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Protective and pathologic immune responses against *Candida albicans* infection

Robert B. Ashman

School of Dentistry, University of Queensland, Brisbane, Queensland 4072, Australia

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1. ABSTRACT

Candida albicans is an important opportunistic fungal pathogen. Clinical observations have indicated that both innate and adaptive immune responses are involved in recovery from initial infection, but analysis in murine models has shown that the contribution of the two arms of the cellular immune response differ in oral, vaginal, and systemic infections. The relative contributions of T cells and phagocytic cells, and the cytokines that mediate their interactions are discussed for each of the different manifestations of the disease, and the consequences of infection, in terms of protection and pathology, are evaluated.

2. INTRODUCTION

The yeast *Candida albicans* is a widespread opportunistic pathogen. Infections of the oral and genital mucosa are frequently encountered in medical and dental practice, and invasive candidiasis represents a continuing threat in the hospital environment. *C. albicans* alone accounts for approximately half of the mortality attributed to systemic forms of the disease (1).

It is generally acknowledged that debility or defects in host resistance predispose to *Candida* infection; however, individuals with defects in cell-mediated immunity tend to be susceptible to mucocutaneous

candidiasis (2), whereas systemic infections are more commonly associated with neutropenia (3) or defects in neutrophil function (4). Despite considerable research, dissecting the mechanisms of host resistance has been an arduous process, attributable, at least in part, to the different manifestations of infection in humans (oral, vaginal, systemic, cutaneous), and the different contributions from the innate and adaptive immune systems to the resolution of each.

Most analysis of the host response has been carried out using mouse models, that closely mimic human oral (5) and systemic disease (6). It is proposed here to review and discuss the mechanisms of recovery from primary infection, induction of protection in the host, and the existence and possible significance of host-damaging responses elicited by *C. albicans* infection.

3. MUCOCUTANEOUS CANDIDIASIS

Candida organisms can be isolated from the oral cavity, gastrointestinal tract, and vagina, of many healthy humans, but few develop signs or symptoms of disease, which typically only appear in association with conditions that lead to alterations in the integrity of the host response.

Although clinical studies have demonstrated a strong correlation between susceptibility to oral or oro-pharyngeal candidiasis and defects in cell-mediated immunity, exemplified particularly by the high incidence of oro-pharyngeal candidiasis in HIV patients with low $CD4^+$ cell counts (7), similar associations with other mucosal infections are less convincing. It must be emphasized also that such correlations are relevant only to secondary infection, as virtually all adults have been exposed to, and developed immunity to, the organism as children.

For this reason, analysis of host responses to primary infection has predominantly been addressed in experimental animals.

3.1. Oral candidiasis

There are few models of oral candidiasis in mice. An early report (8) found that oral infection induced both an inflammatory and a cell-mediated immune response. The number of intraepithelial $CD4^+$ cells was also increased in infected compared to control mice (9). Further studies (10) compared primary immune responses in BALB/c and DBA/2 mice, that carry the same major histocompatibility complex (MHC) haplotype (H-2d). The proportion of $MAC\text{-}1^+$ cells in the oral mucosa was increased in both strains, both demonstrated similar levels of recruitment of $CD4^+$ and $CD8^+$ cells, but infection in BALB/c mice was associated with an earlier influx of □amma□elta T cells when compared with the susceptible DBA/2 strain, that correlated with a more rapid clearance of yeasts from the mucosa (10).

Differential expansion of the □amma□elta T cell population after oral infection was confirmed in an independent study (11), that also demonstrated significantly higher *Candida*-specific T-cell proliferation in resistant BALB/c mice as compared to the susceptible DBA/2 strain. Clearance of *C. albicans* from the oral mucosa of BALB/c mice correlated with early production of interleukin-4 (IL-4), IL-12, and interferon-gamma (IFN-□amma)by cells from the cervical lymph nodes, whereas in the DBA/2 strain, secretion of IL-4 was delayed, and the levels were lower. A role for IL-4 as a mediator of clearance was demonstrated by neutralization using a monoclonal antibody in BALB/c mice (11). This resulted in an increased fungal burden, and delayed clearance of the yeast. Somewhat surprisingly, inhibition of IL-4 production in DBA/2 mice resulted in an increase in IFN–□amma production (12). While consistent with the known reciprocal relationship between expression of Th1 and Th2 cytokines, the result suggests that the action of these cytokines in the control of oral candidiasis may be more complex than has hitherto been described.

A requirement for T cells in recovery from oral candidiasis has been supported by studies in two infectious disease models. When mice were infected with the Du5H(G6T2) strains of mouse leukemia viruses, they developed a disease called murine AIDS (MAIDS), that exhibited many of the immune abnormalities found in human HIV infection. Following oral colonisation with *C. albicans*, retrovirus-infected mice developed a carrier state, which, in about one third of the animals, was punctuated by recurrent episodes of acute *Candida* proliferation, thought to result from virally-induced fluctuations in the levels of CD4+ T cells (13). In a similar experimental system, transgenic mice that expressed HIV type 1 in immune cells developed an increased oral burden of *C. albicans* (14), characterised by penetration of hyphae into the stratified squamous epithelium of the oral cavity, and a mononuclear inflammatory cell infiltrate in the mucosa.

Another approach has used congenitally athymic 'nude' mice. These animals lack functional T cells, and following oral inoculation with *C. albicans*, developed chronic infections that did not clear (5). Histological examination demonstrated extensive infiltration of the oral epithelium and formation of microabscesses. Interestingly, CBA/CaH mice, that are known to develop severe tissue pathology after systemic infection (15), also showed increased severity of oral lesions compared to the more resistant BALB/c mice. This correlated with a higher degree of colonisation of the oral mucosa in these mice (5).

When immune competence was restored by the transfer of syngeneic lymphocytes, established infections were cleared (5); however, the extent of tissue damage in the two strains did not influence recovery, as there was no difference between the 'susceptible' CBA/CaH mice and the 'resistant' BALB/c strain in the rate of clearance of the yeasts from the oral mucosa. Recovery was mediated by the $CD4^+$, but not the $CD8^+$ lymphocyte subset, and was associated with the appearance of IFN-gamma and IL-12 in the cervical and submaxillary lymph nodes of the

