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IFN-γ in *Candida albicans* infections

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1. ABSTRACT

The dimorphic fungus *Candida albicans* is the most frequent etiologic agent that causes opportunistic infections called candidiasis, a disease whose systemic manifestation could prove fatal and whose incidence is increasing as a result of an expanding immunocompromised population. Here we review the role of interferon-gamma (IFN-γ) in the host protection against invasive candidiasis. This cytokine plays an essential role in both the innate and adaptive arms of the immune response to candidiasis. We focus on recent progress on host-pathogen interactions at the molecular level, leading to the production of IFN-γ by host cells. IFN-γ is produced by CD4 Th1, CD8, γδ T, and natural killer (NK) cells, essentially in response to both IL-12 and/or IL-18, and plays an important role in the regulation of the immune system as well as in the control of the infectious process. IFN-γ is required for optimal activation of phagocytes, collaborates in the generation of protective antibody response, and favours the development of a Th1 protective response.

2. INTRODUCTION

Candida species are the most frequent cause of mucosal and invasive fungal infections (1, 2). The leading cause of candidiasis, *Candida albicans*, resides as a commensal of the human mucosae and the gastrointestinal tract. In immunocompromised hosts, saprophytic colonization often leads to opportunistic mucosal or life-threatening deep organ infection. A wide range of factors that lead to various degrees of immunosuppression predispose patients to infections by *Candida*. These factors include a severe underlying disease (i.e. AIDS or leukaemia), impaired phagocytic function (i.e. neutropenia), and exogenous factors (such as wide spectrum antibiotic treatment, i.v. drug use, transplantation medicine, trauma, abdominal surgery). Haematogenous candidiasis is a frequent complication in the treatment of patients with acute leukaemia; oropharyngeal candidiasis, a common mucosal infection, occurs in the majority of AIDS patients, and vaginal candidiasis has been estimated to occur in approximately 75% of women at least once. Morbidity and mortality rates associated with

haematogenous disseminated or invasive candidiasis remain unacceptably high, mainly due to the lack of an early and accurate diagnostic procedure, the limited arsenal of antifungal drugs and the emergence of resistant strains (3-5).

Resistance to candidiasis requires the coordinated action of innate and adaptive immune defenses. The phagocytes, neutrophils and macrophages, can clear the pathogen, and furthermore macrophage activation leads to the release of several key mediators such as proinflammatory cytokines, which are important for protecting the host against disseminated candidasis (6). The specific detection of microbes by macrophages and dendritic cells is mediated by pattern recognition receptors (PRRs), germline-encoded, nonclonal receptors, that recognize microbial structures referred to as pathogen-associated molecular patterns (PAMPs). Phagocytic cells recognize *C. albicans* by a variety of PRRs including receptors for mannosyl/fucosyl glycoconjugates (CR3, MR, DC-SIGN, galectin-3, dectin-2), receptor for β-glucan (dectin-1), and Toll-like receptors (TLRs) (7-10). TLRs are essential PRRs and constitute a family of receptors that mediate recognition of microbial challenges, subsequent inflammatory response and are also regulators of the adaptive responses (11, 12).

It is accepted that antifungal CD4+ T helper 1 (Th1)-mediated responses play a central role in anti-*C. albicans* defenses, providing control of fungal infectivity through production of IFN-γ, which is required for optimal activation of phagocytes and for helping in the generation of protective antibody response (6). Interestingly, although protective immunity to *C. albicans* is mediated by Th1 cells, some Th2 cytokines, such as IL-4 and IL-10, are required for the maintenance of the anti-fungal immune protection (13, 14); in addition, regulatory T cells (Treg), activated by IL-10 producing dendritic cells, are involved in the induction of memory protective immunity by negative regulation of antifungal Th1 reactivity (15).

