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Review

Cytokines and Costimulatory Molecules: Positive and Negative Regulation of the Immune Response to *Cryptococcus neoformans*

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Abstract. Cytokines are small proteins or glycoproteins that transmit information from one cell to another. Most cells in the body secrete and respond to cytokines and their effects have been described on a myriad of cellular functions. Cytokine interactions may not be linear, thus making the system extremely intricate and with unpredictable features. Therefore, each model of disease may be unique, with its own mechanism of autoregulation dictated by positive and negative feedback involving cytokines and costimulatory molecules. The emergence of some cytokines over others in the course of *Cryptococcus neoformans* infection may characterize a positive or negative outcome of cryptococcosis. Much less is known about the influence of costimulatory molecules in regulating *C. neoformans* immune response. The available information indicates a critical role for proinflammatory cytokines such as tumor necrosis factor α and interleukin 12 (IL-12). The positive role of interferon γ in infected tissue as an inducer of antimicrobial function of innate immune cells and as positive feedback for IL-12 induction appears to be indisputable. *In vitro* studies indicate that costimulatory molecule expression appears to be regulated on antigen-presenting cells by *C. neoformans* and increased expression of B7-1 and CD40 on these cells may promote a protective response. These studies await confirmation in an *in vivo* system. The interplay between cytokines and costimulatory molecules has been scarcely explored and additional details are needed to better understand how they convey positive and negative information to immune cells in response to *C. neoformans*.

Key words: *Cryptococcus neoformans*; cytokines; costimulatory molecules.

Introduction

Cryptococcus neoformans is an opportunistic fungus that causes serious infection in immuno-compromised hosts, particularly in patients with AIDS. Although new anti-viral therapies have reduced the incidence of cryptococcal infections in patients with AIDS, cryptococcosis is still a significant problem in Asia and Africa[5, 54]. In addition, there are new groups at high risk for cryptococcosis, including renal trans-

Abbreviations used: APC – antigen-presenting cell, CD40L – CD40 ligand, GXM – glucuronoxylomannan, IFN – interferon, IL – interleukin, mAb – monoclonal antibody, NK – natural killer, NO – nitric oxide, PBMC – peripheral blood mononuclear cells, Th – T helper, TNF – tumor necrosis factor.

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plant patients[14, 25]. The infectious particle enters the host through the respiratory tract and reaches the lung, where the primary infection is contained in immuno--competent hosts. In contrast, the ability to control infection is severely hampered in immuno-suppressed subjects, resulting in dissemination of fungal cells to cause life-threatening meningitis[5]. Therapeutic options are inadequate to eradicate the fungus, as patients with cryptococcosis are in need of life-long suppressive therapy. It is widely recognized that a complex interplay between cell-mediated and humoral immune response plays a pivotal role in the control of *C. neoformans* infection[56]. The immune response to infectious agents is often ascribed to either a dominant cell-mediated or humoral-type effector mechanism which has been attributed to a dichotomy of T helper (Th) $CD4^+$ cytokine production[20]. A Th1-type response has been shown to be important in containing *C. neoformans* infection at lung level[41].

It is known that the generation of a Th1 or Th2 response depends on many factors, including the cytokine milieu, the antigen concentration and the type of antigen-presenting cells (APC). In addition, Th1 and Th2 responses may coexist in the host until one or the other emerges. A switch from a Th1 to a Th2 response has been demonstrated, while a switch from Th2 to Th1 appears improbable. Th1 generation has been associated with high levels of interferon γ (IFN-γ) and IgG2a, whereas a Th2 response correlates with interleukin 4 (IL-4), IL-10 and IgG1 production. Several studies on experimental models have emphasized the pivotal role of a Th1 response[9, 19], the presence of IL-12[9, 33] and a delayed-type hypersensitivity as predictive of a positive outcome to *C. neoformans* infection[1]. On the other hand, convincing studies report on the importance of specific IgG1 in host defense for murine cryptococcosis[2–4, 13, 46]. Thus, it appears that *C. neoformans* infection could be efficiently controlled by the concomitant presence of a Th1 response and specific IgG1. This condition is not likely to be obtained *in vivo* because Th1 development and establishment exclude an appropriate IgG1-specific response that, in contrast, may be achieved in the presence of a dominant Th2-type response[45].

