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

Silvia Bozza, Cecile Clavaud, Gloria Giovannini, Thierry Fontaine, Anne Beauvais, Jacqueline Sarfati, Carmen D'Angelo, Katia Perruccio, Pierluigi Bonifazi, Silvia Zagarella, Silvia Moretti, Francesco Bistoni, Jean-Paul Latgé and Luigina Romani

*J Immunol* 2009; 183:2407-2414; Prepublished online 22 July 2009; doi: 10.4049/jimmunol.0900961
http://www.jimmunol.org/content/183/4/2407

**References** This article cites 51 articles, 23 of which you can access for free at: http://www.jimmunol.org/content/183/4/2407.full#ref-list-1

**Subscription** Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions** Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts** Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
The Journal of Immunology

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

Silvia Bozza,* Cecile Clavaud,† Gloria Giovannini,* Thierry Fontaine,† Anne Beauvais,† Jacqueline Sarfati,† Carmen D’Angelo,* Katia Perruccio,* Pierluigi Bonifazi,* Silvia Zagarella,* Silvia Moretti,* Francesco Bistoni,* Jean-Paul Latgé,† and Luigina Romani†

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 immunomodulatory Aspergillus Ags.

Received for publication March 26, 2009. Accepted for publication May 28, 2009.

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) and Th17 cells are key mediators 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 nonprotective 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 C57BL6 (H-2b) and BALB/c (H-2d) 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 C57BL6

*Department of Experimental Medicine and Biochemical Sciences, University of Perugia, Perugia, Italy; and †Unite des Aspergillus, Institut Pasteur, Paris, France

Received for publication March 26, 2009. Accepted for publication May 28, 2009.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertised in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This study was supported by the Specific Targeted Research Project MANASP (LSHE-CT-2006), contract number 037899 (FP6) (to L.R. and J.P.L.) and the Italian Project PRIN 2007KLCKP8_004 (to L.R.), 2007XYB9T9_001 (to S.B.), and 2007KLCKP8_005 (to F.B.).

2 Address correspondence and reprint requests to Dr. Luigina Romani, Microbiology Section, Department of Experimental Medicine and Biochemical Sciences, University of Perugia, Via del Giochietto, 06126 Perugia, Italy. E-mail address: romani@unipg.it

3 Abbreviations used in this paper: Treg, regulatory T cell; BAL, broncho-alveolar lavage fluid; Cat1p, mycelia catalase 1; Crf1p, β1,6 glucan-chitin linkages; DC, dendritic cell; Ge1p, 1,3-β glucanosyltransferase; GM, galactomannan; GPI, glycosylphosphatidylinositol; GSL, glycosylinositolphosphoceramide; LGM, GPI-anchored lipophosphogalactomannan; Pep1p, aspartic protease; Sod1p, Superoxide dismutase; TLN, thoracic lymph nodes.

Copyright © 2009 by The American Association of Immunologists, Inc. 0022-1767/09/$2.00

www.jimmunol.org/cgi/doi/10.4049/jimmunol.0900961

www.jimmunol.org/cgi/doi/10.4049/jimmunol.0900961
myces cerevisiae and secreted proteins, such as the aspartic protease His Tag in the heterologous host (24) and ribonuclease (RNUp) (25). All proteins used in this study were superoxide dismutase (Sod1p) (23), dipeptidylpeptidase V (Dpp5p) (24), and resuspended at a concentration of 100 \times 10^6 cells/ml. C57BL/6 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–Grunwald 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 informed consent, were pulsed with heat-inactivated conidia or Aspergillus Ags, as described (32). Irradiated (20 Gy) cells were plated in 24-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 SearleLight 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 Biotech) 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 Thet, Gata3, Rorc, Foxp3, ifny 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

Table I. Ags used in this study

<table>
<thead>
<tr>
<th>Proteins (GenBank accession no.)</th>
<th>Production</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gel1p (Afu 2g01170)</td>
<td>Recombinant</td>
<td>18, 19</td>
</tr>
<tr>
<td>Crf1p (Afu 1g16190)</td>
<td>Recombinant</td>
<td>18</td>
</tr>
<tr>
<td>Pep1p (Afu 3g14100)</td>
<td>Recombinant</td>
<td>20</td>
</tr>
<tr>
<td>Cat1p (Afu 3g02270)</td>
<td>Recombinant</td>
<td>22</td>
</tr>
<tr>
<td>Sod1p (Afu 5g09240)</td>
<td>Recombinant</td>
<td>23</td>
</tr>
<tr>
<td>Dpp5p (Afu 2g09030)</td>
<td>Recombinant</td>
<td>24</td>
</tr>
<tr>
<td>RNUp (Afu 5g02330)</td>
<td>Recombinant</td>
<td>25</td>
</tr>
<tr>
<td>Meplp (Afu 5g07080)</td>
<td>Recombinant</td>
<td>21</td>
</tr>
<tr>
<td>Polysaccharides</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α1–3 glucan</td>
<td>Purified</td>
<td>30</td>
</tr>
<tr>
<td>β1–3 glucan</td>
<td>Purified</td>
<td>26</td>
</tr>
<tr>
<td>GM</td>
<td>Purified</td>
<td>29</td>
</tr>
<tr>
<td>Glycolipids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GSL</td>
<td>Purified</td>
<td>27</td>
</tr>
<tr>
<td>LGM</td>
<td>Purified</td>
<td>28</td>
</tr>
</tbody>
</table>

