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Plant production of anti-β-glucan antibodies for immunotherapy of fungal infections in humans

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Summary

There is an increasing interest in the development of therapeutic antibodies (Ab) to improve the control of fungal pathogens, but none of these reagents is available for clinical use. We previously described a murine monoclonal antibody (mAb 2G8) targeting β-glucan, a cell wall polysaccharide common to most pathogenic fungi, which conferred significant protection against *Candida albicans, Aspergillus fumigatus* and *Cryptococcus neoformans* in animal models. Transfer of this wide-spectrum, antifungal mAb into the clinical setting would allow the control of most frequent fungal infections in many different categories of patients. To this aim, two chimeric mouse–human Ab derivatives from mAb 2G8, in the format of complete IgG or scFv-Fc, were generated, transiently expressed in *Nicotiana benthamiana* plants and purified from leaves with high yields (approximately 50 mg Ab/kg of plant tissues). Both recombinant Abs fully retained the β-glucan-binding specificity and the antifungal activities of the cognate murine mAb against *C. albicans*. In fact, they recognized preferentially β1,3-linked glucan molecules present at the fungal cell surface and directly inhibited the growth of *C. albicans* and its adhesion to human epithelial cells *in vitro*. In addition, both the IgG and the scFv-Fc promoted *C. albicans* killing by isolated, human polymorphonuclear neutrophils in *ex vivo* assays and conferred significant antifungal protection in animal models of systemic or vulvovaginal *C. albicans* infection. These recombinant Abs represent valuable molecules for developing novel, plant-derived immunotherapeutics against candidiasis and, possibly, other fungal diseases.

Introduction

Infections caused by *Candida* and other opportunistic fungal species such as *Aspergillus* and *Cryptococcus* represent an increasing health concern, especially for immunocompromised and hospitalized patients. Antifungal therapy is often unable to control these infections and may be associated with severe side effects, high costs and the emergence of resistant fungal strains. In particular, treatment fails in about 42% of patients in the case of disseminated candidiasis, so that the overall mortality from this infection is around 40%–50%, despite administration of the optimal chemotherapeutic regimens (Enoch *et al.*, 2006; Kanafani and Perfect, 2008). Moreover, chronic vulvovaginal candidiasis, which severely affects the quality of life for some million women in the world, is very frequently refractory to antifungal treatments (Sobel, 2003).

In the search of novel therapeutic strategies for fighting candidiasis and other opportunistic fungal diseases, replacement or integration of classical chemotherapy with therapeutic antifungal antibodies (Ab) is considered a feasible and promising approach (Casadevall *et al.*, 2004; Matthews and Burnie, 2004; Cassone *et al.*, 2005; Ostrosky-Zeichner *et al.*, 2010). A variety of possible Ab candidates have been described, including murine or mouse–human chimeric monoclonal Abs (mAbs) and different formats of antibody fragments which have been shown to exert protective activity in preclinical models of invasive and/or mucosal fungal infection (De Bernardis *et al.*, 2007; Cassone, 2008).

We have recently described a murine mAb (mAb 2G8) that is directed against β1,3-glucan, a major cell wall polysaccharide that is crucial for growth and survival of fungal cells. Because β-1,3-glucan is abundantly expressed by the majority of fungi (Masuoka, 2004), mAb 2G8 can target several, most widespread pathogens, such as *Candida albicans*, *Aspergillus fumigatus* and *Cryptococcus neoformans*. Accordingly, mAb 2G8 was shown to provide protection in multiple models of fungal diseases, including vaginal and systemic *Candida* infections and invasive aspergillosis or cryptococcosis (Torosantucci *et al.*, 2005, 2009; Rachini *et al.*, 2007). Properties such as the recognition of an invariant epitope, inhibition of fungal growth and broad spectrum antifungal protection make this antibody a particularly promising therapeutic candidate to treat a wide range of fungal pathogens (Torosantucci *et al.*, 2005, 2009; Casadevall and Pirofski, 2007).