An optimal protective response could be acquired by an early administration of preformed specific IgG1 that 1) may potentiate Th1 generation by optimizing the antigen presentation process via phagocytosis enhancement, FcγR perturbation, and activation of the complement cascade[34, 63] and 2) would facilitate the scavenging of *C. neoformans* and glucuronoxylomannan (GXM) from the host[15]. Although collaboration between the cellular and humoral responses is certainly determinant for the outcome of *C. neoformans* infection, we believe that a strong predictive value for the efficacy of the interconnection of the two branches of immune response is provided by the cytokine milieu and costimulatory molecule interaction. In this report, I discuss experimental approaches to identify positive and negative signals provided by the cytokine network and costimulation.

Cytokine Involvement in the Collaboration between Cellular and Humoral Response

It has been established that the presence of anti-capsular polysaccharide antibodies in the serum helps clear *C. neoformans* from the host[2–4, 46]. Antibodies to *C. neoformans* polysaccharide are found in the serum of healthy subjects as well as in patients with cryptococcosis[10]; the absence of antibodies in the serum may be associated with a poor prognosis in these patients[11]. Thus, both the humoral and the cellular response work in concert to fight yeast invasion. The opsonizing capability of antibodies favors phagocytosis and the killing of *C. neoformans* by natural immune effector cells[3]. Moreover, the presence of protective IgG1 antibodies induces increased synthesis and release of proinflammatory cytokines such as IL-1β and tumor necrosis factor α (TNF-α)[59], promotes T cell proliferation[53, 59], which appears to be a consequence of enhanced phagocytosis of *C. neoformans* by APC[53], and an increased capacity of accessory cells to present cryptococcal antigens. This latter effect is supported by the ability of IgG1 to promote over-expression of major histocompatibility complex class II[43] and the costimulatory molecule B7-1[57]. Increased B7-1 expression has been associated with the generation of a Th1-type response. Our recent results show that IgG1 is capable of increasing IL-12 release from human monocytes[50], likely favoring a protective Th1 response. Differently from B7-1, over-expression of B7-2 appears to favor a Th2 response. By blocking B7-2 expression with monoclonal antibody (mAb) to B7-2, we demonstrated increased Th1 generation. In particular, the concomitant presence of anti-GXM protective antibodies with mAb to B7-2 resulted in the optimization of the anti-B7-2 effects[43]. The collaboration between the humoral and cellular immune responses is clearly demonstrated in an *in vivo* murine model of *C. neoformans* infection showing that T cells are required for antibody-mediated protection[64].

In Vitro Cytokine Response to *C. neoformans*

Proinflammatory and anti-inflammatory cytokines

Several reports have underscored the inhibitory and stimulatory activity of *C. neoformans* on TNF secretion by human cells depending on the type of immune cells. An inhibitory role for *C. neoformans* on TNF-α secretion by natural killer (NK) cells has been observed[48]. In contrast, peripheral blood mononuclear cells (PBMC) or bronchoalveolar macrophages are able to produce TNF-α in response to inactivated *C. neoformans*[37]. Our studies show that acapsular *C. neoformans* is a better inducer of TNF-α than the encapsulated strain. GXM, the principal constituent of the capsular material of *C. neoformans*, is strongly involved in the observed down-regulation[61]. Indeed, human neutrophils are also able to secrete TNF in response to *C. neoformans*. However, unlike monocytes, neutrophils are stimulated by large capsule isolates of *C. neoformans*[51]. Since TNF-α could enhance complement-mediated phagocytosis of encapsulated *C. neoformans* cells[6–8], this cytokine could greatly influence the anti-cryptococcal activity of phagocytic cells.