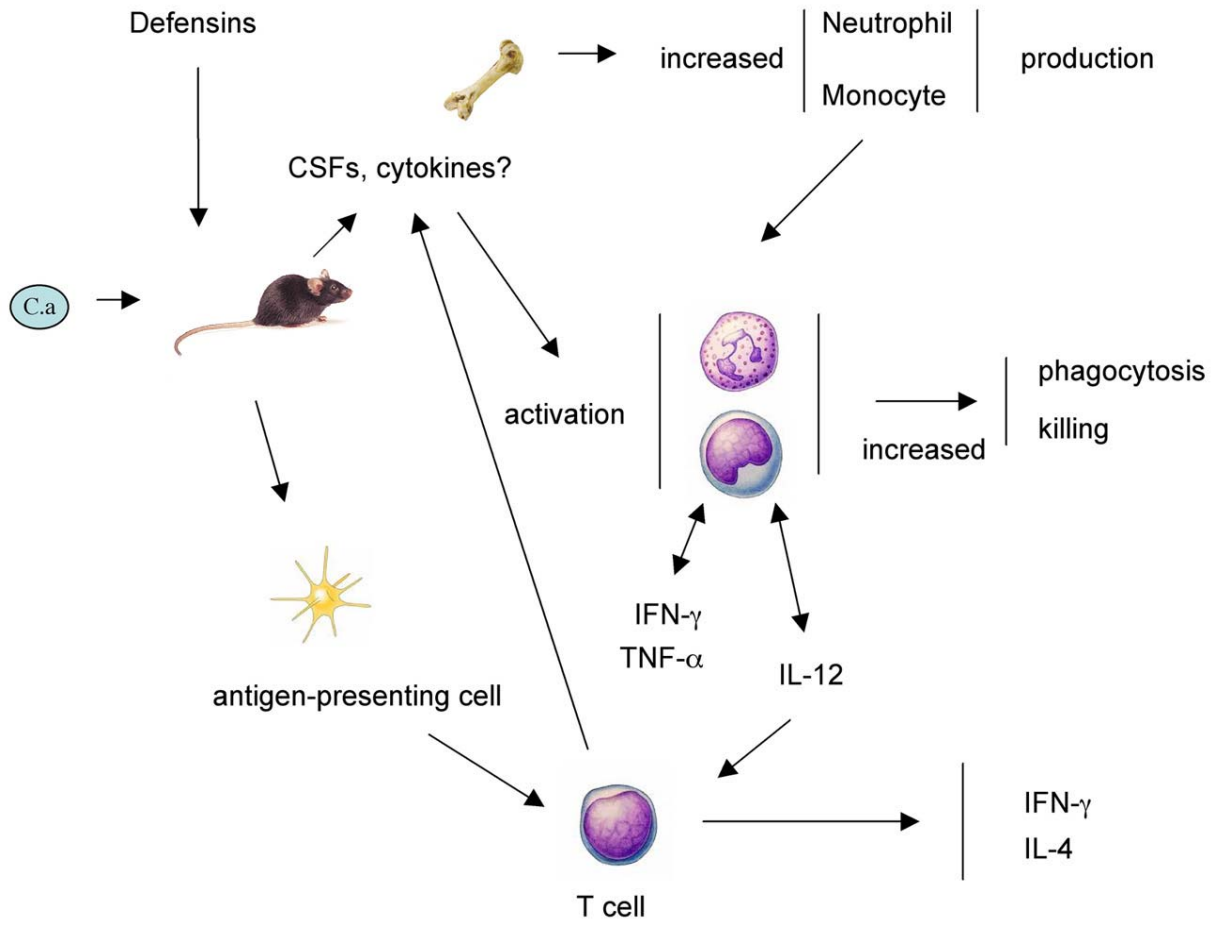

Figure 1. Schematic illustrating pathways of host responsiveness against oral challenge with *C. albicans*.

reconstituted mice. Postulated pathways of host responsiveness are illustrated in Figure 1.

Confirmation of a requirement for T cells in recovery from primary oral candidiasis by antibody depletion of CD4 or CD8 cells from normal mice was unsuccessful; however, in mice that received irradiation to the head and neck, depletion of $CD4^+$, but not $CD8^+$, lymphocytes resulted in a prolongation of infection, with increased severity of the oral lesions (16). As in the nude mice, high levels of IL-12 were produced by cells from the draining lymph nodes, but concentrations of IFN-gamma were not significantly different from those in controls.

Although T cells were essential for recovery to occur, clearance was mediated by the actions of phagocytic cells. Depletion of neutrophils, or inactivation of macrophages/monocytes increased oral colonisation in both BALB/c and CBA/CaH mice (17), though the effect was less marked in the latter (susceptible) strain. Depletion of both phagocytic cell populations further increased infection in BALB/c mice, but dramatically exacerbated the fungal burden in the CBA/CaH strain. Thus, phagocytic activity from either the neutrophil or monocyte/macrophage cell population appears necessary to protect the susceptible CBA/CaH mouse, whereas the more resistant BALB/c strain may utilise other innate mechanisms to control yeast proliferation, or simply present an environment that is less favourable to colonisation.

Candida killing can be mediated by reactive oxygen intermediates (18-20), but nitric oxide (NO) has also been shown to be an effective candidacidal agent (21). In the experiments of Elahi (12), NO was present in the effector phase of the response, with concentrations in the saliva increasing after infection. Saliva from infected mice inhibited the growth of yeast *in vitro*. Administration of NG-monomethyl-L-arginine (MMLA), which inhibits NO synthesis, to orally-infected mice led to an increase in *C. albicans* infection, and a concomitant abrogation of expression of mRNA for IL-4, but not IFN-gamma, in lymphocytes from the draining lymph nodes. Contrary to previous findings (22), mice treated with anti-IL-4 monoclonal antibody showed a marked inhibition of NO production in both saliva, and in cultures of cervical lymph node cells after stimulation with *C. albicans* antigen (12).

The nature of the link between the T cells and the phagocytic cells has not been established. While it is tempting to invoke the simple T helper cell paradigm, with

a Th1-style response leading to activation of the phagocytic cells for increased *Candida* killing (23), the data are not fully consistent with this interpretation. IL-12 and IFN-gamma were the major cytokines secreted by lymphocytes from the draining lymph nodes of recovering animals (17), but levels of IL-4 and IL-10 did not show any association with recovery from oral infection, and tumour necrosis factor-alpha (TNF-alpha) was the only cytokine that was detected specifically in the infected oral mucosa (24).

Further recent studies have shown that recovery from primary oral infection is independent of the presence of IFN-gamma, IL-4 and IL-10 (25), nor was iNOS essential for the host response. In the absence of TNF-alpha, the severity, but not the duration of infection, was increased. IL-12p40 knockout mice, however, developed chronic oro-pharyngeal candidiasis that was of equal, if not greater severity than that developed by the T cell-deficient nude mice. As IL-12 is not produced by T cells, it can be inferred that the recovery from oral infection involves activation of macrophage function via an IL-12-dependent pathway, the nature of which remains to be elucidated. The failure to identify a specific link between the T cells and macrophages may reflect redundancy among the cytokines, or the existence of alternative mechanisms of host defence.

A possibility for which there is some evidence is that T cells act to enhance production of phagocytic cells by the bone marrow. Studies in chronically orally-infected nude mice have demonstrated that bone marrow colony formation *in vitro* is significantly depressed in the CBA/CaH mice compared to BALB/c (26). Reconstitution with T cells significantly increased the colony-forming response in BALB/c, but not in CBA/CaH mice. These data are consistent with the hypothesis that the genetic susceptibility to tissue damage may be related to functional differences in innate immune reactivity between the two strains.

Although the T cell response is essential for recovery from oral candidiasis, other innate mechanisms have the potential to contribute to clearance of the organisms. When nude mice, that develop chronic infections, were challenged with progressively lower doses of the yeast, the magnitude of the fungal burden in the oral cavity decreased proportionally, until eventually, the nude mice were able to clear the infection completely (27). The mechanism of this response is unknown, but may involve either direct inhibitory activity of oral epithelial cells (28), or the ability of these cells to recruit phagocytic cells through the production of inflammatory cytokines and chemokines (29)

3.2. Gastrointestinal candidiasis

Although gastrointestinal candidiasis is not a recognized disease in humans, the mouse model has provided valuable insights into mechanisms of resistance. As in oral candidiasis, an intact T cell response appears to be essential for recovery from primary infection.