In addition to the host status, the pathogenicity of the fungus also depends on a set of virulence factors whose expression is often environmentally regulated, such as the morphological transition from yeast to hyphal form (16). This special feature is strictly associated with virulence, as several agerminative mutants invariably have low systemic pathogenicity (17, 18). Phagocytosis of the yeast form induce murine dendritic cells (DCs) to produce IL-12 and to prime Th1 lymphocytes, whereas ingestion of the hyphal form results in IL-4 production, which favors Th2 cell priming (19).

Here we review the role of IFN-γ in the host protection against invasive candidiasis and focus on recent progress on host-pathogen interactions at the molecular level leading to the production of IFN-γ by host cells.

3. INTERFERON-GAMMA

Immune, type II, or interferon-gamma (IFN-γ) is a pleotropic cytokine secreted by CD4 Th1, CD8, γδ T, and natural killer (NK) cells. Although originally defined as an agent with direct antiviral activity (20), the properties of IFN-γ include regulation of the immune system and the control of infectious disease. This cytokine plays an essential role in both the innate and adaptive phases of an immune response (21).

Human as well as mouse IFN-γ is encoded by a single-copy gene, generating a single 1.2.-kb mRNA-species and a polypeptide of 166 residues containing a cleaved hydrophobic signal sequence of 23 residues. Biologically active IFN-γ is in the form of a noncovalent 34-kDa homodimer. IFN-γ exerts its effects by binding to the IFN-γ receptor, composed of the IFN-γ R1 and R2 chains, present on many lymphoid and nonlymphoid cell types (21). IFN-γ R complex utilizes the JAK/STAT signal pathway, specifically the receptor-associated Janus-family protein kinases Jak1 and Jak2. Upon ligand binding these kinases are activated, causing the phosphorylation, dimerization, and nuclear translocation of the Stat1 transcription factor (22). The signal transduction pathway stemming from IFN-γ is well established, but the manner in which IFN-γ-induced genes mediate this cytokine's pleotropic functions remains unclear. Over 200 genes are known to be regulated by IFN-γ and several IFN-γ-regulated genes are themselves components of transcription factors. Additionally, IFN-γ exerts its effect on a wide variety of cell types owing to the broad expression pattern of the IFN-γR complex. Thus, determination of the cell types and gene products mediating IFN-γ's *in vivo* effects remains difficult.

Il is well established that one essential role of IFN-γ is to activate a number of phagocytic cell functions, including induction of superoxide formation, upregulation of surface molecule display (e.g. major histocompatibility complex (MHC) class II, FcγR, integrins), reduction of phagocytic vacuole pH, degradation of intracellular tryptophan, and enhanced killing of intracellular parasites (21). Mice deficient in either IFN-γ, or IFN-γ R1, or IFN-γ R2, or the signalling molecule Stat1 have severely impaired immune responses *in vivo*, as demonstrated by their increased susceptibility to microbial pathogens and certain viruses (23). Besides, humans with mutations in components of the IFN-γ receptor-signalling pathway have been identified. Such individuals have profound immunodeficiencies, particularly to intracellular microbial infections, with some individuals dying in early childhood as a consequence of mycobacterial infections (23).

3. INTERFERON-GAMMA IN *Candida albicans* INFECTIONS

3.1. IFN-γ is essential for host defence against invasive candidiasis

The important role of IFN-γ in the resistance against invasive candidiasis has been clearly demonstrated several years ago. Knockout mice deficient in either IFN-γ or IFN-γ receptor were more susceptible than wild-type mice to invasive *C. albicans* infection (24-26). These reports demonstrated (i) that IFN-γ is required for development of protective Th1-dependent immunity (24, 25), (ii) that IFN-γ knockout mice produce *C. albicans* specific

immunoglobulins in lower titers than control mice, and (iii) that peritoneal macrophages from these mice have an impaired nitric oxide production (26). The administration of IFN-γ to mice infected with *C. albicans* showed a beneficial effect on the outcome of the infection (27), whereas the injection of a monoclonal antibody to IFN-γ resulted in the development of a nonprotective Th2 rather than a protective Th1 response (28).

