

Immune Sensing of *Aspergillus fumigatus* Proteins, Glycolipids, and Polysaccharides and the Impact on Th Immunity and Vaccination¹

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The ability of the fungus *Aspergillus fumigatus* to activate, suppress, or subvert host immune response during life cycle in vivo through dynamic changing of cell wall structure and secretion implicates discriminative immune sensing of distinct fungal components. In this study, we have comparatively assessed secreted- and membrane-anchored proteins, glycolipids, and polysaccharides for the ability to induce vaccine-dependent protection in transplanted mice and Th cytokine production by human-specific CD4⁺ T cell clones. The results show that the different fungal components are endowed with the distinct capacity to activate Th cell responses in mice and humans, with secreted proteins inducing Th2 cell activation, membrane proteins Th1/Treg, glycolipids Th17, and polysaccharides mostly IL-10 production. Of interest, the side-by-side comparison revealed that at least three fungal components (a protease and two glycosylphosphatidylinositol-anchored proteins) retained their immunodominant Th1/Treg activating potential from mice to humans. This suggests that the broadness and specificity of human T cell repertoire against the fungus could be selectively exploited with defined immunoactive *Aspergillus* Ags. *The Journal of Immunology*, 2009, 183: 2407–2414.

The ubiquitous mold *Aspergillus fumigatus* is known to cause a spectrum of diseases in humans, including allergic syndromes, noninvasive infections, and invasive aspergillosis. The morbidity and mortality associated with invasive aspergillosis is high, although the mortality rate appears to be improving, partly because of the use of effective antifungal agents, most notably the triazoles (1). Compounding the seriousness of the infection, including the emergence of azole-resistant strains spreading in the environment (2), the available arsenal is not ideal. Indeed, the discovery of newer classes of drugs, immune-modulating therapies, and vaccines is an area of extensive research (3, 4).

Prerequisite of successful immunomodulation and candidate vaccine is the appropriate targeting of cells and pathways that lead to protective immune responses to the fungus. The new pathways involving T regulatory cells (Treg)³ and Th17 cells are key medi-

ators of host inflammatory/anti-inflammatory responses to the fungus (5, 6). These novel signaling pathways, in concert with or independent of Th1/Th2 responses, have potentially important implications in the pathogenesis of fungal infections and diseases, mainly for achieving a balanced immune response that enhances host immunity without concomitant pathogenic inflammation (7).

Ags and allergens of *A. fumigatus* have been described as being capable of vaccinating potential (8–11) and of inducing distinct patterns of Th cytokine production in mice and humans (12–15). However, a side-by-side comparison of fungal Ag-induced activation of Th cytokine responses in mice and humans has not been reported. This may have hampered the translation of basic research into clinical realities, including the interpretation of vaccine surrogate markers (16, 17).

In the present study, we have comparatively assessed distinct fungal components for ability to activate adaptive Th cell responses in experimental models of vaccination and Th cytokine production by human peripheral CD4⁺ T lymphocytes. The results show that the different fungal components are endowed with a distinct, yet overlapping, capacity to activate protective and non-protective Th cell responses in mice and Th cytokine production in humans. However, at least three fungal Ags retained their immunodominant Th1/Treg activating potential from mice to humans.

Materials and Methods

Animals

Female, 8- to 10-wk-old inbred C57BL/6 (*H-2^b*) and BALB/c (*H-2^d*) mice were obtained from Charles River Laboratories. Experiments were performed according to the Italian Approved Animal Welfare Assurance A-3143–01.

A. fumigatus strain and infection

The strain of *A. fumigatus* was obtained from a fatal case of pulmonary aspergillosis at the Infectious Diseases Institute of the University of Perugia (Perugia, Italy). Viable conidia (>95%) were obtained by growth on Sabouraud dextrose agar (Difco Laboratories) supplemented with chloramphenicol for 4 days at room temperature. For infection, the C57BL/6

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³ Abbreviations used in this paper: Treg, regulatory T cell; BAL, broncho-alveolar lavage fluid; Cat1p, mycelia catalase 1; Crf1p, β 1,6 glucan-chitin linkages; DC, dendritic cell; Gel1p, 1,3- β glucanoglucantransferase; GM, galactomannan; GPI, glycosylphosphatidylinositol; GSL, glycosylinositolphosphoceramide; LGM, GPI-anchored lipophosphogalactomannan; Pep1p, aspartic protease; Sod1p, Superoxide dismutase; TLN, thoracic lymph nodes.

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Table I. *Ags used in this study*

	Production	Ref.
Proteins (GenBank accession no.)		
Gel1p (Afu 2g01170)	Recombinant secreted	18, 19
Crf1p (Afu 1g16190)	Recombinant secreted	18
Pep1p (Afu 3g11400)	Recombinant	20
Cat1p (Afu 3g02270)	Recombinant	22
Sod1p (Afu 5g09240)	Recombinant	23
Dpp5p (Afu 2g09030)	Recombinant	24
RNUp (Afu 5g02330)	Recombinant	25
Mep1p (Afu 8g07080)	Recombinant	21
Polysaccharides		
α 1–3 glucan	Purified	30
β 1–3 glucan	Purified	26
GM	Purified	29
Glycolipids		
GSL	Purified	27
LGM	Purified	28

mice were lightly anesthetized by inhaled diethyl ether before instillation of a suspension of 2×10^7 viable conidia/20 μ l saline intranasally. Mice were immunosuppressed i.p. with 150 mg/kg of cyclophosphamide a day before infection. Mice were monitored for survival (median survival time, days) and fungal growth (colony forming unit/organ expressed as mean $\pm$ SE) as described (9). Mice dying of fungal challenge routinely underwent necropsy for histopathological confirmation of pulmonary aspergillosis. For histology, sections (3–4 μ m) of paraffin-embedded tissues were stained with the periodic acid-Schiff or Gomori's methenamine silver procedure.

