and Th17 responses in *Candida* infection

1. Autosomaal dominante chronische mucocutane candidiasis wordt veroorzaakt door een mutatie in het CC domain van het *STAT1* gen.

2. Zuurstofradicalen hebben ook een anti-inflammatoire functie, wat betekent dat anti-oxidanten niet alleen ontstekingsremmend werken zoals algemeen wordt aangenomen, maar juist kunnen bijdragen aan een schadelijke ontstekingsreactie.

3. De Th17 afweerreactie is essentieel voor de bescherming tegen mucocutane candidiasis en *Staphyloccoccus aureus* pneumonie.

4. *Candida albicans* en *S. aureus* induceren een zeer uitgesproken Th17 afweerreactie.

5. De Th17 afweerreactie wordt met name geinduceerd door glycoproteinen die worden herkend door mannose bindende receptoren zoals de mannose receptor.

6. NSAIDs werken in ontstekingsziekten zoals reumatoïde artritis doordat ze de Th17 reactie onderdrukken.

7. B-cellen spelen een belangrijke rol in het onderhouden van een pathologische Th17 respons.

8. "Word geen archivarissen van feiten. Tracht hun ontstaan te doorgronden Zoek hardnekkig hun wetten."
   Ivan Petrovich Pavlov (1849-1936)

9. "Het is een teken van een geschoolde geest als iemand kan nadenken over een gedachte zonder ze te aanvaarden."
   Aristoteles (384-322 v.C.)

10. "Fantasie is belangrijker dan kennis."
    Albert Einstein (1879-1955)

Frank Leo van de Veerdonk