Wild-type and T cell-deficient nude mice develop oro-gastric candidiasis (30), but only euthymic mice demonstrate *Candida*-specific lymphoproliferation and DTH responses, that correlate with the clearance of *C. albicans* hyphae from mucosal surfaces. The requirement for T cells was confirmed by the demonstration that T-cell receptor alpha- and delta-chain knockout mice, that are deficient in both alpha/beta and gamma/delta T cells, were highly susceptible to oro-gastric candidiasis (31), as were transgenic epsilon 26 mice, that have defects in both natural killer cells and T cells (32). Conversely, B cell knockout mice showed no increased susceptibility (33).

SCID mice, that lack functional T and B cells, were also shown to be susceptible to gastrointestinal infection (34), but dissemination from the gastrointestinal tract was uncommon. Mice with multiple immunodeficiencies showed different patterns of disease depending on the nature and extent of the immunological deficit. Singly immunodeficient (nu/nu, bg/bg) mice developed minimal to moderate mucosal infections, whereas doubly immunodeficient (bg/bg nu/nu) mice showed extensive yeast and hyphal infection of the oral and gastrointestinal mucosa, as well as a progressive systemic infection (35). Depletion of $CD4^+$ lymphocytes from bg/bg nu/+ mice increased their susceptibility to *Candida* infection of the tongue and esophagus (36), indicating that protection of mice from oro-gastric candidiasis was mediated by $CD4^+$ lymphocytes. However, antibody depletion of IL-2 and IFN-gamma, either separately or combined, did not abrogate their resistance; neither did treatment of susceptible bg/bg nu/nu mice with IFN-gamma or IL-2 protect them.

Extensive studies of host susceptibility and resistance, and the induction of protection using an avirulent isolate of *C. albicans*, have shown that the generation of protective host responses is associated with expression of a T helper type 1 (Th1) cytokine profile by $CD4^+$ T cells (37). Systemic infection of BALB/c mice with a virulent isolate caused early mortality, whereas infection of the gastrointestinal tract resulted in the production of both Th1- and Th2-type cytokines by $CD4^+$ cells from Peyer's patches and mesenteric lymph nodes, at a time when the yeasts were being cleared from the intestine (38). In contrast, DBA/2Cr mice, that develop fatal disseminated candidiasis after intravenous infection with the avirulent strain (37), developed Th1-type cell-mediated immune responses after intragastric inoculation with the virulent strain, and were able to clear the infection (39). When mice with gastrointestinal candidiasis were treated with soluble IL-4 receptor (sIL-4R), a Th1-type response was promoted, and clearance of the fungus from the stomach was accelerated (40). These data suggest that activation of Th1- but not Th2-like responses may be responsible for controlling oro-gastric candidiasis and generating protective immunity.

Mice lacking the IFN-gamma receptor (IFN-gamma $R^{-/-}$) were more susceptible to gastrointestinal infection than wild-type mice (41), showing increased fungal growth in the stomach, and failing to develop Th1-mediated acquired immunity. The impaired resistance correlated with defective IL-12 responsiveness, but was

independent of IL-12 production, suggesting that IFN-gamma is required for the development of IL-12-dependent Th1 immunity. However, IL-12 is required for IFN-gamma production, and IL-12-deficient mice, that showed elevated production of IL-4 with defective production of IFN-gamma, were highly susceptible to primary or secondary gastrointestinal challenge with *C. albicans* (42). There is also evidence for a positive regulatory loop between IL-12 and IL-10 that promotes optimal Th1 responses, even though it may compromise the efficiency of the innate immune response. IL-18 is also a strong stimulator of IFN-gamma production, and treatment of normal mice with IL–18-specific antibodies increased susceptibility to candidiasis (43). However, as neutralization of IL-18 in IFN-gammagene knockout (GKO) mice had no effect, it was concluded that IL-18 acted indirectly, via endogenous IFN-gamma. Comparable results were obtained using IL-18 GKO mice (44). This system also demonstrated a down-regulation of IFN-gamma synthesis and a defect in macrophage inflammatory protein-2 (MIP-2) production, with a reduced recruitment of monocytes to the sites of *Candida* infection

The gamma/delta subset of T cells has been implicated in the defence of the oral mucosa (11), and oral colonization of B cell-deficient mice, which have a normal T cell response, increased the number of both alpha/beta and gamma/delta T cells in the gastrointestinal mucosa (45). Intraperitoneal infection of either inbred, B cell-deficient, or $beta_2$ microglobulin knockout ($CD8^+$ T cell-deficient) mice, resulted in a rapid increase in gamma/delta T cells in the peritoneal cavity (46). These cells were shown to enhance macrophage nitric oxide production and candidacidal activity, and contributed to host defence *in vivo*, as demonstrated by increased susceptibility of mice to *C. albicans* infection after treatment with a monoclonal antibody against the gamma/delta T cell receptor. As gamma/delta T cells have also been shown to be involved in the induction of Th1 and Th2 responses (47), this may also represent a mechanism linking clearance via innate immunity with specific adaptive immune responses to the organism.

It was inferred from the above (46) that nitric oxide production was involved in gamma/delta T cell-mediated resistance to *C. albicans* administered intraperitoneally, and its role in host defence was further evaluated in immunodeficient SCID and C.B-17 mice (48). The candidacidal activity and nitrite-producing capacity *in vitro* of activated resident peritoneal macrophages was reduced by treatment with an inhibitor of NO synthase, and *C. albicans*-monoassociated SCID mice treated with the inhibitor developed more severe oro-gastric candidiasis than controls. Although these results tended to support a direct role for NO in clearance of *Candida* infection, further studies (49) suggested that NO was candidastatic rather than candidacidal, and that it was associated with, or induced, other macrophage candidacidal mechanisms.

The role of both reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) in the control of systemic and mucosal candidiasis was further examined in mice deficient for phagocyte oxidase (Phox) and nitric oxide synthase 2 (NOS2) (50). Doubly deficient ($phox^{-/-}/NOS2^{-/-}$) mice were highly susceptible to infection, and died within 12 to 15 days after oral colonization. However, the candidacidal activities of the phagocytic cells from the peritoneal cavity of either single or double GKO mice were not different from those of the wild-type controls, indicating that factors other than ROI or RNI are competent to control proliferation and growth of *C. albicans*. Infection was associated with up-regulation of expression of IFN-gamma, TNF-alpha, and the chemokines MIP-2 and KC (the mouse ortholog of IL-8) at the site of infection, suggesting that dysregulation of inflammatory reactions might contribute to mortality in this model.