A. *fumigatus* Ags

Table I describes the Ags used in this study. These include glycosylphosphatidylinositol (GPI)-anchored proteins involved in cell wall biosynthesis (18, 19), such as the 1,3- β glucanase (Gel1p) and an ortholog of Crf1p associated in β 1,6 glucan-chitin linkages (Crf1p) in *Saccharomyces cerevisiae* and secreted proteins, such as the aspartic protease (Pep1p) (20), metalloprotease (Mep1p) (21), mycelial catalase 1 (Cat1p) (22), superoxide dismutase (Sod1p) (23), dipeptidylpeptidase V (Dpp5p) (24) and ribonuclease (RNUp) (25). All proteins used in this study were produced as soluble proteins with a 6 $\times$ His Tag in the heterologous host *Pichia pastoris*, as described previously (26). Galactofuranose containing glycolipids, hereafter referred to as glycolipids, such as the glycosylphosphatidylcholine (GSL) B (27) and the GPI-anchored lipophosphogalactomannan (LGM) (28) were purified from total mycelial membranes as described (28, 29). Major polysaccharides of the cell wall such as α 1–3glucan, β 1–3glucan, and galactomannan (GM) were basically purified as described earlier (29–31), with modifications for the production of α 1–3 glucan and β 1–3glucan. Basically, α 1–3 glucans were obtained from an alkali-soluble fraction treated with 100 mM sodium metaperiodate for 3 days at 4°C in darkness and reduced by 250 mM NaBH₄ for 16 h in 100 mM ammonium hydroxide and hydrolyzed with 10% acetic acid at 100°C for 1 h. For β 1–3glucan, the alkali-insoluble fraction was periodate treated as described above and chitin further deacetylated with a mixture of sodium hydroxide 40% containing 1 N sodium borohydride for 3 days at room temperature. The de-acetylated product was then treated with nitrous acid to destroy the glucosamine polymer produced from chitin and produce β 1–3glucan.

Vaccination models

Two vaccination models were used, one using Ags with the immunoadjuvant murine CpG oligodeoxynucleotide 1862 (8) (CpG/Ag model), the other using dendritic cells (DC model) as adjuvant (9). In the CpG/Ag model, mice were injected intranasally with 2×10^7 *Aspergillus* conidia/20 μ l of saline 14 days before the infection or with 5 μ g of Ag + CpG 10 nM/20 μ l of saline, administered 14, 7, and 3 days before the infection. Mice were immunosuppressed i.p. with 150 mg/kg cyclophosphamide a day before infection. In the adoptive transfer model of Ag-pulsed DCs (9), splenic CD11c⁺ DCs, purified by magnetic cell sorting with MicroBeads (Miltenyi Biotec) conjugated to hamster anti-mouse CD11c mAbs (clone N-418), were pulsed with viable *Aspergillus* conidia (1:1 cell/fungus ratio) or different concentrations (5–20 μ g/ml) for 6 h at 37°C (9), and injected

twice (5×10^5 /each injection) i.p., a week apart, before the intranasal *Aspergillus* infection.

Generation of bone marrow transplanted mice

Bone marrow cells from donor BALB/c mice were resuspended in HBSS (Life Technologies) containing 10% FBS (Life Technologies) at a concentration of 100×10^6 cells/ml. T cell depletion was obtained upon incubation of the cells with MicroBeads (Miltenyi Biotec) conjugated to rat anti-mouse CD5 mAb (Ly-1; clone 53–7.3) for 15 min at 4°C followed by magnetic sorting in a magnetic cell separator (CliniMACS cell separation system; Miltenyi Biotec). Unbound cells were carefully aspirated, washed, and resuspended at a concentration of 100×10^6 cells/ml. C57BL6 mice were exposed to a lethal dose of 9 Gy, infused with T cell-depleted donor cells and injected i.p. 1 day later with Ag-pulsed DCs. Intranasal infection with *Aspergillus* conidia was performed 1 week after the DC administration (9).

Collection of broncho-alveolar lavage (BAL) fluid

Lungs were filled thoroughly with 1 ml aliquots of pyrogen-free saline through a 22-gauge bead-tipped feeding needle introduced into the trachea. The lavage fluid was collected in a plastic tube on ice and centrifuged at 400 g, 4°C, for 5 min. For differential BAL cell counts, cytospin preparations were made and stained with May-Grünwald Giemsa reagents (Sigma-Aldrich). At least 200 cells per cytospin preparation were counted and the absolute number of each cell type was calculated. Photographs were taken using a high-resolution Microscopy Olympus DP71 (Olympus).

Proliferative activity and cytokine production by human T lymphocyte clones

Human PBL, obtained from nine healthy donors upon written informal consent, were pulsed with heat-inactivated conidia or *Aspergillus* Ags, as described (32). Irradiated (20 Gy) cells were plated in U-bottom 96-well plates in RPMI 1640 medium containing 10% autologous human serum and autologous irradiated PBL at limiting dilution concentrations (range: 12,000–1,500 cells/well) was added. Exogenous IL-2 (125 U/ml; Chiron) was added on day 14 of culture. Growing cultures were split and supplemented with IL-2 as necessary. Frequency of Ag-specific CD45RO⁺ CD4⁺ T cell (as determined by FACS analysis) clones was determined as described (32). Clones were rested in recombinant human IL-2-free medium for 24 h and divided into three aliquots; one to detect specificity against the *Aspergillus* Ags, one against autologous irradiated cells (as a negative control), and one assessed for proliferation to 0.5% PHA (PHA-L; Biochrom) as a positive control. Proliferation was measured 3 days after pulsing by [³H]thymidine labeling (Amersham Biosciences) for 6 h. In parallel experiments, T cell clones supernatants were collected for determination of cytokine content (Pierce SearchLight Products; Thermo Fisher Scientific).

