Challenges in the Control of Environmental Pathogenic Microbes

Review

Vaccines and Protective Immune Memory against Cryptococcosis

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Cryptococcosis is a potentially lethal disease caused by fungal pathogens including *Cryptococcus neoformans* and *Cryptococcus gattii* species complex. These fungal pathogens live in the environment and are associated with certain tree species and bird droppings. This infectious disease is not contagious, and healthy individuals may contract cryptococcal infections by inhaling the airborne pathogens from the environment. Although cleaning a contaminated environment is a feasible approach to control environmental fungal pathogens, prophylactic immunization is also considered a promising method to regulate cryptococcal infections. We review the history of the development of cryptococcal vaccines, vaccine components, and the various forms of immune memory induced by cryptococcal vaccines.

Key words antifungal vaccine; dendritic cell-based vaccine; memory T cell; lung-resident memory T cell; innate immune memory; cryptococcosis

1. INTRODUCTION

Several mycoses, including cryptococcosis, aspergillosis, and imported mycoses, are caused by pathogenic fungi living in the environment. Although disseminated fungal infections are generally opportunistic, immunocompetent individuals can also contract systemic infections by several fungal pathogens including *Cryptococcus gattii* and *Coccidioides immitis* which inhabit the soil, trees, and dust in certain regions.^{1,2)}

Cryptococcosis is a life-threatening infection caused by inhaling the encapsulated yeasts in *Cryptococcus neoformans* and *C. gattii* species complexes.^{3,4)} Cryptococcal cells often disseminate to the central nervous system, leading to meningoencephalitis. *C. neoformans* generally infects immunocompromised hosts such as AIDS patients, and approximately 220000 cases of cryptococcal meningitis occur among AIDS patients worldwide each year, leading to 180000 deaths annually.⁵⁾ A retrospective investigation revealed that immunocompetent individuals can also contract *C. neoformans* infection in Japan.⁶⁾ In Australia and New Zealand, the original hotspots of *C. gattii*, immunocompetent individuals are more likely to be infected by *C. gattii* than immunocompromised hosts.⁷⁾ Although *C. gattii* infection has been considered a type of tropical disease, outbreaks of *C. gattii* infection were reported in Canada and the USA, and immunocompetent individuals also died of *C. gattii* infection. In the outbreaks, the maximum annual incidence was estimated to be 38 cases (9 cases per million) in British Columbia, Canada, and the mortality rate was in the range of 8.7–33%.^{8–10)}

Systemic cryptococcosis and coccidioidomycosis are designated as category IV and V infectious diseases, respectively, in the Japanese Law of Infectious Disease Control; thus, physicians diagnosing these mycoses must report the occurrence

to public health centers. Although the diagnosis and treatment method of mycoses still require specialized experience and knowledge, specific clinical techniques have been established. As a prophylactic approach, vaccines against those mycoses are still under development and are not available in the worldwide clinical setting.

Previous studies developed many experimental vaccines and investigated vaccine-mediated protective immunity against cryptococcosis.^{11,12)} We also developed a dendritic cell (DC)-based vaccine against cryptococcosis caused by the highly virulent *C. gattii*.^{13–16)} We demonstrated that the DC vaccine improves the survival rate and ameliorates the fungal burden in the lungs after *C. gattii* infection and that lung resident-memory Th17 cells (lung TRM17) play a role in the vaccine-mediated protective effect.¹³⁾ Each research group has utilized different antigens, adjuvants, and delivery systems to activate the protective immune memory, including T cells, B cells, and other novel memory cells, against fungal infection. As the progress of vaccine research and development is so rapid, regular overviews of current knowledge are required.

This review focuses on prophylactic vaccines against cryptococcosis. The following sections cover: 1) the history of vaccine development in animal models; 2) antigens and carriers; 3) adjuvants and adoptive cell transfer (ACT) for immunization; and 4) the immune memory contributing to vaccine-mediated protection.