Many different effector mechanisms have been implicated in the host response, but phagocytic cells play a major role. Immunodeficient SCID mice treated with cyclophosphamide, which causes severe neutropenia, demonstrated increased susceptibility to mucosal candidiasis (51), although infection remained confined to the mucosal surfaces, and did not disseminate. In contrast, impairment of macrophage function by administration of poly (I-C), that is a potent inducer of interferons, increased susceptibility to disseminated candidiasis of endogenous (gastrointestinal tract) origin (52, 53). The resistance of immunocompetent controls to mucosal candidiasis was not altered by treatment with poly(I-C) alone, and interference with both macrophage and neutrophil function was necessary to render these mice susceptible to the disease (54). In the absence of activation, *Candida* killing by phagocytic cells (both neutrophils and macrophages) is relatively inefficient, but the candidacidal potential of both cell types is significantly enhanced by exposure to Th1-type cytokines, such as IFN-□□□□□ and TNF-alpha(55-57).

It is widely acknowledged that many functions of the innate and adaptive immune responses are mediated by cytokines, which also orchestrate interactions between them in responses to infections. The study of candidiasis in GKO mice has contributed to the debate about the relevance of various cytokine pathways, but it has done little to clarify the issue, as studies have not been concordant. The pro-inflammatory cytokine IL-17A is important in activation of the innate immune response, and deletion of this gene dramatically decreased survival, with substantial increases in the fungal burden in the kidneys (58). In contrast, mice lacking IFN-gamma have been variously reported to show increased susceptibility to both gastric and systemic candidiasis (59); to show no altered response to either form of the disease (60), and to show decreased survival after intraperitoneal infection (61), although the mortality did not correlate with the extent of organ colonization. IL-10 GKO mice were more resistant than controls to both gastrointestinal (62) and systemic (63) candidiasis, but the susceptibility of mice lacking IL-4 was unchanged (61).

Early studies (64) had suggested that TNF-alpha might serve a protective function against *C. albicans*

infection *in vivo*, and consistent with this, TNF–alphą GKO mice showed an increase in the severity, (65), but not the duration (25) of both oral and systemic disease. Mice lacking both TNF-□lphąand lymphotoxin-□lphą (LT) showed markedly increased susceptibility to both gastrointestinal and systemic candidiasis (66, 67). This was attributed to delayed recruitment of neutrophils and a reduced phagocytic capacity in these mice; however, oxidative metabolism and candidacidal activity were unaltered (66). Susceptibility to infection was correlated with impaired development of protective Th1 responses (67), although in these experiments, all functions were improved by treatment with recombinant TNF-alpha. The increased susceptibility of TNF-alpha/LT double knockout mice to *Candida* infection after intra-abdominal infection (68) was also associated with impaired neutrophil function and a delayed Th1 response.

A role for Th1 cells, particularly in the later stages of the response, could be inferred from the increased susceptibility of mice from which the CD40 ligand had been deleted (69). The increased susceptibility was associated with reduced concentrations of circulating TNF-□lpha, and was attributed to decreased candidacidal activity of macrophages as a consequence of lower nitric oxide production by the GKO mice. However, the importance of recruitment of inflammatory cells was confirmed by a reduction in the candidacidal capacity of polymorphonuclear leukocytes, and a slower recruitment into infected tissues, in mice lacking the homologue for the IL-8 receptor (70).

Given the complex relationships between them, conflicting data concerning cytokine pathways relevant for protection against *Candida* infections can be attributed not only to differences between experimental systems, but also to differential expression of pathogen-associated molecular patterns (PAMPs) on different strains or isolates of the yeast, and to real differences in the host response against particular manifestations of the disease. In addition, inbred mice not only express significantly different background levels of cytokines such as beta defensins, TNF-alpha and IL-12 in the gastric mucosa (71), but also different patterns of response to challenge, even though they were equally susceptible to infection.

3.3. Vaginal candidiasis

Approximately twenty percent of otherwise healthy women carry *Candida* in the vagina (72), and three to five percent of these are susceptible to frequent recurrences, the basis for which is still unknown. No satisfactory animal model of recurrent *Candida* vaginitis has yet been developed, and most experimental studies have utilized oestrogen-conditioned mice. A persistent infection could be established after treatment with physiological or near physiological concentrations of estrogen (73), but pseudoestrus had to be maintained for the infection to persist.

Most inbred and outbred strains of mice were found to be similarly susceptible to vaginal candidiasis (74). The only exceptions were the outbred strain CD-1, that was markedly resistant, and CBA/J that was moderately resistant. The basis for the resistance of these strains is unknown, although CD-1 is known to be resistant to endocrine disruption by oestrogen (75), and thus may not respond to the oestrogen priming necessary to establish experimental infection.

The severity of vulvovaginal candidiasis in nude mice was comparable to that in euthymic controls (76), however, in about thirty percent of nude mice infected intravaginally, *Candida* was recovered from extra-vaginal sites, such as the kidney, liver, and small intestine (77). In these studies, adoptive transfer of either immune or non-immune syngeneic T cells into the infected nude mice had no affect on either the magnitude of infection or vaginal inflammation, but protected the animals from extra-vaginal dissemination. These results suggested that T cells might play a role, albeit perhaps minor, in constraining dissemination of the yeast from the vaginal mucosa.

After vaginal infection, both oestrogen-conditioned and control mice developed equivalent levels of cell-mediated immune reactivity, and T cell memory (78), even though the severity and duration of the infection was much less in the control mice (79). Both groups developed delayed-type hypersensitivity responses, and demonstrated synthesis of IL-2 and IFN-gamma, but no IL-10 and only small concentrations of IL-4, in the draining lymph nodes. These data were consistent with the elicitation of a Th1-type response in the peripheral circulation; however, although depletion of $CD4^+$ cells reduced both *Candida*-specific DTH, and lymph node cell Th1-type cytokine production during a primary vaginal infection, and depletion of $CD8^+$ cells reduced gamma interferon production (80), neither affected the severity or course of infection. This suggested that there might be limited lymphocyte traffic between the periphery and the vaginal mucosa.

Vaginal inoculation of *C. albicans* into oestrogen-conditioned mice resulted in a rapid increase in the total number of $CD3^+$ T lymphocytes in the vaginal mucosa, which persisted throughout the period of infection (81). The majority of the T cells belonged to the $CD8^+$ subset; however, significant numbers of both $CD4^+$ and $CD4^+CD8^+$ cells were also present throughout the infection period. Depletion studies demonstrated a low level of circulation of T cells from the periphery to the vaginal mucosa (82), and the expression of homing receptors and integrins on T cells from lymph nodes draining the infected vagina was reduced compared to uninfected mice (83).

Although this suggested that T cell recruitment might be hindered by lack of appropriate recognition molecules on the infiltrating lymphocytes, another study has demonstrated high levels of the immunoregulatory cytokine, transforming growth factor beta (TGF-beta), in the vaginal tissue of naive mice (84). TGF-beta was expressed at higher levels in the vagina as compared to other areas of the genital tract, was significantly increased as a as a result of pseudoestrus and/or infection, and

predominated in the draining, but not in the non-draining, lymph nodes during infection.

Unlike the situation in other modes of infection, depletion of neutrophils had no effect on the magnitude of the fungal burden or on the rate of clearance of yeasts from the vagina (85), although vaginal inflammation was significantly decreased in neutrophil-depleted animals. In this context, Fidel (86) has postulated that recurrent vaginal candidiasis is part of a spectrum of innate immune reactivity against vaginal infection with the yeast.