Quantification of cytokines by real-time RT-PCR and ELISA

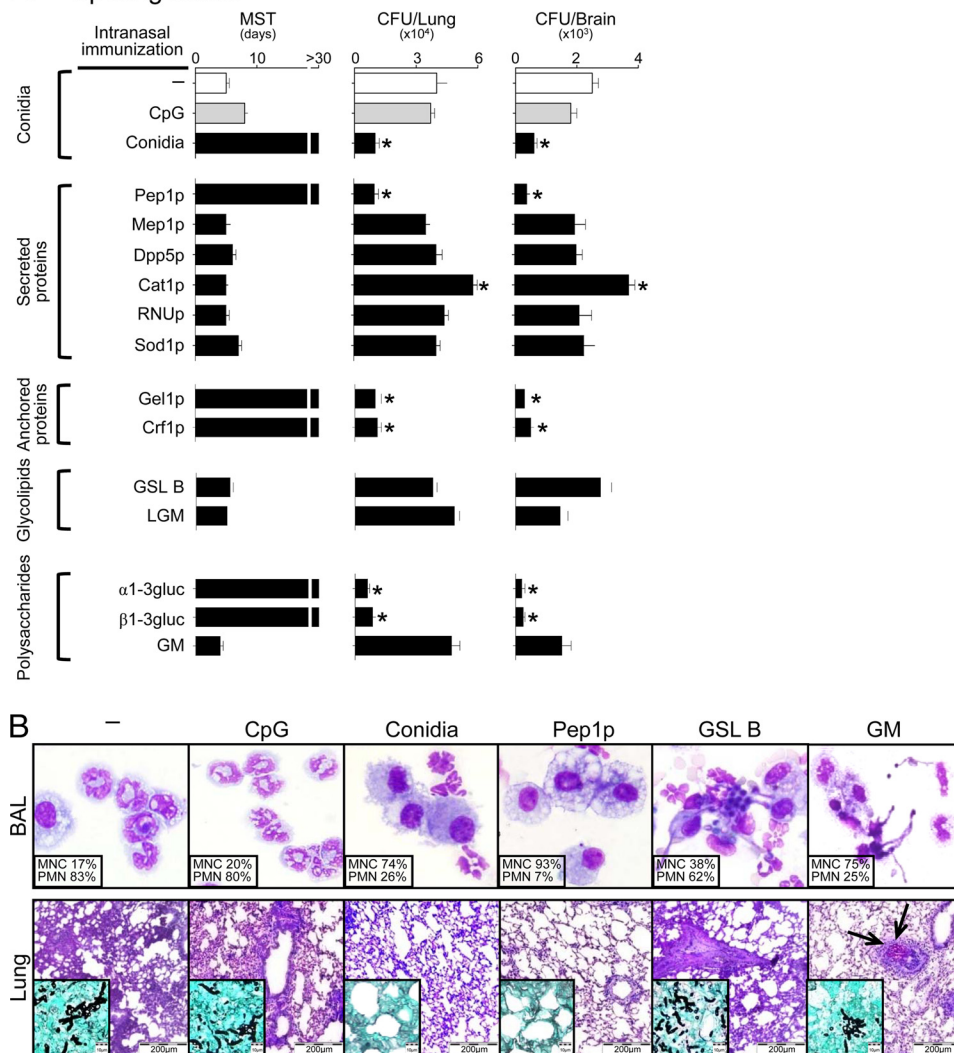
Real-time RT-PCR was performed using the Mx3000P QPCR System and SYBR Green chemistry (Stratagene). CD4⁺ T cells were separated by magnetic cell sorting with MicroBeads and MidiMacs (Miltenyi Biotec) from thoracic lymph nodes (TLNs) were recovered a week after the infection, lysed, and total RNA was extracted using RNeasy Mini Kit (Qiagen) and reverse transcribed with SensiScript Reverse Transcriptase (Qiagen), according to manufacturer's directions. The PCR primers for genes encoding *Tbet*, *Gata3*, *Rorc*, *Foxp3*, *ifn γ* and *il10* were as described (33, 34). Amplification efficiencies were validated and normalized against *Gapdh*. The thermal profile for SYBR Green real-time PCR was at 95°C for 10 min, followed by 40 cycles of denaturation for 30 s at 95°C and an annealing/extension step of 30 s at 72°C. Each data point was examined for integrity by analysis of the amplification plot. The mRNA-normalized data were expressed as relative cytokine mRNA in treated cells compared with that of mock-infected cells. Cytokine content was assessed by ELISA (R&D Systems; for IL-23, eBioscience) on lung homogenates 3 days after the infection. The detection limits (pg/ml) of the assays were <10 for IFN- γ , <5 for IL-4, <10 for IL-17A/F, and <3 for IL-10.

Statistical analysis

Student's *t* test was used to determine significance of values among experimental groups (significance was defined as *p* < 0.05). In vivo groups consisted of six to eight animals. The data reported were pooled from three to five experiments. The data reported are either from one representative

A - CpG/Ag model

FIGURE 1. Vaccinating potential of the different *Aspergillus* Ags against invasive pulmonary aspergillosis in the CpG/Ag model. A, C57BL/6 mice were injected with 2×10^7 *Aspergillus* conidia 14 days before or with the different Ags (5 μ g) and CpG (10 nM) 14, 7, and 3 days before the intranasal infection with 2×10^7 live, resting conidia. Fungal growth (CFU/organ) is expressed as mean $\pm$ SE. Bars indicate SE. *, $p < 0.05$, treated vs untreated (–) mice. B, Percentages of BAL cells obtained by lung lavage and lung histology (periodic acid-Schiff-staining and Gomori's methenamine silver-staining in the inset) of mice treated as indicated above, 3 days after infection. Note the reduced inflammatory cell recruitment and amelioration of lung inflammatory pathology in mice vaccinated with conidia or Pep1p, as opposed to neutrophil recruitment and inflammatory pathology seen in untreated or GSL B-treated mice. Note the limited inflammatory cell recruitment and unrestricted fungal growth (arrows) in GM-treated mice. Bars indicated magnifications. Shown are the results pooled from four experiments (six animals/group). Photographs were taken using a high-resolution Microscopy Olympus DP71. MST, median survival time (days); PMN, polymorphonuclear cells; MNC, mononuclear cells.



experiment of three independent experiments (histology and RT-PCR) or pooled from three to five experiments. Data were analyzed by GraphPad Prism 5.0 program (GraphPad Software).