In addition to TNF-α, *C. neoformans* and its capsular component have been shown to regulate IL-1 secretion by monocytes and neutrophils. Production of IL-1 has been demonstrated in PBMC exposed to heat-inactivated *C. neoformans*[38]. In agreement with these observations, we demonstrated that monocytes are the major source of IL-1 and that the capsular material acts as a negative regulator[61]. Indeed, IL-1 release is limited by the presence of IL-10[44]. On the other hand, this deactivating cytokine is produced by human monocytes in response to the capsular material of *C. neoformans*[60]. In contrast to monocytes, human neutrophils produce the largest amount of IL-1 when stimulated with heavily encapsulated strains. An explanation for this effect is provided by the ability of *C. neoformans* and its capsular material to activate the complement cascade[34, 35] by generating bioactive complement fractions, such as C3a, and C5a, that mediate IL-1 release[58]. The fact that monocytes and neutrophils exhibit opposite behavior with regards to TNF-α and IL-1 production is an intriguing result that highlights how human cells can respond very differently to fungal components.

Among the inflammatory cytokines, IL-6 is produced in response to *C. neoformans* by alveolar macrophages and monocytes. IL-6 mRNA levels are significantly increased in the presence of antisera to *C. neoformans*[39]. Peripheral blood monocytes were also found to produce IL-6 in response to *C. neoformans* or its capsular components[62].

C. neoformans is also able to induce IL-8 release from neutrophils[58] and it is conceivable that endogenous IL-8, in turn, could enhance the antifungal activity of phagocytic cells[52]. However, TNF-α, IL-1, IL-6 and IL-8, produced largely by phagocytic cells, have been studied with respect to their effects on the inflammatory process and their ability to regulate, in an autocrine manner, natural effector cells, but their influence on the lymphocyte response to *C. neoformans* is currently unknown. Moreover, the primary role of phagocytic cells as a first line of defense against microorganisms includes the capacity to direct the generation of a subsequent T cell response, thus determining the success or failure of the *C. neoformans* invasion.

A bridge between innate and adaptive immunity is represented by IL-12, a proinflammatory cytokine that is a key factor in the induction of a Th1-type response by governing the development of IFN-γ-dependent host resistance in *C. neoformans* infection[29].

Cytokines regulating T cell function

Phagocytic cells are the major biological source of IL-12. Secretion may be induced by direct microbial stimulation in a T cell-independent manner or in a T cell-dependent manner, depending on the interaction of CD40 on monocytes with CD40 ligand (CD40L) on T cells and on the presence of IFN-α[55].

Human monocytes produce low levels of IL-12 even when mRNA for IL-12 is detected after 16 h of stimulation[17]. However, considerable IL-12 is produced later after *C. neoformans* stimulation and is dependent on the presence of T cells. This implies a requirement for the interaction of CD40 molecules on monocytes with CD40L expressed on T cells and the presence of IFN-γ[50]. The presence of endogenous IFN-γ could be provided by T cell interaction with *C. neoformans*. This is supported by previous results showing that human lymphocytes are capable of being directly stimulated to release IFN-γ after exposure to *C. neoformans*[36]. However, regulation of IL-12 production by phagocytic cells in response to *C. neoformans* is extremely complicated and appears to be dependent on the status of activation and on the type of immune cells. Priming of human monocytes with IFN-γ has been shown to facilitate IL-12 release to *C. neoformans*[18]. In contrast, *C. neoformans* suppresses IL-12 p40 production in the murine macrophage cell line J774 even after stimulation with lipopolysaccharide and IFN-γ[27].

IL-15 is another cytokine produced by phagocytic cells that affects T cells and has IL-2-like activity. An elegant paper by MODY et al.[42] shows that IL-15 is

produced by human monocytes stimulated with *C. neoformans* and that the acapsular strain is a better inducer than the encapsulated strain, suggesting phagocytosis as a potential mechanism for IL-15 induction. This paper shows that IL-15 has a double role against *C. neoformans*: 1) it makes an essential contribution to lymphocyte activation and 2) has an important role in the induction of the lymphocyte effector function.

IL-18 is also involved in the induction of the T cell response[12] and effector function of immune cells. IL-18 in combination with IL-12 stimulates the anti-cryptococcal activity of peritoneal exudate cells via IFN-γ production by NK cells[65] or via TNF-α-induced nitric oxide (NO) production[28].

Much of what we know about the cytokine response to *C. neoformans* comes from *in vitro* studies. *In vitro* studies allow control of many variables and provide excellent systems for the study of these interactions. However, the degree to which *in vitro* systems are predictive of *in vivo* effects is unknown.