2. HISTORY OF CRYPTOCOCCAL VACCINES

Previous reviews described experimental antifungal vaccines in detail.^{11,12)} Several veterinary vaccines against dermatomycosis, including Insol Dermatophyton, Biocan-M, and RIVAC Mikroderm, are clinically available.^{17,18)} Although

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Table 1. Chronology of the Development of Cryptococcal Vaccines and Analysis of Vaccine-Induced Protective Immunity

Year	Antigen	Adjuvant, carrier, and ACT	Vaccine injection route	Vaccine injection frequency	Suggested protective immunity	Challenge strain	Challenge route	Challenge after vaccination	Protection	Animal strain	Ref.
1958	CPs	Sterile resin, Amberlite XE-67	s.c.	3 times on alternate days	ND	<i>C. neoformans</i> 3723, 1523, DU, and RE	i.v.	Day 14	+	Swiss Webster	94
1960	Live thin capsular <i>Cn</i>	—	i.p.	3 times every 10d	ND	<i>C. neoformans</i> 1148	i.v.	Day 14	+	Swiss Webster	60
1960	Enzymatic de-capsulated <i>Cn</i>	—	s.c.	3 times on alternate days	AMI	<i>C. neoformans</i> 3723	i.v.	Day 14	+	Swiss Webster	59
1960	Formalin-killed <i>Cn</i>	—	s.c or i.p.	4–8 times every 14d	ND	<i>C. neoformans</i> strain C	i.v.	Day 7	+	Swiss Webster	61
1963	CPs	—	i.v.	Once	AMI	<i>C. neoformans</i> BRI, TRE, and BRY	i.v.	Day 7	+	ICR	95
1967	CPs	BGG	s.c or i.p.	>2 times	—	<i>C. neoformans</i> serotype A	i.v.	Day 14	—	CF-1	44
1976	Irradiated acapsular <i>Cn</i> mutant	FA	s.c. or i.d.	4 times every 4–5 d	ND	<i>C. neoformans</i> P36	i.p.	Day 12	+	Swiss Webster	62
1978	Live or HK <i>Cn</i>	—	s.c.	Once	ND	<i>C. neoformans</i> 145	i.v.	Day 21	+	CD-1	96
1979	Avirulent pseudohyphal <i>Cn</i> mutant, NU-2-P	—	i.p.	8 times weekly	CMI (DTH response)	<i>C. neoformans</i> NU-2	i.v.	Day 14	+	C57BL/6	63
1982	Live <i>Cn</i>	—	s.c.	Once	CMI (DTH response)	<i>C. neoformans</i> 145	i.v.	Day 21	+	CBA/J	97
1982	Fractionated culture supernatant	FA	s.c.	2 times every week	CMI (DTH response)	<i>C. neoformans</i> serotype A	i.p.	Day 14	+	BALB/c	98
1983	Live acapsular <i>Cn</i> mutants 64, 67, and 70 HK capsular <i>Cn</i> strain	—	i.p. or s.c.	8 times every 3–4 d	CMI	<i>C. neoformans</i> 9–401	i.v.	Day 14	+	C3H/HeN	64
1994	Live capsular <i>Cn</i> strain	—	i.t.	Once	CD4 ⁺ T cells	<i>C. neoformans</i> 184	i.v.	Days 42–70	+	BALB/c	87
1995	Live capsular <i>Cn</i> strain	—	i.t.	Once	TNF- α and IFN- γ dependent	<i>C. neoformans</i> 184 or <i>ura5</i>	i.v.	Days 5–42	+	C.B-17 and BALB/c	88
1996	GXM	TT, MPL	s.c.	3 times every 2 weeks	AMI	<i>C. neoformans</i> serotype A	i.v.	Day 9	+	Swiss Webster	42
1997	Live capsular <i>Cn</i> strain	—	i.t.	Once	B independent, CD4 ⁺ T cell dependent	<i>C. neoformans</i> <i>ura5</i>	i.v.	Day 56	+	C57BL/6 and μ MT	20
1998	CneF	FA	s.c.	Once	CMI (DTH response)	<i>C. neoformans</i> 184A	i.v.	Day 8	+	CBA/J	57
2001	P13	TT, BSA, Alhydrogel	s.c.	>3 times every 2 weeks	AMI	<i>C. neoformans</i> ATCC 24067	i.v.	Day 24	+	BALB/c and CBA/n	43
2002	d25	FA	s.c.	Once	CMI (DTH response)	<i>C. neoformans</i> H99	i.v.	Day 7	+	BALB/c	55
2003	d25	FA	s.c.	Once	Th1, IFN- γ dependent, IL-2 independent	<i>C. neoformans</i> H99	i.v.	Day 7	+	C57BL/6 and BALB/c	56
2004	Live capsular <i>Cn</i> strain	—	s.c.	Once	CD8 ⁺ T cell dependent	<i>C. neoformans</i> <i>ura5</i>	i.v.	Day 70	+	A β Null mice	99
2004	MP	Ribi	i.p.	2 times every 3 weeks	T cell dependent, B cell independent	<i>C. neoformans</i> B3501	i.v.	Day 7	+	C57BL/6 and CBA/J	21
2005	Live <i>Cn</i> mutant Δ <i>cnal</i>	—	i.n.	Once	—	<i>C. neoformans</i> H99	i.n.	Day 28	—	A/J	100
2006	HK <i>Cn</i>	FA	s.c.	Once	Th1	<i>C. neoformans</i> 102/85	i.p.	Day 4	+	Wistar rat	101
2007	Live <i>Cn</i> expressing IFN- γ (H99 γ)	—	i.n.	Once	Th1	<i>C. neoformans</i> H99	i.n.	Day 70	+	A/J and BALB/c	65
2009	Live <i>Cn</i> expressing IFN- γ (H99 γ)	—	i.n.	Once	B cell and IL-4R independent, IFN- γ and IL-12 dependent, Th1	<i>C. neoformans</i> H99	i.n.	Day 100	+	BALB/c	22