Results

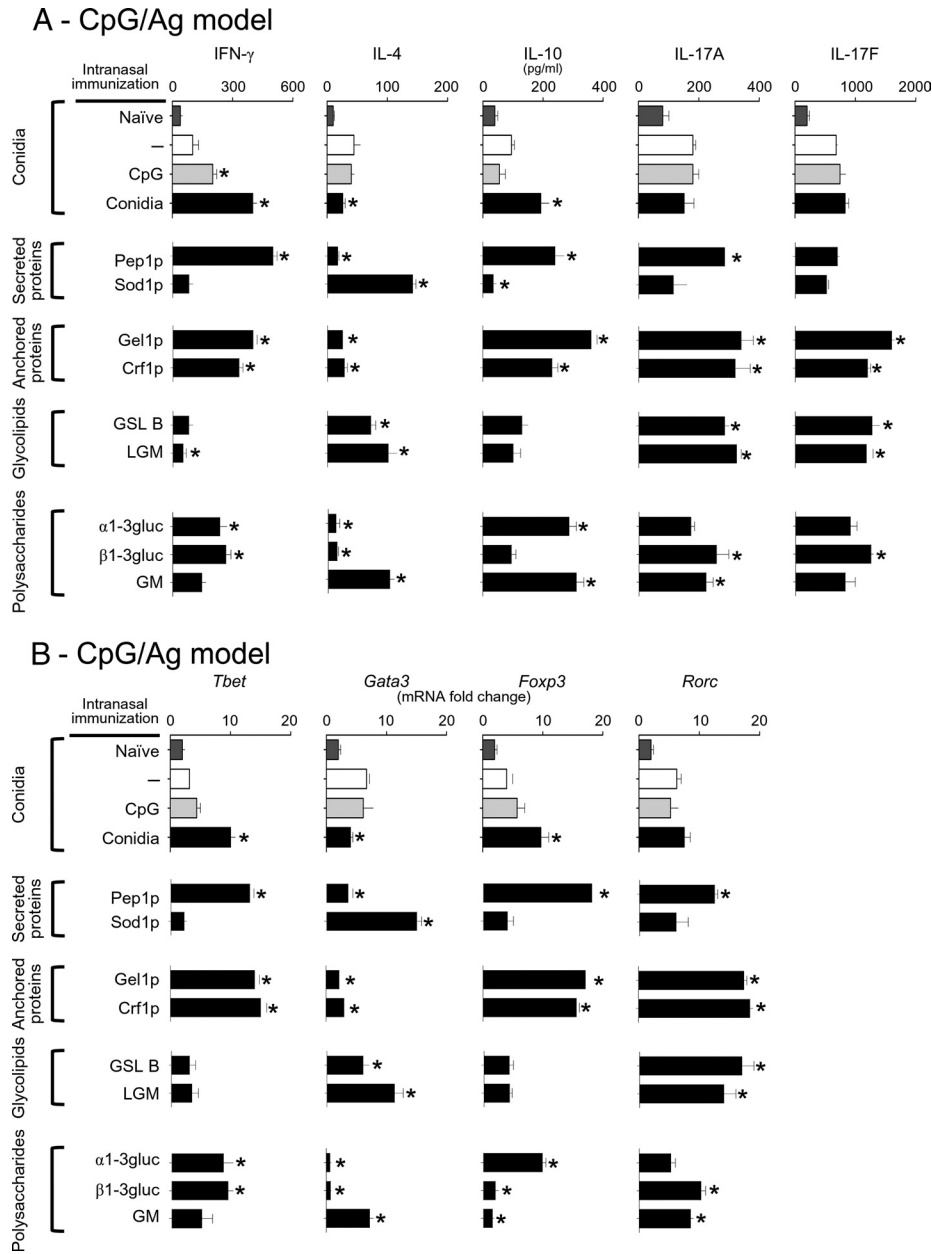
Vaccination studies in mice

CpG/Ag model. Proteins and polysaccharides indicated in Table I were comparatively analyzed in a well established experimental model of Ag-induced protection (8). Mice were treated with Ags and CpG as adjuvant before the assessment of protection against pulmonary aspergillosis in terms of survival, local fungal growth and dissemination, inflammatory cell recruitment and histopathology, and pattern of Th cell activation. Figs. 1 and 2 show the cumulative results obtained with the different Ags. Similar to the protection afforded by conidia, the protease Pep1p, the GPI-anchored proteins Gel1p and Crf1p, and the polysaccharides α 1-3glucan and β 1-3glucan all conferred protection against the infection, as indicated by the long-term survival, restriction of fungal growth in the lung, and reduced dissemination to the brain (Fig. 1A). Protection was associated with reduced neutrophil recruitment in the BAL and lungs as well as reduced inflammatory pathology in the lung for conidia and Pep1p as representative examples (Fig. 1B). In contrast, most secreted proteins tested and galactofuranose containing glycolipids (GSL and LGM) and polysaccharide galactomannan (GM) failed to confer protection. This was associated

with fungal growth and dissemination as well as with neutrophil and mononuclear cell recruitment, as seen with glycolipids and proteins (see GSL B as a representative example, Fig. 1B). With GM, the uncontrolled fungal growth occurred in the relative absence of inflammatory cell recruitment, at least in the lung (Fig. 1B). The patterns of protection and non-protection observed with the different Ags did not vary upon using a higher (i.e., 10 μ g) dose of each Ags (data not shown).