Several mechanisms have been shown to mediate the protective effect of IFN-γ against *C. albicans* infections. *In vitro* studies have demonstrated stimulatory effects of IFN-γ on the phagocytosis and killing of *C. albicans* by neutrophils and macrophages (29, 30). Exposure of adult macrophages to IFN-γ resulted in increased phagocytosis and killing of *C. albicans (*30); however, no enhancement with cord macrophages could be detected under the same experimental conditions (31). These data suggested that neonatal macrophages have a normal capacity to ingest and kill *Candida* but cannot be fully activated by IFN-γ, a finding that could not be attributed to lower expression or binding of IFN-γ receptor to its ligand on neonatal cells. Remarkably, a significantly decreased Stat-1 phosphorylation was detected in neonatal cells in response to IFN-γ, suggesting the possible existence of a negative regulation of IFN-γ receptor signaling in newborns (31). More recently, Donini *et al.*, (32) have described that NADPH oxidase, the enzyme responsible for ROS production, is involved in *C. albicans* killing by human DC. Interestingly, *C. albicans* cells did not activate NADPH oxidase in DC, and were poorly killed by these cells; however, the *C. albicans* killing activity increased upon treatment of DC with IFN-γ, indicating that IFN- γ probably modulates NADPH oxidase activity.

The IFN-γ induced by the fungus during experimental candidiasis acts on the metabolic pathway involved in tryptophan catabolism by mediating the activation of the enzyme indoleamine 2,3-dioxygenase (IDO) in neutrophils and dendritic cells at the sites of infection. IDO activation in neutrophils leads to the activation of the killing machinery while sparing the inflammatory response, whereas in dendritic cells results in tipping the balance between IL-12 and IL-10 production leading to Th1 and Treg-mediated protective immunity. IDO blockade by an enzyme inhibitor exacerbates the infection process as well as the associated inflammatory pathology, and in addition, abolishes resistance to reinfection caused by deregulated innate and adaptive immune responses. Therefore, IDO activation represents one of the mechanisms by which IFN-γ exerts a fine control on the inflammatory and adaptive antifungal responses (33, 34).

4.2. IFN-γ production by NK cells

A crucial step in the control of intracellular pathogens is the early production of IFN-γ by NK cells. NK cells are an essential component of the innate immune response and are recruited to the site of infection within minutes following pathogen invasion. At the site of infection, and as a consequence of interacting with various microbial products, macrophages and dendritic cells produce proinflammatory cytokines (TNF-α, IL-12, IL-15 and IL-18) which bind to their receptors on NK cells, leading to their activation and to the rapid production and secretion of IFN-γ. This IFN-γ produced by NK cells not only serves as a first line of defence against invading pathogens, but may also contribute to the induction of the appropriate adaptive immune response. Moreover, during the last years the expression of TLRs on NK cells has been reported and several data indicate that NK cells can directly recognize and respond to pathogen components through TLRs by secreting IFN-γ (35-40).

Interaction of lymphokine activated killer (LAK) cells with *C. albicans* does not inhibit or kill the fungal pathogen by means of the LAK lytic machinery, but through secretion of cytokines which have stimulatory effects on phagocytic cells (41). Therefore, the antifungal immunity mediated by NK cells appears to occur by secretion of cytokines that activate phagocytic cells (41, 42). However, whether NK cells are stimulated directly by the fungus or indirectly in response to signals generated by activated bystander host cells remains to be established (43). Interestingly, when the role of TLRs in triggering IFN-γ secretion by NK cells in response to yeasts and hyphae of *C. albicans* was studied, it was unexpectedly found that fungal cells cause an inhibition of IFN-γ secretion by murine NK cells. Killed yeasts and hyphae of *C. albicans* inhibit IFN-γ secretion by highly purified murine NK cells in response to TLR4 and TLR2 ligands, LPS and zymosan, respectively, and this effect was also observed in the presence of NK activating cytokines (IL-2, IL-12 and IL-15) (44). Since the role of NK cells in resistance to *C. albicans* appears to be basically mediated by cytokine production (including IFN-γ) that activates phagocytic cells (30, 41), its inhibition by *C. albicans* may favor the survival of the pathogen. Besides, this inhibitory effect may also: (i) inhibit IFN-γ production by NK cells in response to cytokines secreted by activated bystander cells (37), and (ii) disturb the crosstalk between NK and dendritic cells, as the IFN-γ secreted by NK cells controls dendritic cell maturation and their T cell stimulatory activity (45, 46). This finding may represent a novel mechanism of immune evasion, based on inhibition of NK cells that contributes to the virulence of *C. albicans.*