Table 1. Continued

Year	Antigen	Adjuvant, carrier, and ACT	Vaccine injection route	Vaccine injection frequency	Suggested protective immunity	Challenge strain	Challenge route	Challenge after vaccination	Protection	Animal strain	Ref.
2011	Opsonized live <i>Cn</i>	Eosinophils	i.p.	Once	Th1	<i>C. neoformans</i> 102/85	i.p.	Day 10	+	Wistar rat	78, 79
2011	GalXM	FA, BSA conjugate	i.p.	4 times every 2 weeks	—	<i>C. neoformans</i> ATCC 24067	i.v.	Day 1	—	BALB/c	45
2011	Live <i>Cn</i> expressing IFN- γ (H99 γ)	—	i.n.	Once	T cell dependent and independent immunity	<i>C. neoformans</i> H99	i.n.	Day 70	+	BALB/c	93
2013	Crude proteins extracted from <i>Cn</i>	—	i.n.	3 times every 4 weeks	AMI, CMI	<i>C. neoformans</i> H99	i.n.	Day 10	+	BALB/c	51
2014	Crude proteins extracted from <i>Cg</i>	—	i.n.	3 times every 4 weeks	AMI, CMI	<i>C. gattii</i> R265	i.n.	Day 10	+	BALB/c	52
2015	Live <i>Cn</i> mutants Δ <i>sgl1</i>	—	i.n.	Once	CD4 ⁺ T cell independent	<i>C. neoformans</i> H99, and <i>C. gattii</i> R265	i.n.	Day 30	+	CBA/J	66
2015	HK <i>Cg</i> mutant Δ <i>cap60</i>	DC	i.v.	2 times every 2 weeks	Th1, Th17	<i>C. gattii</i> R265	i.t.	Day 1	+	C57BL/6	14
2015	Live and HK <i>Cn</i> strain overexpressing <i>ZNF2</i>	—	i.n.	Once	Th1, Th17	<i>C. neoformans</i> H99	i.n.	Day 25 or 48	+	A/J	67
2015	1. Live <i>Cn</i> mutant Δ <i>cdal1/2/3</i> or Δ <i>cap59</i> 2. Alkaline-extracted crude proteins derived from <i>Cn</i>	GP	s.c.	3 times every 2 weeks	Th1, Th17	<i>C. neoformans</i> KN99, and <i>C. gattii</i> R265	i.n.	Day 14	+	C57BL/6J	53
2016	Live or HK <i>Cn</i> mutant Δ <i>cdal1/2/3</i>	—	i.n.	Once	Th1	<i>C. neoformans</i> KN99, <i>C. gattii</i> R265, and WM276	i.n.	Day 40	+	CBA/J, C57BL/6, BALB/c, A/J, and 129	68
2017	Live <i>Cn</i> expressing IFN- γ (H99 γ)	—	i.n.	Once	Th1	<i>C. neoformans</i> H99, <i>C. gattii</i> R265, <i>C. bacillisporus</i> WSA87, and <i>C. deneoformans</i> R4247	i.n.	Day 70	+	BALB/c	33
2017	Recombinant proteins, Sod1, Cda1, Cda2, and/or Cda3	GP	s.c.	3 times every 2 weeks	ND	<i>C. neoformans</i> KN99, and <i>C. gattii</i> R265	i.t.	Day 14	+	C57BL/6, BALB/c, and humanized DR4 mice	54
2018	Live <i>Cn</i> expressing IFN- γ (H99 γ)	—	i.n.	Once	Independent of T cells, B cells, neutrophils, and NK cells, trained M ϕ	<i>C. neoformans</i> H99, and <i>C. gattii</i> R265	i.n.	Day 70	+	BALB/c	25
2019	HK <i>Cg</i> mutant Δ <i>cap60</i>	DC	i.v.	2 times every 2 weeks	IL-17A dependent, lung TRM17	<i>C. gattii</i> R265	i.t.	Days 60–180	+	C57BL/6	13
2019	Live <i>Cn</i> mutant Δ <i>sgl1/\Delta</i> <i>cap59</i>	—	i.n.	Once	—	<i>C. neoformans</i> H99	i.n.	Day 30	—	CBA/J	102
2019	Live <i>Cn</i> expressing IFN- γ (H99 γ)	—	i.n.	Once	Trained DCs	<i>C. neoformans</i> H99	i.n.	Day 70	+	BALB/c	24