To correlate protection with pattern of Th cytokine production in the lungs and Th cell activation in the TLNs, the levels of Th1 (IFN- γ in the lungs and *Tbet* expression in TLNs), Th2 (IL-4/*Gata3*), Th17 (IL-17A and *F/Rorc*), and Treg (IL-10/*Foxp3*) activation were assessed. In line with previous findings (33), protection by conidia was associated with Th1/Treg cell activation and concomitant inhibition of Th2 cells (Fig. 2). Similar to conidia, α 1-3glucan also conferred a Th1/Treg protection and concomitant Th2 inhibition. Interestingly, protection afforded by the different Ags was associated with Th1 activation variably occurring with both Treg and Th17 cell activation (for Pep1p, Gel1p, and Crf1p) or Th17 activation alone (for β 1-3glucan). In contrast, defective Th1 activation associated with Th2 activation was observed with failure to induce protection, as seen with Sod1p (as a representative member of the secreted proteins), glycolipids (GSL B and LGM) and the GM polysaccharide. Interestingly, Th17 activation

FIGURE 2. Vaccinating potential of the different *Aspergillus* Ags against invasive pulmonary aspergillosis in the CpG/Ag model. *A* and *B*, Cytokine production in lung (*A*) and transcription factor mRNA expression in CD4⁺ T cells from TLNs (*B*) of mice injected as in Fig. 1. Cytokines were determined in lung homogenates 3 days after the infection by ELISA and mRNA expression was measured by RT-PCR a week after infection. *, *p* < 0.05, treated vs untreated mice. Shown are the results pooled from four experiments, each assessed in triplicates. Naive, uninfected mice; –, untreated. Bars indicate SE.



was also concomitant with Th2 activation in condition of failure to confer protection, particularly with the glycolipids GSL and LGM (Fig. 2), a finding suggesting that Th17 cell activation induced by

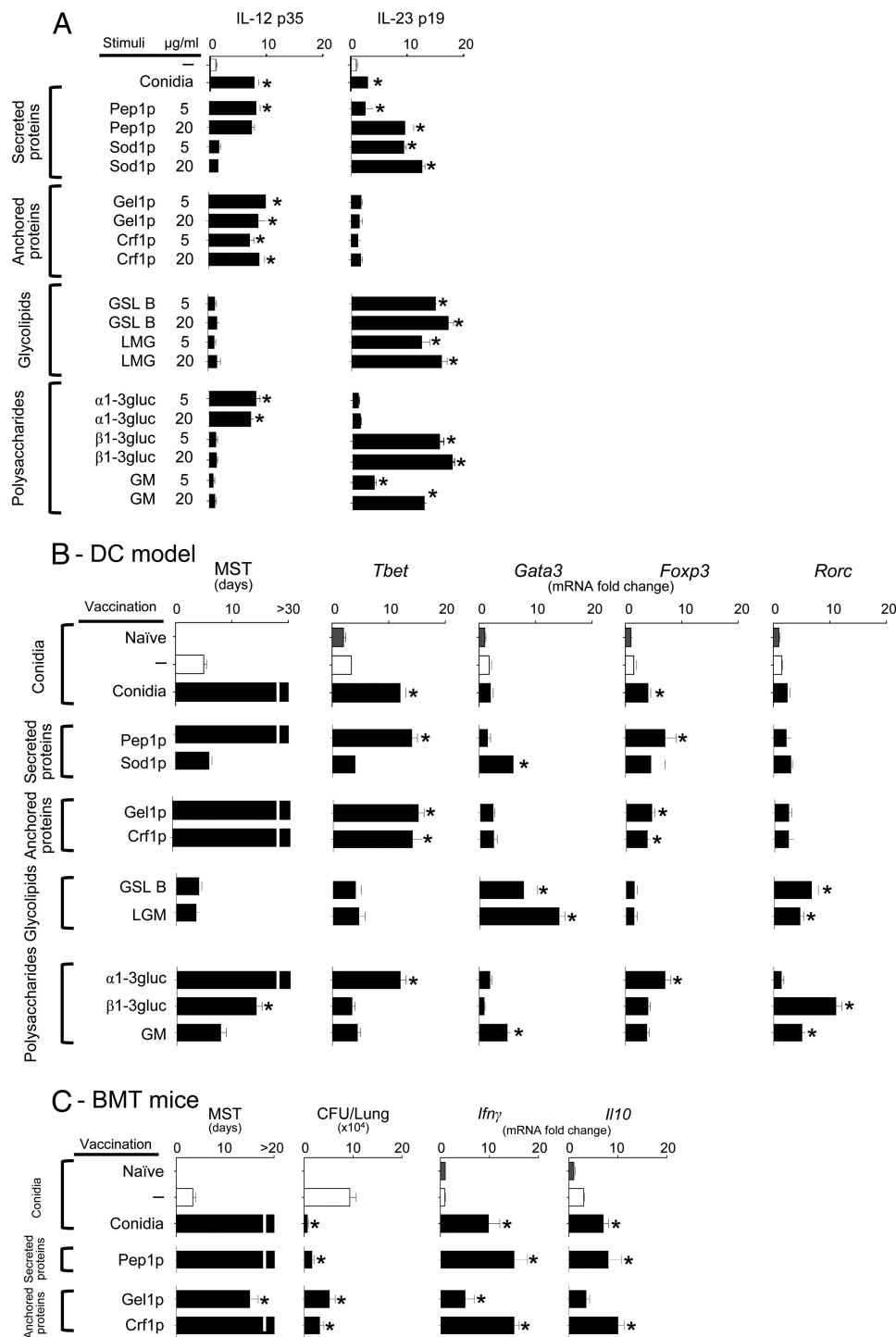
glycolipids is not discriminative of protective/non-protective anti-*Aspergillus* responses upon vaccination. Table II summarizes the immunoactivating properties of the different Ags in the CpG/Ag model.