mice were lightly anesthetized by inhaled diethyl ether before instillation of a suspension of 2 \times 10^7 viable conidia/20 µl 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 ± SE) as described (9). Mice dying of fungal challenge routinely underwent necropsy for histopathological confirmation of pulmonary aspergillosis. For histology, sections (3–4 µm) 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-glucan synthetase (Gel1p) and an ortholog of Crf1p associated in β1,6 glucan-chitin linkages (Crf1p) in Saccharomyces cerevisiae and secreted proteins, such as the aspartic protease (Pep1p) (20), metalloprotease (Meplp) (21), mycelial catalase 1 (Cat1p) (22), superoxide dismutase (Sod1p) (23), and ribonuclease (RNUp) (24). All proteins used in this study were produced as soluble proteins with a 6xHis Tag in the heterologous host Pichia pastoris, as described previously (26). Galactofuranose containing glycolipids, hereafter referred to as glycolipids, such as the glycosylisotolylphosphocheramide (GSL) B (27) and the GPI-anchored lipophosphagactomannan (LGM) (28) were purified from total mycelial membranes as described (28, 29). Major polysaccharides of the cell wall such as α1–3glucan, β1–3glucan, and galactomannan (GM) were basically purified as described earlier (29–31), with modifications for the production of α1–3 glucan and β1–3 glucan. Basically, α1–3 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 in 100 mM sodium hydroxide and hydrolyzed with 10% acetic acid at 100°C for 1 h. For β1–3glucan, the alkali-insoluble fraction was periodate treated and resuspended in 6xH2O at the target concentration of 100 µM and SYBR Green chemistry (Stratagene). CD4+ T cells were separated by magnetic cell sorting with MicroBeads and MidiMacs (Miltenyi Biotech) 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 Thet, Gata3, Rorc, Foxp3, ifny 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...
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 α1–3glucan and β1–3glucan 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 μg) 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, α1–3glucan 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 β1–3glucan). 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...
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 im-munoactivating properties of the different Ags in the CpG/Ag model.

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

<table>
<thead>
<tr>
<th>Protective</th>
<th>Non-protective</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Conidia</td>
</tr>
<tr>
<td>Cpg model</td>
<td>α1–3glucan</td>
</tr>
<tr>
<td>DC model</td>
<td>Pep1p</td>
</tr>
</tbody>
</table>

α Partially protective in the DC model.
β Cat1p, Mep1p, RNUp, and Dpp5p were not tested in 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, Crf1p and α1-3glucan at both concentrations, 5 and 20 μg, but not by the non-protective Sod1p, GSL B, LGM, GM, or the poorly protective β1-3glucan at any concentration. In contrast, the IL-23p19 mRNA message was induced by all non-protective galactofuranose containing glycolipids, polysaccharides, and secreted proteins, as well as by the protective Pep1p and β1-3glucan (Fig. 3A). IL-23 was not induced by protective Gel1p, Crf1p, and α1-3glucan (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 β1-3glucan 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 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 × 10⁵ 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 were 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.
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 immunomodulating 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 Crf1p) 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 Crf1p 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 dipetidylpeptidase 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 Crf1p, 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 Crf1p, 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 Crf1p 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 RNu1p, 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 (Crl1) 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 explains the stronger Th17-inducing activity of LGM (data not shown) and the additional GPI-anchored Crf1p protein (this study) and its homologue in yeasts (Crl1) were similarly protective against murine aspergillosis (data not shown).

In conclusion, the results of the present study show that different fungal components are endowed with a 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.

FIGURE 5. Immunology of *Aspergillus* cell wall: a symphony of multiple components with distinct immunomodulating 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.

### Acknowledgments

We thank Dr. Cristina Massi Benedetti for editorial support.

### Disclosures

The authors have no financial conflict of interest.

### References