Cn: *C. neoformans*; *Cg*: *C. gattii*; HK: heat-killed; ND: not determined; Δ *cnal1*: disruptant of gene coding calcineurin A1, avirulent temperature-sensitive mutant; Δ *cdal1/2/3*: disruptant of three genes coding chitin deacetylase, chitosan-deficient avirulent strain; Δ *sgl1*: disruptant of gene coding sterylglucosidase-1, avirulent strain accumulating sterylglucosides; Δ *cap59*, Δ *cap60*: disruptant of capsule synthesis-related gene, acapsular mutant; *ZNF2*: gene coding the transcriptional factor, zinc finger protein (the overexpression strain forms hyphae in *C. neoformans*, and this strain strongly induced inflammatory response in the lungs after infection); *ura5*: mutant of orotidine monophosphate pyrophosphorylase which is uracil auxotrophic and can proliferate in medium containing 5-fluoroorotic acid (5-FOA) (wild-type cryptococcal cells used for immunization cannot grow in the medium and thus cryptococcal cells for immunization and challenge are distinguishable on plates containing 5-FOA); Th1: CD4⁺ helper T cells producing IFN- γ ; Th17: CD4⁺ helper T cells producing IL-17A; μ MT mice: B-cell deficient mice; DR4 mice: humanized mice expressing human HLA-DR4 (DRB*0401) and lacking endogenous mouse MHC class II; A β null mice: MHC class II-deficient mice in which CD4⁺ T cells are absent; Protection (+/-): +=immunization improved survival rates or reduced fungal burdens after challenge. Other abbreviations are defined in the main text.

vaccines against candidiasis is in human clinical trials, no antifungal human vaccines are yet commercially available.

Experimental vaccines against cryptococcosis are listed in Table 1. As many as possible are included, but there may be some omissions. Cryptococcal vaccines have not yet progressed to clinical trials or for which clinical trials have been discontinued. Note that each vaccine can be characterized by antigen, adjuvant, antigen carrier, route of injection, duration of antifungal effect, and induced protective immune memory.

In the early days, whole cryptococcal cells and capsular polysaccharides (CPs) were used as vaccination antigens; in recent years, recombinant cryptococcal cells and antigenic proteins have become available for immunization, as described below. The vaccinations have been administered *via* the intradermal (i.d.), subcutaneous (s.c.), intraperitoneal (i.p.), intravenous, and intranasal (i.n.) injection routes. To augment pulmonary local immunity more efficiently, intranasal forms are being used in the development of experimental and clinical vaccines against pulmonary infectious diseases including influenza, tuberculosis, pertussis, and pneumococcal infection.¹⁹⁾

Vaccines generally induce memory B cells and antibody-mediated immunity (AMI), while several cryptococcal vaccines depend on T cell-mediated immunity (CMI) including the delayed-type hypersensitivity (DTH) response in a B cell-independent manner.^{20–22)} Since *C. neoformans* often infects immunocompromised hosts such as AIDS patients, vaccines have also been evaluated in immunodeficient mice lacking T cells, B cells, and/or other immune cells. Those studies provided new insights into what is termed “innate memory” or “trained immunity,” where innate immune cells, including macrophages (M ϕ), DCs, and natural killer (NK) cells, exert memory like-responses to antigen reexposure.^{23–26)}