4. SYSTEMIC CANDIDIASIS

The brain and kidney are common targets of disseminated *Candida* infections in humans (86, 87), and these organs are also major foci of infection in mice (6, 89). Early after systemic inoculation, yeasts can be detected in the tissues in the absence of any inflammatory responses ((6), but abscesses, consisting of mixed inflammatory cells together with yeast and hyphal forms of the organism, gradually form, and the infection eventually resolves.

With increasing challenge doses, mice of all strains begin to die. Mortality is a good correlate of susceptibility and resistance in viral and bacterial infections; however, in *Candida* infections, the situation is more complex. When inbred strains are ranked by mortality or colony counts in the kidney, those deficient in the fifth component of complement are highly susceptible, and die after challenge with substantially lower doses of *C. albicans* than C5-sufficient mice (90, 91). However, in this latter group, there is no correlation between mortality or colonisation patterns and other known genetic markers, (92, 93).

In contrast, histopathological assessment found two discrete patterns of tissue damage in inbred mice of different strains (15). Severe lesions were characterised by numerous large abscesses containing yeast and mycelial growth forms within the necrotic debris, together with an inflammatory infiltrate consisting of mononuclear and polymorphonuclear leucocytes, whereas mild lesions showed similar characteristics, but were small and infrequent. The two patterns of tissue destruction do not show a strong correlation with either mortality, or quantitation of viable organisms in the brain (91), although this latter was generally greater in the susceptible strains. However, the kinetics of fungal growth and clearance were similar in both groups of mice, peaking on day 4 after infection, and falling away rapidly thereafter.

The propensity to develop mild or severe tissue damage is heritable, and studies in F1 hybrid and backcross mice have shown that this is controlled by a single codominant gene (currently termed *Candida albicans* resistance gene 1) that segregates in Mendelian fashion (94). Analysis in the AKXL recombinant inbred set (94) demonstrated that the *Carg1* was probably located between 42.5 and 51.7Mb on chromosome 14 (96). The mechanisms by which this gene determines the expression of mild or severe tissue damage have not yet been identified, but as the phenotypes are expressed in nude mice (97), they probably involve qualitative or quantitative differences in the effector functions of bone marrow-derived phagocytic cells. Data from the AKXL recombinant inbred mice indicated that a second gene was also involved in the regulation of tissue damage, and this was later confirmed in [C57BL/6 x C57L] x C57L backcross mice (98). This gene (*Carg2*) was also a Mendelian co-dominant; however, its effect appeared to be most prominent in the kidney. It regulated the severity of tissue damage but not the magnitude of the fungal burden.

BALB/c mice, that develop only mild lesions in the tissues after intravenous infection, also show a greater recruitment of inflammatory/immune cells to the draining lymph nodes following subcutaneous challenge than do CBA/CaH mice (99). However, the more susceptible CBA/CaH strain clears the yeast more rapidly from the spleen. This may reflect a disjunction between immune responses and effector activity in various anatomical regions, comparable to that reported by Spellberg (100). In those experiments, a Th1-type cytokine response developed in the kidney after sub-lethal infection, whereas a Th2 response was seen after lethal challenge. However, the spleen showed Th2 responses only.

4.1. Role of complement

Despite the above, mapping studies have confirmed that the dominant genetic influence on mortality can be attributed to the fifth component of complement (101, 102), although the magnitude of the effect of C5 deficiency differs depending on the route of infection and the strain of mouse.

Strains such as A/J and DBA/2 are ten to one hundred-fold more susceptible to lethal challenge than C5-sufficient mice (90), and demonstrate a greatly increased fungal burden in the kidneys (91). The essential role of C5 in enabling these mice to resist lethal infection was demonstrated by passive transfer of C5-sufficient serum prior to challenge (103). The fungal burden in the kidneys of reconstituted mice was reduced, and survival was prolonged. Other C5-deficient strains, such as AKR, show a lower mortality (91, 104), and in mice bred on the B10 background, the effect of C5-deficiency, although still significant, was much less marked (105), and did not affect the ultimate outcome of challenge.

Although increased kidney colonisation is a feature of infection in C5-deficient mice, it may not be the immediate cause of mortality. After lethal infection, A/J mice became moribund within twenty-four hours (106), but displayed little if any kidney damage despite an inability to mobilize granulocytes and a high fungal load in the kidney. The heart was the organ most affected, exhibiting tissue damage associated with cellular infiltration, which was not seen in the kidney (107); and a strong inflammatory response, with elevated levels of circulating TNF-alpha, IL-6, monocyte chemotactic protein 1 (MCP-1), MCP-5, and eotaxin (106). In contrast, C57BL/6J mice died late in the course of infection, showing severe kidney pathology, with

extensive fungal replication and tissue damage associated with a neutrophil-based inflammatory response (106).

It follows that C5 is crucial in limiting the initial fungal growth in the kidney (103, 104); however, as the candidacidal activity of phagocytic cells from normal and C5-deficient mice is equivalent (108), it is clear that the biological properties of C5 are critical factors in the early containment and elimination of the yeast. C5-deficient mice were less efficient in clearing cutaneous infection, but their initial responses, and the recruitment of neutrophils to the skin lesions, were normal (109). Interestingly, the severity of mucosal colonisation in C5 deficient mice was unaltered (103).

A rapid recruitment of inflammatory cells may be essential for protection of organs, such as the kidney, that are highly susceptible to fungal invasion, whereas in other anatomical regions, such as the skin or oral cavity, the proliferative capacity of the yeast may be more limited, and other host resistance mechanisms may assume a more important role.

4.2. Cellular responses

It has long been known that phagocytic cells, neutrophils (110, 111) and macrophages (112, 113) in particular, are crucial for recovery from initial infections with *C. albicans*, and that the adaptive immunity and the T cell response play only a minor role (114).

This latter conclusion has been reinforced by repeated demonstrations that immunodeficient 'nude' (97, 115, 116) and SCID (117) mice are no more susceptible to systemic infection than euthymic controls. However, as nude mice raised under germfree conditions demonstrated an increased susceptibility to infection compared to mice bred in a conventional environment (118), it was thought that the resistance to disseminated candidiasis of T cell-deficient mice could be attributed to the non-specific activation of macrophages known to occur in these animals (119). Nevertheless, macrophage activation by treatment with BCG did not enhance resistance (120), and it was later shown that mice lacking both □lpha/□eta and □amma/□elta T cells were as resistant to acute systemic candidiasis as appropriate controls (31). In addition, transgenic epsilon 26 mice, that have defects in both natural killer cells and T cells, were resistant to acute systemic candidiasis (32), and granulocytes were shown to be the major effector population responsible for protection in this model.

There are, however, numerous reports of the T cell-dependence of host responses against disseminated *Candida* infection (reviewed in (23). Although an involvement of T cells in the generation of protection against re-infection is not contentious, this model has also used various combinations of yeasts (attenuated and virulent), and mouse strains (DBA/2 and BALB/c), to produce infections that either resolved with the development of resistance to re-infection, or that led to chronic disease and death. Resolution of infection and the development of resistance was associated with the generation of a Th1 cytokine profile by $CD4^+$ spleen cells *in vitro*, whereas cells from animals with chronic disease exhibited a Th2 profile (121). Neutralization of IFN-gamma *in vivo* prevented the development of protective Th1 responses (122) whereas neutralization of IL-4 reduced mortality, with a concomitant induction of a Th1 cytokine profile and development of protective immunity (123). Nevertheless, activation of phagocytes by T cell-derived cytokines was necessary for the full expression of resistance against the disease (23).