Table II. Summary of the immunoactivating properties of the different *Aspergillus* Ags

	Protective			Non-protective	
	Th1/Treg	Th1/Th17/Treg	Th1/Th17	Th2	Th2/Th17
CpG model	Conidia α1-3glucan	Pep1p Gel1p Crf1p	β1-3glucan	Sod1p Cat1p Metalloendopeptidase RNase Dipeptidylpeptidase Sod1p ^b	GSL B LGM GM
DC model	Conidia Pep1p Gel1p Crf1p α1-3glucan		β1-3glucan ^a		GSL B LGM GM

^a Partially protective in the DC model.
^b Cat1p, Mep1p, RNUp, and Dpp5p were not tested in DC model.

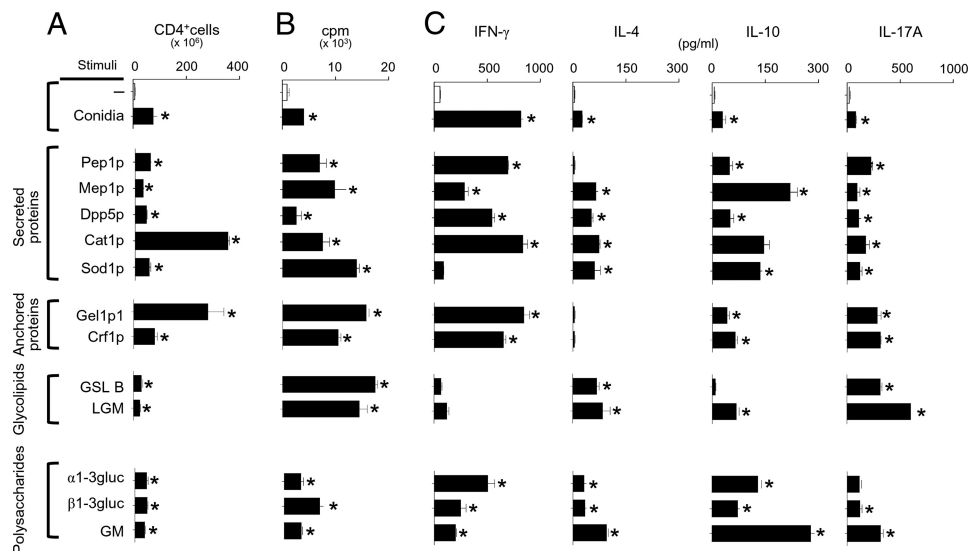
FIGURE 3. Vaccinating potential of the different *Aspergillus* Ags against invasive pulmonary aspergillosis in the DC model. **A**, Purified DCs from spleens were pulsed with viable *Aspergillus* conidia or the Ags at the indicated concentrations before the assessment of IL-12p35 or IL-23p19 mRNA expression by RT-PCR 6 h after pulsing. *, $p < 0.05$, pulsed vs unpulsed (–) DCs. **B** and **C**, The vaccinating potential of Ag-pulsed DCs injected twice (5×10^5 /each injection), a week apart, before the intranasal infection with *Aspergillus* conidia in untransplanted (**B**) or transplanted (**C**) mice. Colony-forming units (CFU) were evaluated a week after the infection; gene expression analysis for *Tbet*, *Rorc*, *Gata3*, and *Foxp3* on purified CD4⁺T cells from TLNs or for *ifn γ* and *il10* in lungs was done by RT-PCR a week after the infection. Shown are the results pooled from four experiments (six animals/group), each assessed in triplicates (for RT-PCR). *, $p < 0.05$, treated vs untreated (–) mice. MST, median survival time (days); naive, uninfected mice; BMT, bone marrow transplanted.



DC model. The protective/non-protective activity of the different Ags was also assessed in the DC-based vaccination model (9) in which untransplanted or transplanted mice are subjected to adoptive therapy with DCs pulsed in vitro with the relevant Ag. Ag-pulsed DCs were also assessed for Ag-induced activation in terms of IL-12p35 or IL-23p19 mRNA expression, being IL-12p70 or IL-23 production signatures of Th1- or Th17-inducing DCs (34). The results showed a clear distinction in cytokine mRNA expression among the different Ags and IL-12p35 mRNA expression being induced by protective Pep1p, Gel1p, Crflp and α 1-3glucan at both concentrations, 5 and 20 μ g, but not by the non-protective Sod1p, GSL B, LGM, GM, or the poorly protective β 1-3glucan at any concentration. In contrast, the IL-23p19 mRNA message was in-

duced by all non-protective galactofuranose containing glycolipids, polysaccharides, and secreted proteins, as well as by the protective Pep1p and β 1-3glucan (Fig. 3A). IL-23 was not induced by protective Gel1p, Crflp, and α 1-3glucan (Fig. 3A). In untransplanted mice, DCs pulsed with the protective Ags induced a long-term protection (median survival time > 30 days) similar to that induced by conidia-pulsed DCs as opposed to the failure to increase survival over the untreated controls by DCs pulsed with the non-protective Ags. Of interest, DCs pulsed with β 1-3glucan conferred only a limited, albeit significant, protection in terms of survival (Fig. 3B). The pattern of Th cell activation revealed that protection occurred with the expression of *Tbet* and *Foxp3* in CD4⁺ T cells from TLNs, whereas non-protection occurred with