The immune response and susceptibility to infection often differ in various mouse strains, and different cryptococcal strains show different virulence. Outbred mice such as ICR and Swiss mice were used for early vaccine protection assays, but inbred mice, including C57BL/6, BALB/c, and CBA/J strains, have been widely used in more recent studies. In the pulmonary infection model with *C. neoformans*, C57BL/6 mice showed a higher fungal burden in the lungs 4 weeks postinfection than BALB/c and CBA/J mice, and BALB/c mice showed longer survival times than CBA/J mice.^{27,28)} Because genome-wide studies were performed against standard strains including *C. neoformans* H99 and *C. gattii* R265, those standard strains have been commonly used in recent studies evaluating vaccine efficacy. Although there are contradictory data, it is believed that *C. gattii* R265 isolated in the Vancouver, Canada, outbreak is a more virulent strain than *C. neoformans* H99.^{29–33)} At present, protective vaccines can ameliorate the fungal burden and improve the survival rate after *C. gattii* infection, although no vaccine offers complete protection from cryptococcosis caused by highly virulent *C. gattii* R265.

3. ANTIGENS AND CARRIERS

3.1. CPs Cryptococcal cells form capsular layers on the cell surface which can be visualized with the Indian ink method under a microscope. The capsules consist of CPs including glucuronoxylomannan (GXM) and galactoxylomannan (GalXM).³⁴⁾ CPs enable cryptococcal cells to evade

anticytotoxic immune systems.^{35,36)} We demonstrated that capsular cryptococcal cells could not be recognized by immune cells without opsonization and that acapsular mutants are immediately engulfed by innate immune cells.^{14,37,38)} Cryptococcal cells can be opsonized with anti-GXM antibody, and opsonized capsular cells are phagocytized by innate immune cells.^{39,40)} Thus, it is reasonable to use GXM or GalXM as vaccine antigens to induce specific antibodies and AMI.

CPs are categorized into the T cell-independent type-2 (TI-2) antigens and poorly immunogenic antigen.⁴¹⁾ To enhance the immunogenicity of CPs, previous studies developed CP-protein carrier-conjugated vaccines, and tetanus toxoid (TT),^{42,43)} bovine γ -globulin (BGG),⁴⁴⁾ and bovine serum albumin (BSA)^{43,45)} have been used as protein carriers. Ten-base peptides mimicking the GXM epitope were screened from a peptide library, and peptide P13 was used for a conjugation vaccine.^{43,46)} Although the GalXM-conjugated vaccine did not prolong survival times when challenged with *C. neoformans*,⁴⁵⁾ GXM- or P13-conjugated vaccine significantly increased the survival rate after challenge.^{42,43)} It has not been demonstrated whether CP-based vaccines could protect against *C. gattii* infection.

3.2. Mannoproteins As well as common pathogenic fungi, cryptococcal cells express highly mannoseylated proteins, referred to as mannoproteins (MPs), on the cell surface and can secrete them to the extracellular space during infection.^{47,48)} Cryptococcal cells require MPs for infection to progress and therefore MPs are feasible targets for vaccine antigens. In contrast to CPs, MPs are T-cell dependent (TD) antigens, and vaccines containing cryptococcal MPs likely induce CMI and AMI for protection against cryptococcosis.^{49,50)} Both crude and single recombinant MPs have been used as vaccine antigens, and it was found that those vaccines significantly improved survival rates and decreased fungal burdens when challenged with *C. neoformans* and highly virulent *C. gattii*.^{21,51–56)}

C. neoformans culture filtrate antigen (CneF) is a crude antigen containing GXM, GalXM, and MPs, and MPs can be enriched from the culture filtrate *via* concanavalin A (ConA) affinity columns.^{21,57)} The following MPs have been identified as protective vaccine antigens: chitin deacetylase (Cda1, Cda2, also known as MP98 and Cda3); and a 25-kDa chitin deacetylase homologue (d25).^{54–56)}

3.3. Other Antigenic Proteins Non-MPs in the cell wall and cytoplasm were also identified as protective vaccine antigens. Those proteins can be extracted from cryptococcal cells *via* a mild alkaline extraction technique, the 2-mercaptoethanol method, or a manufactured kit. Superoxide dismutase (Sod1) was identified as the protective vaccine antigen that was the most abundant protein in the mild alkaline extract of *C. neoformans* acapsular mutant $\Delta cap59$.^{53,54)} It was demonstrated that vaccination with the cytoplasmic proteins significantly prolonged survival times and reduced the fungal burden after the infection challenge with highly virulent *C. gattii*.^{52,54)}