The apparent conflict between the mechanisms demonstrated in different models has yet to be resolved, but at present, the weight of evidence appears to favour components of the innate immune response as the dominant mechanisms responsible for clearance of yeasts from the tissues after initial systemic infection (Figure 2). The pathways by which this occurs are poorly understood, but almost certainly involve recognition of pathogen-associated molecular patterns (PAMPs) on the yeast by receptors on the phagocytic cell, leading to the activation and release of cytokine that initiate inflammatory responses. The role of Toll-like receptors (TLRs) in host resistance is currently a major focus for investigation.

4.3. Role of Toll-like receptors

Toll-like receptors are a large class of recognition molecules for phagocytic cells, and mediate macrophage recognition of many different microbial ligands. When the genes for TLR2, TLR4 and TLR6 were deleted from a mouse macrophage cell line, only ablation of the gene for TLR2 completely abolished the cytokine production by murine macrophages in response to *C. albicans* phospholipomannan (124). Contrary to this, macrophage cell lines generated from the bone marrow of TLR2 GKO mice showed an increased ability to contain *C. albicans* infection compared to $TLR2^{+/+}$ controls (125), although cytokine production was equivalent. The interpretation of these latter findings has been disputed (126).

Studies on the role of TLR2 using GKO mice have given conflicting results. One demonstrated significantly increased mortality after either intravenous or intraperitoneal infection (127), associated with a decreased macrophage production of TNF-alpha and macrophage inhibitory protein-2 (MIP-2). However, phagocytosis and production of reactive oxygen intermediates by these cells was unaltered. Another (128) found that GKO mice were more resistant to disseminated infection, and concluded that signaling through TLR2 enhanced IL–10 production and promoted the survival of a population of regulatory T cells that reduced the efficiency of the innate immune response. TLR4 GKO mice were also found to be highly susceptible to *C. albicans* infection (129), although the candidacidal potential of the neutrophils and macrophages was normal, and cytokines synthesis by macrophages was unaffected. The increased susceptibility of the infected mice appeared to be due primarily to impaired chemokine expression and recruitment of neutrophils.

A different group (130) compared susceptibility to intravenous infection with either low-virulence *Candida*

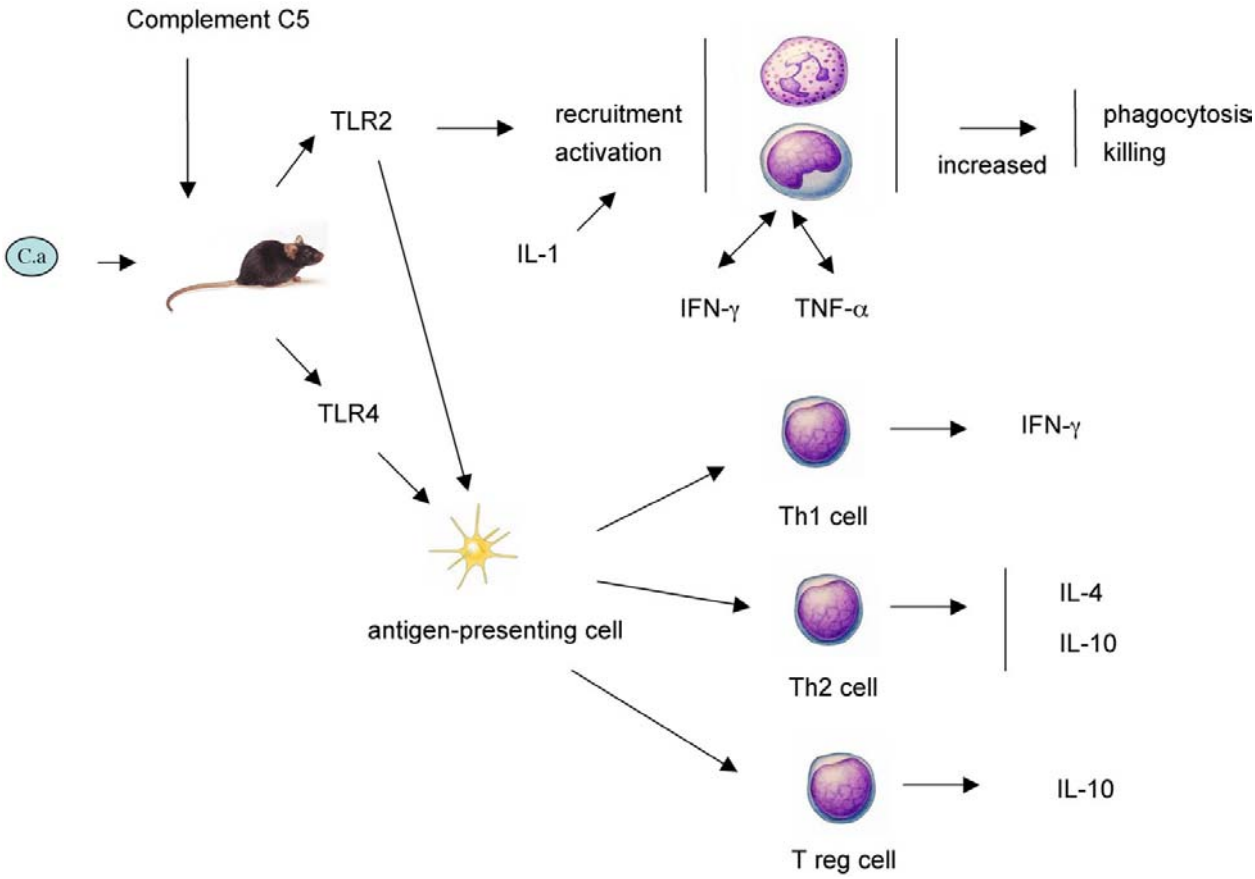

Figure 2. Schematic illustrating pathways of host responsiveness against systemic challenge with *C. albicans*.

yeasts, or virulent *Candida* hyphae, in mice with deletions in genes coding for various TLRs. In TLR2 GKO mice, survival was unchanged, but the fungal burden in the organs was significantly decreased. Deletion of TLR4 had no effect on the severity of systemic infection with either virulent or avirulent *Candida*, whereas mice TLR9 GKO animals showed increased resistance. Surprisingly, MyD88 GKO mice, in which production of TNF-alpha following activation of TLR2 was impaired, developed an increased fungal burden following challenge with the avirulent, but not the virulent strain. Other studies, also using macrophages from MyD88 GKO mice, found that recognition of *C. albicans* (131), as well as phagocytosis and intracellular killing (132), was impaired compared to controls, and cytokine production substantially decreased. Activation of macrophages for cytokine production via stimulation of the TLRs is usually thought to involve the transcription factor NF(kappa)B, but other paths have also been implicated. The leucine zipper transcription factor C/EBP(beta) is known to play a part, as demonstrated by the increased susceptibility to *C. albicans* infection of C/EBP(beta) GKO mice (133), and the impaired expression of the genes for TNF-alpha, IL-6, and iNOS (134), even though activation of NF(kappa)B was normal.