Experimental animal models have been used to study the role of cytokines in the pathogenesis of cryptococcosis. It is likely that the outcome of cryptococcal infections is strongly influenced by the cytokine milieu at the site of the immune response. The *in vivo* studies related to cytokine response to *C. neoformans* *in vitro* and *in vivo* experimental systems are reported below.

In Vivo Cytokine Response to *C. neoformans*

The bulk of results on cytokine involvement in the positive and negative regulation of *C. neoformans* infection has been acquired using an experimental mouse model. In the lung, the control of infection is related to a Th1 response. This is supported by the observation that the administration of mAb to IFN-γ and of mAb to IL-12 inhibits clearance of the fungus[19]. The pivotal role of IL-12 in inducing protection against *C. neoformans* was highlighted by recent studies showing that mice with targeted disruption of the genes IL-12 p35 and IL-12 p40 were more susceptible to *C. neoformans* infection than wild-type mice. Furthermore, IL-12 p40 mice died earlier with respect to IL-12 p35 mice, suggesting a protective role for IL-12 p40 heterodimer. The increased susceptibility of IL-12 p40 and IL-12 p35 mice appeared to correlate with marginal production of IFN-γ and elevated levels of IL-4[9].

A possible role for IL-18 in protection against *C. neoformans* infection has been suggested by murine studies. In a mouse model of cryptococcosis, administration of the cytokine was shown to enhance elimination of live organisms from the lungs, to prevent dissemination to the brain and to reduce the level of capsular polysaccharide in serum. The beneficial effect of IL-18 appeared to be mediated by an increase of IFN-γ. Administration of mAb to IFN-γ abrogates the protective effect of IL-18[31]. In agreement with these results, IFN-γ has been shown to promote the clearance of *C. neoformans* from the lung. In fact, administration of mAb to IFN-γ reduces clearance of the fungus possibly by blocking NO production of lung macrophages[41].

A granulomatous inflammatory process is often associated with protection versus *C. neoformans* infection[2] and TNF-α is considered a prototype of proinflammatory cytokines. The role of TNF-α has been extensively studied in experimental animal models. HUFFNAGLE et al.[24] showed that TNF-α is required in the afferent phase for a protective T cell immune response to *C. neoformans*. TNF-α mRNA and protein precede the inflammatory response in the lungs of mice undergoing intratracheal challenge with *C. neoformans*[24]. The production of TNF-α was also required to prevent colonization of the central nervous system[23]. The administration of mAb to TNF drastically reduced the inflammatory response and clearance of the fungus from the lungs[24]. Consistent with a critical role for TNF-α, mice deficient in both TNF-α and lymphotoxin-α gene were more susceptible to systemic cryptococcosis than their wild-type counterpart[49]. During the course of cryptococcosis, exogenous TNF-α favors clearance of the fungus. The production of TNF-α correlates with fungal burden in the blood and spleen[40] and appears to play a critical role in regulating the immune response against *C. neoformans*. The suppression of TNF-α synthesis by highly virulent strains could provide an explanation for their ability to elude host immune defense. Besides TNF-α, highly virulent strains have been shown to decrease IL-12, IL-18 and IFN-γ production in the initial phase of the immune response[22, 24, 26, 32].

A difference in the Th1-type response to highly or weakly virulent isolates of *C. neoformans* was observed in a murine model of pulmonary cryptococcosis. The cytokine profiles of spleens from infected mice showed production of IL-2 and IFN-γ early after infection with either isolate. However, the weakly virulent strain retained its ability to induce these cytokines, while the highly virulent strain lost it. In addition, IL-10 was produced by the spleens of mice infected with the highly virulent strain; IL-10-deficient mice survived longer than wild-type mice[1]. Thus, IL-10 is involved in the suppression of the protective response to *C. neofor-*

mans and in the *in vivo* inhibition of TNF secretion observed with the highly virulent strain. A human parallel for this result may be the report that TNF-α and IL-10 were detected in the plasma of AIDS patients with cryptococcosis[40].