3.4. Whole-Cell Antigens Like vaccines against other infectious diseases, live or inactivated whole cryptococcal cells have been used for the vaccines.⁵⁸⁾ In early research, wild-type capsular strains, spontaneous mutants, and mutants generated with random mutagenesis were used for vaccination, and every approach prolonged survival rates after infection challenge.^{59–64)} More recently, the following strains

Fig. 1. Paradigm of Cryptococcal Vaccine and Vaccine-Mediated Protective Immunity

In cryptococcal vaccines, various antigens including CPs, MPs, cytosolic proteins, and whole cells have been used with the vaccine enhancers shown in Table 1. It was demonstrated that DCs and Eos could be used for ACT to induce protective CMI. Although CP-based vaccines protect against *C. neoformans* infection via AMI, there is no evidence that CP-based vaccines protect against cryptococcosis caused by highly virulent *C. gattii*. *Cryptococcus*-specific antibody and T cell responses cannot directly remove cryptococcal cells, and those immune responses induce innate immunity to suppress fungal growth. Vaccine-induced AMI can increase opsonization of cryptococcal cells via specific antibodies, and vaccine-induced CMI generally requires inflammatory cytokines including IFN- γ , TNF- α , and IL-17A to recruit and activate innate immune cells, including granulocytes, M ϕ , and DCs. Granuloma is a typical pathological finding in the lungs of protected mice that received cryptococcal vaccines. Granuloma likely suppresses fungal growth through the tight mononuclear infiltrates surrounding unremovable cryptococcal cells. It was shown that novel immune memory such as lung-resident CD4⁺ TRMs and innate immune memory were induced by cryptococcal vaccines, in addition to conventional immune memory.

have been constructed using the gene-targeting approach and used for immunization: *C. neoformans* interferon- γ (IFN- γ)-expressing strain (H99 γ); *ZNF2* overexpression strain; Δ *sgl1* strain; Δ *cda1/2/3* strain; Δ *cap59* strain; and *C. gattii* Δ *cap60* strain.^{14,53,65–68} Interestingly, all groups immunized with *C. neoformans* recombinants had significantly improved survival rates when challenged with highly virulent *C. gattii* as well as with *C. neoformans*. Thus, it appears that these immunizations are cross-reactive to *C. neoformans* and *C. gattii* infection.

4. ADJUVANTS AND ACT

4.1. Adjuvants On MPs, the mannose polymer mannan is a pathogen-associated molecular pattern that can be recognized by pattern recognition receptors (PRRs) such as a dectin-2 on innate immune cells.⁶⁹ It was shown that *C. neoformans* Cda2 recombinant protein prepared in *Pichia pastoris* was recognized by dectin-2 in B3Z reporter cells and DCs, and recombinant soluble dectin-2 physically bound to *C. gattii* cells.^{38,70} Antigen-presenting cells (APCs) such as DCs can uptake MPs via PRRs and process MPs for antigen presentation to naive T cells.^{71,72} Recombinant proteins generated in *Escherichia coli* (rCda1, rCda2, rCda3, rd25, and rSod1) are likely lacking glycosylation and similar to CPs are less recognizable as foreign antigens. Adjuvants are substances to compensate for the weak antigenicity of vaccine antigens,

and the following adjuvants have been added to cryptococcal vaccines: glucan particles (GPs); Freund's adjuvant (FA); Ribi adjuvant; monophosphoryl lipid A (MPL); and alhydrogel.^{73,74}

4.2. ACT for Immunization ACT is used to administer immune cells to a host. It is commonly used in cancer therapy and is a candidate for antifungal immunotherapy or immunization.^{75–77}

We have developed a DC vaccine against cryptococcosis caused by highly virulent *C. gattii*.¹⁴ In this approach, DCs were pulsed with the heat-inactivated *C. gattii* acapsular mutant Δ *cap60*, and then the antigen-pulsed DCs were transferred to the host. DCs are major APCs, and the antigen-loaded DCs potentially induce the cytokine-producing T cells that are responsible for CMI against cryptococcosis.^{13,14}

It was shown that eosinophils (Eos) can also be used for immunization against cryptococcosis in a rat model.^{78,79} Eos can phagocytize opsonized cryptococcal cells and present antigens to T cells in a major histocompatibility complex (MHC)-dependent manner.⁷⁸ The transfer of cryptococcal cell-pulsed Eos (Cn-pulsed Eos) significantly decreased the fungal burden after challenge with *C. neoformans*.⁷⁹ Interestingly, Cn-pulsed Eos promoted the proliferation of *C. neoformans*-specific T cells producing IFN- γ but not interleukin (IL)-4.⁷⁸ Although there is an advantage that these approaches can induce protective CMI, ACT-based vaccines will not be immediately applied in the clinical setting due to the cost-

effectiveness problem.