4.3. IFN-γ production by Th1 cells

The inflammatory environment established by the innate response influences the adaptive immune response: activation, expansion, and selection of pathogen specific lymphocytes. Besides cell-cell contacts that provide activation signals via peptide-MHC class II/TCR and classical costimulatory interactions (B7/CD28), APCs communicate with T cells via cytokine production that is responsible for the expansion and differentiation of naïve T cells to generate mature phenotype such as Th1 and Th2 cells. Th1 cells secrete IFN-γ, IL-2, TNF-α and TNF-β, whereas Th2 cells secrete IL-4, IL-5, IL-6, IL-10, and IL-13, and down regulate Th1 responses. IL-12, discovered in 1989, is the signature cytokine produced by cells of the innate immune system that influences adaptive cell immunity. IL-12 plays a central role in promoting the differentiation of naïve CD4+ T cells into mature Th1

effector cells and is a potent stimulus for natural killer cells and CD8+ T cells to produce IFN-γ.

It is well established that Th1, and not Th2, response confers protection against candidiasis. The activation of Th1 cells is instrumental in the optimal activation of phagocytes at the sites of infection through the production of IFN-γ and in the help for the generation of opsonizing antibodies (6). Romani *et al* (47, 48) developed a murine model of systemic candidiasis in which resistance to reinfection with virulent wild-type cells is induced by prior exposure of mice to a low-virulence agerminative strain of *C. albicans* (PCA strain). In this experimental model, the development of a protective anticandidal response correlates with the detection of Th1-associated immunity (48, 49). Using this model in MyD88-/- mice, (which are defective in the adaptor molecule MyD88 that is essential for signal transduction through TLRs except TLR3) (12), it was shown a default of production of Th1 cytokines (IFN-γ, IL-12, TNF-α) in these mice, which correlated with a diminished frequency of IFN-γ producing CD4+ T lymphocytes (50). These results indicate that TLR/MyD88-dependent inflammatory pathway is essential for the development of a protective Th1 response against *C. albicans*. These observations agree with those reported by Bellocchio *et al* (51) showing that MyD88-dependent signalling is crucial for antifungal responses. Since TLR2 and TLR4 have been implicated in anti-*Candida* responses (8, 9, 51), we performed similar studies in TLR2-/- and TLR4-/- mice (9). IL-12 could not be detected in TLR2-/- mice as opposed to wild type mice, and the IFN-γ production was significantly lower in TLR2-/- mice than in control mice in response to both *C. albicans* yeasts and hyphae (52). Moreover, TLR2 -/- mice showed a lower frequency of Th1 cells than control mice (unpublished results). By contrast, TLR4-/- and C3H/HeJ mice (carrying a mutant TLR4 allele which confers defective TLR4-mediated signalling) showed an IL-12 and IFN-γ production similar to their respective control mice and accordingly a similar frequency of IFN-γ producing CD4+ T lymphocytes (53). Therefore, these results indicate that TLR2 is the most important TLR involved in the interaction with *C. albicans* leading to a Th1 protective response, whereas TLR4 appears to be dispensable (9). However some discrepancies about the recognition of *C. albicans* by TLR2 and TLR4 and its consequences in host protection against infection have been reported (9, 54, 55). Results from other groups partially agree with our hypothesis (51) or even support a different model of involvement of TLR2 and TLR4 in interaction with *C. albicans* (54). According to this model, TLR2 may confer host susceptibility to infection and yeast cells signal through both TLR2 and TLR4, whereas hyphae signal only through TLR2 (54).