TLR ligands can be recognized as soluble proteins (135), and may not function as phagocytic receptors, but as regulators of phagocytosis. Although the TLR pathway provides highly conserved responses to infectious agents, flexibility may be conferred by cooperative interactions between TLRs and other receptors. Thus, Dectin-1 has been shown to be a major macrophage receptor for □eta-glucans (136), and both DC-SIGN and other c-type lectins appear to be important for phagocytosis of non-opsonised *C. albicans* (137). SIGNR1 is a mannan-inhibitable receptor, distinct from the mannose receptor, that cooperates with Dectin-1 in internalization of particles (137), but neither binding of Dectin-1 nor SIGNR1 appear to be essential for TNF-□lpha production. Although beta-glucan is a major component of yeast cell walls, its recognition depends on the form of the yeast, as it is exposed only at bud scars (138). It is as yet uncertain whether Dectin-1 is (139) or is not (140) required for recovery from *C. albicans* infection *in vivo*.

Although the results of experiments using either GKO mice or cells have not as yet been definitive, recent gene-profiling studies in both humans (141, 142) and mice (Wells et al, unpublished), have demonstrated up-regulation of genes encoding pro-inflammatory cytokines, such as TNF-alpha, IL1 and IL-6, as well as increased expression of genes for a number of chemokines. Given the emphasis on T cells in the evolution of host responses against the yeast (23), it was surprising that there was no evidence of differentiation along the lineage leading to antigen presentation, and that expression of genes encoding T cell regulatory molecules such as IL-12, IFN-□amma and transforming growth factor-beta was not significantly

altered. As it is known that TLR2 signaling through NF(□□□□□B is sufficient for the differentiation of monocytes/dendritic cells towards the antigen-presentation pathway (143), this provides indirect evidence that TLR2/ NF(□□□□□B may not be the primary transcriptional target of pattern recognition receptors detecting yeast PAMPs, and is consistent with a lack of lymphocyte involvement in the early stages of the response.

TLR2 and dectin-1 can act synergistically to induce synthesis of TNF-□lpha and IL-12, but can also activate alternate syk-dependent kinases, leading to production of IL-2 and IL-10 (144). However, $syk^{-/-}$ dendritic cells do not make IL-10 or IL-2 upon yeast stimulation but produce IL-12, indicating that the Dectin-1/Syk and Dectin-1/TLR2 pathways can operate independently, and may represent a mechanism by which innate host responses could direct the adaptive response towards the development of T helper type 1 versus regulatory T cells. This issue is not straightforward, as gene profiling has not produced any evidence for up-regulation of cytokines such as IL-12 or IL-10, that are representative of either the Th1 or Th2 style of adaptive immune response. However, binding through other receptors, such as the mannose receptor or Fc receptors, can also modulate patterns of cytokine production (135).

The patterns of gene activation in human monocytes did not appear to differ markedly after exposure to opsonised (141) or unopsonised (142) yeasts, suggesting a possible role for the mannose receptor in initiating early host inflammatory responses. The mannose receptor has been shown to be implicated in *Candida* killing and cytokine secretion by mouse macrophages (61), and it has also been reported that engagement of this receptor results in fungal degradation, the production of pro-inflammatory cytokines, and up-regulation of co-stimulatory molecules and MHC class II, indicative of a protective Th1-type response (145). However, when tested directly, mice lacking the mannose receptor were found to be no more susceptible than wild-type (146). This probably reflects significant redundancy in receptor recognition of the yeast and/or in pathways of activation (147). Phagocytosis by peritoneal macrophages was equivalent in the two groups, and was inhibitable by glucan, but not by mannan.

5. GENERATION OF PROTECTION

In humans, oral and gastrointestinal colonization occurs shortly after birth, and most healthy individuals develop both cell-mediated and humoral immunity against the yeast. Progression from colonization to disease is almost invariably associated with a defect or deficiency in the cellular arm of the immune response, demonstrating that antibodies elicited by natural infection are not effective in conferring resistance. However, apart from the early demonstrations (148, 149) that persistent kidney colonization with an avirulent strain of *C. albicans* conferred non-specific protection against challenge with a virulent strain, little attention has been paid to enhancement of innate immune responses as a means of 'cellular immunization' against the yeast.

Recovery from initial oral or systemic infection induced significant protection against a second challenge. Oral infection resulted in the development of systemic immunity, although responses were different in susceptible and resistant mice. *Candida*-specific DTH responses were demonstrable in the susceptible DBA/2, but not in the more resistant BALB/c mice (10), but levels of serum IgG and salivary IgA antibodies were higher in BALB/c than in DBA/2 mice (11). Oral immunization with viable *C. albicans* was effective in protecting both BALB/c and CBA/CaH mice from secondary oral challenge, reducing both fungal burden and duration of infection (150), but systemic immunisation was ineffective. IgM was the predominant antibody detected in serum following both primary and secondary oral challenge; however, in contrast to the experiments of Elahi (11), *Candida*-specific salivary IgA was not detectable. Protection from oral challenge could not conferred by passive transfer of either lymphocytes or immune serum (150).

Protective antibody responses are also elicited after recovery from systemic infection, and these are again related to tissue susceptibility or resistance in the host (151). Protection could be passively transferred by serum from immune mice (152) but was expressed more strongly in the susceptible CBA/CaH mice as compared with the resistant BALB/c strain. It is yet unclear whether the greater protective effect seen in CBA/CaH mice is related to the greater range of antibody specificities produced by these mice (153-155), or to intrinsic differences between the two strains in tissue susceptibility to infection. Analysis of antibody profiles in BALB/c and CBA/H mice has permitted the identification of numerous immunogenic proteins of *C. albicans*; however, those that are specifically protective remain elusive. Both human and mouse monoclonal antibodies against hsp 90 protected mice against systemic challenge (156), and protection could also be conferred by polyclonal and monoclonal mouse antibodies against the adhesin fraction of *C. albicans* cell surface mannans (157). Immunisation with an immunosuppressive mitogenic protein (p43) of *C. albicans* fully protected the mice against challenge (158), whereas immunization with sonicates of the yeast was not protective, and actually facilitated infection. Some enhancement of infection was also observed after immunization of mice with the 70 kDa heat shock protein of *C. albicans* (159).

Clinical observations suggest that antibodies play a minimal role in protection against vaginal candidiasis; however, protective effects of antibodies have been demonstrated in some animal models. *Candida*-specific IgA antibodies protected against vaginal candidiasis in rats (160), and a monoclonal antibody specific for a beta-1,2-mannotriose also conferred protection against vaginal infection in the mouse (161). In contrast, relatively low levels of antibody were produced after primary vaginal infection in mice (162), and these had little protective effect. The role of cell-mediated immunity is contentious. Induced systemic cell-mediated immunity was ineffective in enhancing resistance against vaginal challenge (163), and was unrelated to the susceptibility to vaginal infection

of inbred strains of mice (164). However, later research demonstrated that protection could be conferred on BALB/c but not C57BL/6 mice by passive transfer of $CD3^+$ or $CD4^+$, but not $CD8^+$, syngeneic immune spleen cells (165).