5. IMMUNE MEMORY FOR VACCINE-MEDIATED PROTECTION

5.1. B Cells CP-based vaccine produces plasma B cells that secrete CP-specific antibodies including immunoglobulin M (IgM) and IgG, and some B cells likely become memory B cells that can immediately respond to secondary exposure to CPs via B cell receptors (BCRs).^{23,80} The unique antigen binding sites of BCRs are identical to those of the secreted antibodies recognizing the CP epitope (<https://www.ncbi.nlm.nih.gov/books/NBK26884/>). CP-based vaccine significantly increases the serum titer of CP-specific antibody, and passive immunization with CP vaccine-induced antiserum significantly prolongs survival time after challenge.^{42,43} Therefore, AMI appears to be involved in the protective effects of CP-based vaccine.

Anti-GXM monoclonal antibodies (mAbs) including 18B7 and 2H1 were generated from splenocytes of BALB/c mice that received the GXM-TT conjugate vaccine.^{81,82} Those mAbs can opsonize capsular cryptococcal cells and enhance phagocytes to uptake fungal cells.^{39,40} Intraperitoneal administration of 18B7 or 2H1 mAb significantly decreased the serum GXM concentration and increased the survival rate after intraperitoneal infection with *C. neoformans*.^{81,82} Thus, vaccine-induced AMI may be involved in the opsonization of cryptococcal cells and help the innate immune cells including neutrophils and M ϕ to uptake and kill cryptococcal cells.

Similar to CP-based vaccine, immunization with cryptococcal proteins or whole cells likely induces the production of antigen-specific B cells that are helped by cognate T cells.^{49,50} However, those vaccines exert sufficient protective effects in B cell-deficient mice when challenged with *C. neoformans*.^{20–22} Thus, rather than AMI, CMI has been the subject of recent major cryptococcal vaccine studies.

5.2. T Cells T cells can be separated into the following different subsets: 1) conventional T cells (CD4⁺ helper T cells and CD8⁺ cytotoxic T cells); and 2) unconventional innate-like T cells ($\gamma\delta$ T cells, NKT cells, and MAIT cells).^{83–85} In general, conventional T cells differentiate into memory T cells after immunization with protein antigens, and memory T cells can be divided into the stem cell-like memory, central memory, effector memory, and tissue-resident memory subsets.⁸⁶ Memory T cells immediately proliferate or produce effector molecules including cytokines and cytotoxic effectors when T cell receptors (TCRs) receive the epitope of protein antigen by APCs during reinfection.⁸⁶ Protective immunization with cryptococcal proteins or whole cells clearly activates T cells, mainly CD4⁺ helper T cells, producing inflammatory cytokines including IFN- γ , tumor necrosis factor (TNF)- α , and IL-17A, and vaccine efficacy was reported to be significantly attenuated under cytokine-deficient conditions.^{13,14,20–22,56,87,88} Thus, the findings suggest that T-cell-related cytokines including IFN- γ , TNF- α , and IL-17A are required for the protective effect of cryptococcal vaccines using TD antigen.

In general, IFN- γ , TNF- α , and IL-17A play a crucial role in leukocyte recruitment, activation, and granuloma formation. Granuloma consists of tight mononuclear infiltrates surrounding unremovable foreign substances such as cryptococcal cells and it differs from the loose inflammatory infiltrates that are a

characteristic pathology of allergic bronchopulmonary mycosis.²² Granuloma is often found in the lungs of protected mice that received a cryptococcal vaccine.^{13,14,22,89} For example, activated neutrophils expressing the activation markers Siglec-F and CD11c and granuloma are induced 7–14 d postinfection in the lungs of C57BL/6 mice that received the DC vaccine, but not in the lungs of IL-17A knockout (KO) mice. Furthermore, DC vaccine cannot reduce the fungal burden in the lungs of IL-17A KO mice. These results suggest that the DC vaccine requires an IL-17A response, mainly produced by lung TRM17 (described in further detail below), for the protective effect after challenge.¹³

H99 γ whole-cell vaccine protects against *C. neoformans* infection in IL-4R KO mice as well as in C57BL/6 mice, but not in IL-12p40, IL-12p35, and INF- γ KO mice.²² The cryptococcal d25-vaccine significantly improved the survival rate of mice in which IL-2 was depleted, but not of INF- γ KO mice.⁵⁶ These results suggest that several cryptococcal vaccines require CD4⁺ Th1-related cytokines, including IFN- γ and IL-12.