One important advantage of *C. albicans* hyphae appears to be its ability to inappropriately polarize the adaptative immunity towards a nonprotective Th2 response. Although the mechanism for this effect is not completely understood, some relevant information has been achieved. The most important difference in the secretory response to both fungal morphotypes is the well-documented higher production of IL-12 in response to yeasts that is accompanied by a Th1 type response. IL-12 production by human blood monocytes as well as mouse and human dendritic cells basically occurs in response to the yeast form, whereas a reduced IL-12 production is elicited upon exposure to hyphae (6, 19, 56-58). Romani and co-workers observed that dendritic cells pulsed *in vitro* with yeast and transferred back into mice, stimulated Th1 immune responses, while dendritic cells exposed to fungal filaments induced Th2 responses (19, 58, 59). On the other hand, it has been demonstrated that dectin-1, a phagocytic receptor for β-glucan, either alone or in collaboration with TLR2, elicits a strong inflammatory response (enhanced stimulation of TNF-α and IL-12) to yeasts (60, 61). Gantner *et al*, (62) have shown that dectin-1 mediates macrophage recognition of *C. albicans* yeasts but not hyphae, since hyphal cells do not appear to expose the β-glucan at the cell surface, suggesting that failure of hyphae to activate dectin-1 might contribute to an impaired Th1 response (62, 63).

The Th1-versus Th2 dichotomy has dominated T cell biology for many years. However, another T helper subset, the Th17 lineage that secretes IL-17, has now risen to prominence (64). Th17 cell development occurs in the presence of TGF-β and IL-6 and is opposed by Th1 cytokines. Th17 cells are maintained in the presence of the IL-12 related cytokine IL-23. IL-17 induces chemokine production at the sites of infection and causes recruitment of neutrophils. IL-17 receptor-deficient mice are more susceptible than control mice to systemic candidiasis and this effect may be attributed to a decreased influx of neutrophils to infected organs (65). Recently, it has been described that the Th17 pathway do develop in response to *C. albicans* (66, 67) and that IL-23 and the Th17 pathway acted as negative regulators of the Th1-mediated immune resistance to *C. albicans* (68). Acosta-Rodriguez *et al* (66) showed in an *in vitro* T helper cell priming assay that yeasts induced more IL-12 than IL-23 and a strong Th1 response, whereas hyphae induced only IL-23 and a strong Th17 response. In this context, as hyphae and not yeasts stimulates dectin-2 (10), Palm & Medzhitov (64) have suggested that this PRR may have a propensity to promote IL-23 secretion and a Th17 response. It has been also suggested that dectin-1, which binds to yeasts and signals through the Syk kinase and the CARD9 adaptor, induces the secretion of IL-23 and the development of Th17 responses (67). TLR2 and TLR4 receptors signalling through MyD88 have also been implicated in IL-23 secretion and development of Th17 pathway in response to *C. albicans* (68). In this study, IL-17 neutralization increased fungal clearance, ameliorated inflammatory pathology and restored protective Th1 antifungal resistance (68). Therefore, the Th17 pathway may promote inflammation and subvert antifungal immunity, acting as negative regulator of protective Th1-mediated immune resistance to *C. albicans* (68). These new studies represent an important advance in understanding the relationship between the interaction of PAMPs and PRRs and the induction of tailored adaptive immune responses.

4.4. IFN-γ production by γδ T cells/ CD8 T cells

A minority of T cells expresses a TCR consisting of γ and δ chains and those cells are primarily CD4+. These

chains are encoded by very few genes; the γδ repertoire is accordingly very limited. Despite sharing many similarities with αβ T cells, their method of activation differs markedly as the recognition of antigen by γδ T cells is not MHC-restricted. γδ T cells comprise a very small percentage of circulating lymphocytes that primarily populate epithelial tissues such as skin, intestine, and reproductive tract. Although the function of γδ T cells appears to be dependent on several factors, including tissue distribution, local microenvironment, and stage of the immune response, one attribute of these cells is the production of IFN-γ upon activation. It has been shown that the γδ T cells enhance macrophage nitric oxide production and anti-*Candida* activity in vitro (69). Although γδ T cells may contribute to immunosurveillance at the body surface, as mice deficient in γδ T cells or depleted in these cells are highly susceptible to orogastric candidiasis little is known of the possible role of these cells in resistance to invasive candidiasis (70).