FIGURE 4. Activation of human Th cells by *Aspergillus* Ags. **A**, Frequencies of Ag-specific CD4⁺ T cell clones in human PBL from healthy donors. Frequencies of Ag-specific T cell clones were determined in PBL cultured at limiting dilution condition (from 12,000 to 1,500 cells/well) on irradiated autologous cells, as described in Ref. 32. **B**, Ag-specific proliferation of T cell clones was measured 3 days after pulsing by [³H]thymidine labeling. cpm, mean cpm $\pm$ SE of triplicate samples. **C**, Cytokine production by CD4⁺ T cell clones, quantified by specific ELISA. *, $p < 0.05$, stimulated vs unstimulated (–) cells. Shown are pooled results of three independent experiments, each involving PBL from three healthy donors, each assessed in triplicates.



the expression of *Gata3* and/or *Rorc* (Fig. 3B). Thus, in both models of vaccination, protection is associated with Th1/Treg activation and non-protection with Th2, with or without Th17 activation. In the DC-based vaccination model, Th17 cells were not concomitantly activated with Th1 cells, a finding suggesting that the contribution of Th17 cells to Th1-mediated protection may depend on the mode and site of fungal Ag presentation. Table II summarizes the immunoactivating properties of the different Ags in the DC model.

Of interest, DCs pulsed with the protective Ags retained their vaccinating potential in hematopoietic transplanted mice, where a long-term protection (Pep1p and Crflp) or an increased survival (Gel1p) was observed. This was associated with the activation of *ifnγ* and *il10* in the lungs (Fig. 3C). Thus, the three protective *Aspergillus* Ags retained their vaccinating potential in condition of immunodeficiency.

Th cell activation in human

To correlate the murine findings with human Th cell activation, the different Ags were assessed for ability to activate Ag-specific proliferation and Th cytokine production by human T cell clones from healthy donors. The frequency of Ag-specific T clones greatly varied with the different Ags, irrespective of their chemical nature and their vaccinating potential in mice. For example, the frequency of specific T cell clone was very high with in the non-protective Cat1p or the protective Gel1p, whereas the number of T cell clones induced by the protective Pep1p and Crflp was low (Fig. 4A). Despite the low frequency of clones, the majority of the secreted proteins and glycolipids were the most potent activators of proliferation, whereas polysaccharides induced the lowest proliferation (Fig. 4B). However, irrespective of the frequency of Ag-specific clones, the Ag-specific proliferation and, most importantly, the production of IFN-γ was associated with IL-10, but not IL-4 production, in response to the protective protein Ags.

The majority of the clones produced IFN-γ, except the Sod1p-, GSL-B-, and LGM-specific clones, which instead produced a noticeable amount of IL-4, similar to the non-protective secreted proteins. Among these, although non-protective in mice, both dipeptidylpeptidase V- and Cat1p-specific clones produced high levels of IFN-γ in addition to IL-4 and IL-10. At variance with murine IL-10 production by CD4⁺ T cells, which mostly occurs in association with Th1 cell activation, IL-10 production by Ag-specific clones mostly occurred in response to secreted proteins and

polysaccharides, particularly GM. Similarly, IL-17A was produced in response to the different fungal Ags, including protective Pep1p, Gel1p, and Crflp, and non-protective components, such as glycolipids and GM (Fig. 4C). To our knowledge, this is the first observation of the occurrence of IL-17-producing cells in the human peripheral Th repertoire against *Aspergillus*.

Discussion

Vaccines targeting invasive fungal infections are an extremely promising area of research and development (35, 36). In this study, by comparatively assessing a number of *Aspergillus* Ags for immune activation, we have identified several fungal components that acted as immunodominant Th1/Treg-inducing Ags in mice and humans. Our study shows the broadness and specificity of human T cell repertoire against *Aspergillus* Ags that could be usefully exploited to selective probe antifungal Th effector responses in the different clinical settings. In fact, secreted proteins mostly activate IL-4-producing clones, glycolipids mostly activate IL-17-producing clones, and polysaccharides variably activate IFN-γ-, IL-17-, or IL-10-producing clones. Furthermore and importantly, at least three unrelated fungal components, Pep1p and the GPI-anchored Gel1p and Crflp, retained their immunodominant Th1/Treg activating potential from mice to humans. As most of the Ags tested also elicited a humoral response in mice (14) and humans (12, 26), this demands a clear definition of the Ag(s) that better correlates with immune protection.

Pep1p shares with the GPI-anchored Gel1p and Crflp the ability to elicit protective Th1/Treg antifungal responses in mice and humans. Pep1p is a member of the aspartic proteases (20) that share with other secreted proteins, such as Mep1p, Sod1p, and RNUP, the ability to act as allergen associated with type 1 hypersensitivity. By showing their Th2 promoting activity, the present study confirms the allergenic potential of the secreted proteins (12, 14). However, the vaccinating potential of some allergens, including secreted proteases (37), have also been described (8, 11). Although the secretion of aspartic proteases has long been recognized as a virulence-associated trait of *Candida* spp. (38), the vaccinating potential of secreted proteases from *Candida* (37), and now from *Aspergillus*, further adds to the biological functions of proteases at the fungus/host interface, as already suggested (39). In this regard, we have recently shown a crucial role for host protease-activated

receptors in murine aspergillosis (39). The emerging role of protease-induced activation of inflammasome and associated signaling pathways in immunity and vaccination (40), as well as the ability of metalloproteases to inhibit inflammasome activation in infection (40), suggests a further immunomodulatory role for microbial proteases.