In summary, no general picture has emerged. Protection was achievable in models of all modes of infection, but a more complete understanding of mechanisms may await characterization of genes coding for susceptibility and resistance in mice, as well as those influencing the virulence of the organism in different anatomical sites.

6. PATHOLOGICAL RESPONSES

Local infection with *C. albicans* induces an inflammatory response, and elicits both cell-mediated and humoral immunity: however, the magnitude of the leucocyte response in the draining lymph nodes, and the nature and evolution of both cellular and humoral immune responses differ between strains of mice that are genetically susceptible or resistant to tissue damage.

BALB/c mice, that develop mild lesions, show a significantly greater recruitment of immune/inflammatory cells to the draining lymph node after primary subcutaneous challenge with live *Candida* yeasts than do CBA/CaH mice, in which the lesions are more severe (99). However, congenic resistant BALB/k mice, that possess the same MHC alleles as CBA/CaH (H-2k), show a significantly reduced inflammatory response in the popliteal lymph node after footpad immunisation (99), even though the lesion severity in these mice is comparable to BALB/c (mild). This demonstrated that magnitude of the local immune/inflammatory response was determined by genes within the mouse MHC (99). An assessment of lymph node responses in [BALB/c ($H\text{-}2^d$) x BALB/k ($H\text{-}2^k$)] F1 hybrid mice showed that low responsiveness was dominant (166).

A further analysis of congenic resistant B10 strains demonstrated that the magnitude of the lymph node responses was controlled, at least in part, by Class I MHC genes (167); however, a gene or genes present in the B10 background appeared either to modify or override the MHC-linked regulation of host responses. Diseases in which there is a significant immunopathological component have been linked to the presence of specific MHC alleles (168), but non-MHC genes are generally of more importance in determining the outcome of infection in both human infectious disease, and in animal models. In murine candidiasis, both BALB/c and BALB/k mice make strong proliferative and DTH responses when challenged after recovery from a primary systemic infection (169), whereas the response of CBA/CaH lymphocytes remains low. The reduction in T cell memory responses in CBA/CaH mice correlates with the presence of the susceptible allele of the *Carg1* gene in this strain, suggesting that the genetically-susceptible strain is subject to some form of differential immune regulation, that inhibits the development or expression of *Candida*-specific T cell activity without influencing T-B cell collaboration in the generation of antibody responses.

The attenuated immunological memory in CBA/CaH mice was associated with functional abnormalities in responsiveness of both $CD4^+$ and $CD8^+$ T lymphocytes. Depletion of $CD4^+$ cells did not influence the magnitude of fungal colonization in either the susceptible or resistant mouse strain, nor did it alter the outcome of infection (170). In contrast, the effect on the severity of tissue damage was strikingly different in BALB/c and CBA/CaH mice. Depletion of $CD4^+$ cells caused a marked increase in tissue damage after infection of BALB/c mice, whereas depletion of $CD4^+$ cells from CBA/CaH mice completely abrogated tissue damage in the early stages of the infection (170). This effect was transient, and after day 4, tissue damage was exacerbated in the same way, and to the same extent, as in the BALB/c mice.

Spleen cells taken from CBA/CaH mice in the early stages of infection, and transferred into infected syngeneic recipients, markedly increased tissue damage when compared to controls (170), and also caused a significant increase in fungal colonization in the brain. A similar transfer in BALB/c mice resulted in an increased recruitment of inflammatory cells in and around the lesions, but had no effect on the fungal burden in brain and kidney. The results demonstrated that systemic infection in both inbred strains resulted in the generation of $CD4^+$ T cell populations that act to limit tissue damage and/or enhance healing, but that do not directly augment clearance of the organism from infected tissues. However, in CBA/CaH mice, there appears to exist early in infection, a population of $CD4^+$ lymphocytes that are essential for recruitment of inflammatory cells to the sites of infection. Whether this represents a stage-specific functionality in the maturation of $CD4^+$ cells in these mice, or the generation of a functionally distinct sub-population, remains to be determined.

In this context, further investigations revealed a small but significant increase in IL-10 positive, $CD4^+CD25^+$ T cells early after infection in CBA/CaH mice (Wanasaengsakul and Ashman, unpublished). This correlated with increased concentrations of IL-10 in the serum of infected mice at this time. A population of regulatory $CD4^+CD25^+$ T cells that produce IL-4, IL-10, and TGF-□eta, has been reported in mice with candidiasis (171), and this study also found that mice lacking these cells were capable of efficiently restricting fungal growth, but demonstrated increased inflammatory pathology. However, this functionality appears quite different to that observed in the CBA/CaH mice, in which removal of the $CD4^+$ cells actually decreased inflammatory pathology, and seems more akin to the reduction in *Candida* growth associated with systemic administration of TLR2 ligand during the acute phase of infection (172). The interaction between TLR2 and *Candida*-specific immune responses clearly merits further investigation. The early abundance of IL-10 in CBA/CaH compared to BALB/c mice might favour the generation of regulatory cells that drive the immune response towards a predominantly antibody response, and the production of *Candida*-specific IgG1 and IgG2a antibodies in CBA/CaH mice, whereas high levels of IFN-gamma and a relative lack of IL–10 might promote

cell-mediated reactivity BALB/c mice, and tend to drive antibody production towards a predominantly IgG1 isotype (153, 155).

Anomalies in reactivity were also observed in the $CD8^+$ cell population. Splenocytes from mice that had recovered from an initial infection with the yeast could be activated by *in vitro* culture with *Candida* antigens, but the proliferative responses of BALB/c memory cells were much stronger than those of cells from CBA/CaH mice (169). However, when these lymphocytes were tested for reactivity by inoculation *in vivo*, $CD8^+$ cells from CBA/CaH mice showed specific reactivity against uninfected syngeneic tissue, whereas cells from BALB/c mice were unresponsive (173)

In the case of *C. albicans*, the abnormal immunological reactivity does not appear to influence the ability of the animal to recover from infection, but does clearly illustrate that the genetic context in which an immune response evolves can influence the effector function of both T cell subsets. Understanding the mechanisms by which these effects are initiated or maintained may provide indications as to the ways in which immune reactivity may be manipulated in other infectious or autoimmune diseases.

7. PERSPECTIVE

Although infection with *C. albicans*, by any route, elicits both cellular and humoral immune responses, it is becoming clear that the anatomical site(s) at which infection occurs dictates the host strategies adopted in clearance of the yeast. Oral and gastrointestinal infections require a predominantly T cell-mediated response, whereas the phagocytic cells of the innate immune system appear competent to eradicate primary systemic infections. Neither T lymphocytes nor phagocytes could be shown to play a role in vaginal infection, and the demonstration of high levels of immunoregulatory cytokines in vaginal tissue suggests that control of inflammation in this area may be an immunological concession to the reproductive system.

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Send correspondence to: Dr Robert B. Ashman, Oral Biology and Pathology, University of Queensland, Brisbane. 4072, Queensland, Australia, Tel: 6173365-3039, Fax: 6173365-1109, E-mail: r.ashman@uq.edu.au