The role of Th2 cytokines, IL-4 and IL-5, has been reported in a murine experimental model of intratracheal infection with *C. neoformans*. IL-4 suppressed host protection and reduced local IFN-γ production, whereas an opposite effect was obtained with administration of mAb to IL-4[30]. HUFFNAGLE et al.[21] used a murine model of pulmonary cryptococcosis to study the involvement of IL-5 in promoting pulmonary eosinophilia and inflammatory damage. Genetically susceptible C57BL/6 mice developed eosinophilia in the lung following *C. neoformans* infection which was associated to IL-5 mRNA in the lung. Administration of mAb to IL-5 prevented eosinophil recruitment. A similar phenomenon was observed by depleting CD4, which ablated IL-5 production. Pulmonary eosinophilia appears to be associated with an inability to clear *C. neoformans* infection[21].

Many details on cytokine interaction have been elucidated and the effect on the immune response to *C. neoformans* has been described, characterizing the behavior of protective or non-protective immune responses to the fungus. However, the majority of studies were performed in an experimental mouse model. Mice are more susceptible to *C. neoformans* infection than humans, while rats and rabbits appear relatively more resistant and are more similar to humans[4]. This could be due to the fact that mice rely entirely on cellular immunity and have difficulty in mounting an adequate antibody response. In contrast, like humans[10], rabbits mount an antibody response to capsular polysaccharide[16], which probably contributes to the resistance of this species to cryptococcosis.

Costimulatory Signals in Immune Regulation to *C. neoformans*

The relationship between the innate and adaptive immune responses to microbial products is interactive via cross-talk between natural effector cells, including phagocytic cells, and B and T cells. Communication between these two types of cells involves both cytokines as soluble factors and costimulatory molecules, the latter implying cell-to-cell contact. Cytokines are known to have multiple and diverse regulatory effects on costimulatory molecules. Although much information has been reported on cytokine involvement in the regulation of costimulatory molecules in experimental models, data on their involvement and role in the immune response to *C. neoformans* are scarce.

We recently reported that B7-1 costimulatory molecules are up-regulated on human monocytes treated with a low virulent acapsular strain of *C. neoformans*. In contrast, weak modulation was observed using a virulent encapsulated strain. It seems unlikely that the simple difference in phagocytosis between the acapsular and encapsulated strains could provide an explanation for B7-1 regulation. Costimulatory molecules are involved in T cell activation, as demonstrated by the decreased T cell proliferation using mAb to B7-1 and mAb to B7-2. In particular, B7-2 expression was required to initiate and maintain T cell activation to *C. neoformans*[43].

B7-1 and B7-2 molecules provide a major costimulatory signal, their natural ligands on T cells being CD28 and CTLA-4. Positive and negative regulation of the immune response could be a consequence of the interaction of B7 molecules with CD28 and CTLA-4. B7/CD28 ligation mediates a positive immune response. In contrast, B7/CTLA4 interaction may predict negative immunoregulation. Recently, MURPHY and MCGABA[47] described up-regulation of the delayed-type hypersensitivity response and IFN-γ production in a murine model of cryptococcosis in mice treated with anti-CTLA-4 Fab fragments. Unpublished results from our group suggest a correlation between virulence and the ability to up-regulate CTLA-4 on T cells *in vitro*. The over-expression of different sets of costimulatory molecules may cause opposite effects in the T cell response[45]. However, a subtle and unstable equilibrium between the cytokine response and costimulatory molecules may determine the outcome of *C. neoformans* infection. The cross-regulatory effect between costimulatory molecules and cytokines in response to *C. neoformans* has received limited attention. Further studies are needed to designate the exact role for each component in order to clarify the immunopathological events following *C. neoformans*-host interaction.

Concluding Remarks

A remarkable understanding has been achieved regarding the cytokine response to *C. neoformans*. This has contributed extensively to elucidating the induction, regulation and maintenance of the immune response to the fungus. First, the cytokine profile associated to the Th1 response is critical in clearing the fungus from infected tissue in mice and, probably, in humans. In-

flammatory cytokines play an important role by recruiting immune cells to the site of infection and by activating phagocytic antimicrobial functions. Secondly, there is evidence that antibody to capsular polysaccharide favors an efficient T cell response, likely a Th1-type response. Thus, the presence or absence of specific antibodies could influence the dominant cytokine profile. Thirdly, based on the dynamics of protective cytokine production, immunotherapeutical approaches may be designed to fight the virulence of the fungus at the appropriate phase of the immune response.

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