The vaccinating potential of fungal GPI-anchored proteins, putatively involved in cell wall morphogenesis and strictly in contact with host cells, is also of interest. Arroyo et al. have demonstrated that the GPI-anchored cross-linking enzymes are immunologically reactive during human fungal infections because they induce the production of specific Abs (18). They are highly enriched in the fungal cell wall and can be released into the environment of the host (15), which may explain why these Ags are targets for humoral and cellular adaptive immunity (12, 26). In mice, not only was the Gel1p homologue of *Coccidioides posadasii* protective against coccidioidomycosis (41), but the yeast homologue, Gas1p, was similarly protective against aspergillosis (data not shown) and the additional GPI-anchored Crf1p protein (this study) and its homologue in yeasts (Crh1) were similarly protective against murine aspergillosis (data not shown).

One interesting observation of the present study is the relative contribution of glycolipids to the activation of Th17 responses to the fungus. Although the production of IL-17A also occurred in response to the other *Aspergillus* Ags, we have recently shown that both pathogenic and nonpathogenic Th17 responses are activated in experimental fungal infections (5, 6, 34). Among glycolipids, GSLs, composed of an oligosaccharide and a ceramide hydrophobic moiety, are essential components of the fungal cell membrane and can be strongly immunogenic when associated with other cell membrane components, but also immunosuppressive (27). We found that GSL B, one of the GSLs identified (27), greatly promoted the activation of pathogenic Th17 responses, as revealed by the unrestricted fungal growth, dissemination, and the recruitment of inflammatory neutrophils. Glycolipids potently induced the activation of CD4⁺Th17, and, to some degree, Th2 cells, in mice and humans as well as the expression of IL-23p19 in DCs, which are known to contribute to antifungal Th17 cell activation (34). This may explain the stronger Th17-inducing activity of LGM, the GM with a lipid anchor (28). Mechanistically, glycolipids are known activators of the CD1d-restricted NK T cells (42), which greatly contribute to Th17 activation and IL-17 production in the lungs.

Our study shows for the first time that α 1-3glucan conferred vaccine-induced resistance to infection that was associated with the activation of Th1 responses occurring with Treg cell activation. Thus, not only α 1-3glucan masks β 1-3glucan recognition in infecting morphotypes (43), but it is also endowed with immunoactivating properties. Interestingly, the ability of β 1-3glucan, known to stimulate the Dectin-1/TLR2/Syk pathway (44), to activate DCs for Th17 priming in vivo conferred only limited antifungal protection, a finding suggesting that Th17 cells contribute to Th1-mediated protection. Abs to β 1-3glucan also mediated, albeit partially, protection against the fungus (45), a finding confirming the immunodominant role of this polysaccharide in infection. Similar results were obtained when mice were injected with curdlan, a bacterial β 1-3glucan (data not shown).

In contrast to the protective effects of some polysaccharides, GM, the most useful diagnostic marker in patients with invasive aspergillosis (46), never induced protection against the fungus and mostly activated Th2/Th17 cells in mice and humans. Of interest, elevated levels of IL-10, with no concomitant activation of Treg cells, were observed in response to GM, a finding suggesting that glycosylation, by inhibiting DC maturation and silencing inflammation via IL-10 production (47), may facilitate the initial estab-

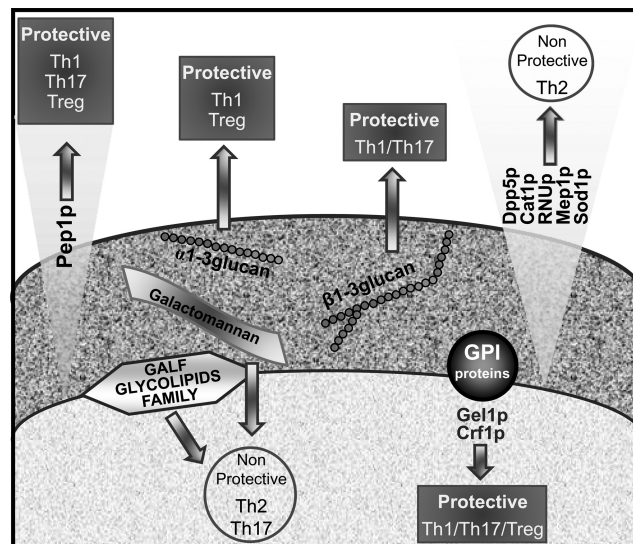


FIGURE 5. Immunology of *Aspergillus* cell wall: a symphony of multiple components with distinct immunoactivating signatures. Different fungal components and secreted proteins are endowed with distinct, yet overlapping, capacity to activate Th cell responses in mice and humans, with secreted proteins inducing Th2 cell activation, anchored proteins inducing Th1/Treg, glycolipids inducing Th17, and polysaccharides, mostly IL-10 production, variably associated with protection. Pep1p, Gel1p, and Crf1p retained their immunodominant Th1/Treg activating potential from mice to humans.

lishment of *A. fumigatus* in the lung. That galactofuranose, which is present in the fungus with no equivalent in human cells, is the component that exacerbates diseases is one current working hypothesis.

Overall, the data obtained with the glycome are consistent with the notion that the specificity of C-type lectin receptors is determined by the type of glycan, the branching of the carbohydrates, and the glycan multimerization on the protein/lipid backbone (48), and this balances immune activation and inhibition of DC function (49). It has been suggested that glycans might have been used to sense the presence of nonself since the beginning of multicellular life (50). Thus, the remarkable structural diversity of glycan repertoire may have allowed fungi to adapt to the vertebrate immune system.

In conclusion, the results of the present study show that different fungal components are endowed with a distinct, yet overlapping, capacity to activate protective and non-protective Th cell responses in mice and Th cytokine production in humans (Fig. 5), a finding consistent with the ability of the fungus to activate, suppress, or subvert host immune response during life cycle in vivo through dynamic changing of cell wall structure and secretion (15, 51) and the exploitation of receptors with opposing modulatory effects on DC functions, such as TLRs and C-type lectin receptors (52).

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Disclosures

The authors have no financial conflict of interest.

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