$CD8^+$ T cells play a pivotal role in immune responses against many viruses and tumors, and may also contribute to immunity to fungi (3, 71). These T cells recognize antigens that are presented on the surface of host cells by MHC class I molecules, leading to their destruction, and therefore are also known as cytotoxic T cells. The possible role of CD8+ T cells in resistance to candidiasis has been somewhat controversial, although some early reports suggested a role of these cells during infection (72, 73). It is accepted that both CD4+ and CD8+ T cells are necessary for elimination of some fungal pathogens; in the primary stages of many fungal diseases the presence of CD4+ T cells is essential for host survival, whereas CD8+ T cells are only necessary to restrict infection (71, 74). The main effector mechanisms of CD8+ T cells are cytotoxicity and cytokine secretion (IFN-γ). The role of cytotoxicity in host defence against fungi is not well delineated, whereas the activity of cytokines is better understood. The Th1 cytokine IL-12 is also a potent stimulus for CD8+ T cells to produce IFN-γ. The frequency of IFN-γ producing CD8+ T lymphocytes is diminished in MyD88-deficient mice as compared with wild-type mice, in agreement with the default of production of Th1 cytokines (IFN-γ, IL-12, TNF-α) detected in these mice, as well as with their strong susceptibility to candidiasis (50).

Both CD8 and γδ T cells may have a role in protection against candidiasis. Probably the major role of these cells is the cytokine production, which is instrumental in mobilizing and activating antifungal effectors, thus providing prompt and effective control of infectivity once the fungus has been established itself in mucosal tissues or spread to internal organs.

5. PERSPECTIVE

The level of our understanding of fungal-host interactions has clearly progressed in the last few years. Although the important role of IFN-γ secreting Th1 responses in protection against *C. albicans* infections has been well established several years ago, recently there has raised increasing interest in how adaptive immune responses are controlled by the innate immune system. Researchers have focused on the discovery of PRRs that upon recognition of PAMPs on fungal cells, initiate adaptive responses through activation and maturation of dendritic cells. Considerably new information has been gained in this field concerning the role of TLRs, dectin-1 and dectin-2, as well as cooperation between dectin-1 and TLR2, although we are far to completely discerning the intricate molecular mechanisms leading to the elicitation of a protective immune response to *C. albicans* infections.

The recently discovered IL-23/Th17 pathway in response to *C. albicans* as a negative regulator of Th1-mediated immune resistance represents a significant advance in the field of adaptive immune response. Modulation of this inflammatory Th17 response is a potential target for development of new strategies to stimulate protective immunity to *C. albicans*.

The ultimate objective is to apply our knowledge of how *C. albicans* stimulates the immune response to improve the therapy of candidiasis and thus the clinical control of fungal infections in the at-risk population. There are several different strategies for immunotherapy related to IFN-γ; one of them is the use of recombinant (r) IFN-γ as adjunctive therapeutic. Despite the widespread use of prophylactic rIFN-γ in chronic granulomatous disease (a genetic disorder characterized by recurrent bacterial and fungal infections and tissue granuloma formation), invasive fungal infection has remained a persistent problem in these patients, with an incidence of 0.1. fungal infections per patient-year (75). However, on the basis of a large volume of others preclinical studies and phase I and II clinical trials, adjunctive rIFN-γ (in combination with antifungal agents) merits evaluation in trials powered to address efficacy (76). Alternatively, the generation of functionally active anti-*C. albicans* IFN-γ secreting T cells is feasible, and may be a promising treatment option for patients with candidiasis after allergenic haematopoietic stem-cell transplant (77).

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