Tissue-resident memory T cells (TRMs) are newly identified nonvascular T cells found in various nonlymphoid tissues including the lungs, brain, liver, and intestine. Various types of local infection induce the differentiation of circulating memory T cells and TRMs, and CD4⁺ TRMs can quickly secrete cytokines after responding to secondary exposure to antigen proteins presented by APCs.⁸⁶ Recent studies have demonstrated that lung TRMs are involved in the protection from pulmonary infectious diseases, and researchers have developed vaccines inducing protective lung TRMs against pulmonary infectious diseases.⁹⁰ We developed the DC vaccine that strongly induces lung CD4⁺ TRM secreting IL-17A (lung TRM17).¹³ Lung TRM17 is detectable in the lung parenchyma for at least 6 months after immunization, and this subset can be reactivated by cryptococcal antigens but not by unrelated antigens derived from *Candida albicans*, suggesting that lung TRM17 is an antigen-specific T cell. DC vaccine can ameliorate fungal burden in the lungs when infected with *C. gattii* within 6 months after the final vaccination; suggesting that the long life of lung TRM17 is correlated with the long-term protective effect of DC vaccine.¹³

Administration of the immunosuppressive agent FTY720 inhibits lymphocyte egress from lymph nodes and critically decreases circulating T and B cells in blood.⁹¹ Although the administration of FTY720 induces lymphopenia, substantial levels of TRMs can be detected in various organs of FTY720-treated hosts.⁹² DC vaccine exerts protective effects even if the mice receive FTY720 during infection, suggesting that circulating lymphocytes are dispensable and that the protective effects of DC vaccine likely depend on the TRM subset.¹³

5.3. Trained Innate Immune Cells There is a new paradigm referred to as “innate immune memory” or “trained immunity,” where innate immune cells exert memory-like responses against reexposure to antigens in the absence of T cells and B cells.²⁶ Surprisingly, H99 γ vaccine significantly prolonged survival time in B cell-deficient mice in which CD4⁺ and CD8⁺ T cells, neutrophils, and NK cells were also depleted by specific antibodies, suggesting that nonconventional memory cells are involved in vaccine-mediated protection from cryptococcosis.^{25,93} In this model, spleen M ϕ or DCs were isolated 70 d after the final immunization with H99 γ , and then those immune cells were restimulated by cryp-

tococcal cells or unrelated antigens, including lipopolysaccharide (LPS), *C. albicans*, and *Streptococcus aureus*. As a result, the vaccine induced-M ϕ and DCs secreted greater amounts of inflammatory cytokines, including IL-2, IL-4, and IFN- γ , than in the case of M ϕ and DCs isolated from nonimmunized mice.^{24,25)} In general, innate immune memory has less strict antigen specificity compared with conventional acquired immunity and can cross-react against other pathogens.²⁶⁾ However, unrelated antigens, including LPS, *C. albicans*, and *S. aureus*, did not induce the cytokine recall response in H99 γ -trained M ϕ and DCs. These results suggest that H99 γ -trained M ϕ and DCs can be specifically recalled by cryptococcal antigens.^{24,25)} This trained immunity may be a new solution to protect against cryptococcosis in immunocompromised hosts lacking CD4⁺ T cells.

6. CONCLUSION

The outline of the mechanism of cryptococcal vaccine is shown in Fig. 1. TI-2 and TD antigens have been used in combination with various adjuvants, nanoparticles, and ACT to develop cryptococcal-protective vaccines. TI-2 antigen-based vaccines produce cryptococcus-specific antibodies and enhance AMI including opsonization of cryptococcal cells after reinfection. However, it has not been demonstrated whether vaccines inducing AMI can protect against highly pathogenic *C. gattii* infection. Recent studies have focused on cryptococcal vaccines inducing CMI. It was demonstrated that several vaccines inducing CMI significantly increase survival rates and decrease fungal burdens in various mice strains when infected with *C. gattii*. Those vaccine studies also newly identified the potentially protective immune memory, including lung TRM17 and H99 γ -trained M ϕ /DCs.^{13,24,25)} Future studies will be needed to develop vaccines that are effective in immunodeficient hosts lacking CD4⁺ T cells, such as AIDS patients, and can offer complete protection against cryptococcosis caused by highly virulent *C. gattii*.

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Conflict of Interest The authors declare no conflict of interest.

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