Recombinant, mouse–human chimeric Abs, constructed by fusing human C regions to mouse V regions, are definitely preferred to murine Abs for therapeutic use in humans because of their reduced immunogenicity and substantially longer half-lives (LoBuglio *et al.*, 1989; Clark, 2000). Although 'humanization' in general does not substantially modify the

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therapeutically relevant biological properties of the Ab when compared to the original mAb, data have been reported demonstrating that changes in the C regions can affect, in some instances, the structure of the V regions, modifying mAb affinity and specificity (Torres and Casadevall, 2008). This stands true, in particular, for most mAbs that binds multivalent antigens such as the fungal polysaccharides and, to an extreme, change of the Fc portion can convert a protective mAb into a nonprotective or even into a disease-exacerbating molecule (McFadden and Casadevall, 2004; Beenhover *et al.*, 2007; Torosantucci *et al.*, 2009). Thus, a careful assessment of fine specificity, avidity and relevant biological activities of chimeric recombinants generated from murine mAbs is needed before considering clinical application.

Mouse–human chimeric mAbs have proven excellent biotherapeutics for a wide spectrum of human diseases, but a general limitation to their application for human therapy is the high costs of production and industrial scaling-up. In this regard, plants are now increasingly accepted bioreactors, as alternatives to currently used microbial or mammalian cell cultures, because of the lack of animal pathogenic contaminants, the low cost of production and the relative ease of agricultural scale-up (Twyman *et al.*, 2003; Tiwari *et al.*, 2009). To date, different mAbs for human therapy against HIV (Sack *et al.*, 2007; Strasser *et al.*, 2009), rabies (Ko *et al.*, 2003) or cancer (Ko *et al.*, 2005; Brodzik *et al.*, 2006) have been expressed in plants, and some of them are undergoing clinical trials (Basaran and Rodríguez-Cerezo, 2008). Particular efforts are currently focused on maximizing expression levels and accumulation of functional product in plants. While mAb expression was initially achieved by transgenic plants, more efficient virus- or *Agrobacterium tumefaciens*-mediated transient expression systems are now preferred (Wroblewski *et al.*, 2005; Giritch *et al.*, 2006). Recently, we optimized an *Agrobacterium*-mediated transient expression system in *Nicotiana benthamiana* plants and obtained yields in the order of hundred milligrams of human antibody per kilogram of fresh plant tissue, clearly overwhelming those obtained from stable transgenics (Villani *et al.*, 2009).

On this basis, we addressed engineering, plant expression and biological properties of chimeric, human–murine mAbs derived from the anti-β-glucan mAb 2G8, to obtain potential candidates for wide-spectrum immunotherapy and/or prophylaxis of human fungal diseases. In particular, we constructed two recombinant formats, namely a chimeric complete IgG and a scFv-Fc fragment, that were transiently expressed in *N. benthamiana* plants and purified from leaves at high yields. Both Ab formats were compared to the original murine mAb for fine specificity and antigen-binding activity, as well as for antifungal activities *in vitro* and protection *in vivo* against the most harmful and pervasive pathogen *C. albicans*.

Results

Construction of genes for the recombinant anti-β-glucan Abs

V_H and V_L genes were cloned from the cDNA of the hybridoma producing the 2G8 mAb and were engineered as to obtain a chimeric IgG and a scFv-Fc construct (Figure 1). The chimeric IgG was produced by fusing V_H and V_L regions to the constant sequences of a human IgG1 γ and λ chain, respectively. In addition, the same variable regions were assembled in a scFv format and further fused to the Fc-encoding sequence of the human IgG1 γ chain (including hinge, CH_2 and CH_3 domains). Irrelevant controls were engineered in analogous formats using variable regions from a murine mAb with different, fungus-unrelated specificity (Tavladoraki *et al.*, 1993).

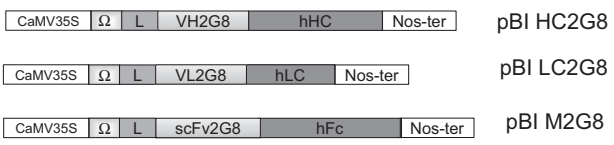

Figure 1 Schematic representation of recombinant Ab constructs for plant expression. For the chimeric IgG, the sequence encoding the variable heavy chain (HC) of 2G8 mAb (VH2G8) was fused to the sequence encoding the constant HC of a human IgG1 (hHC), and the sequence encoding the variable light chain of 2G8 mAb (VL2G8) was fused to the sequence of the constant light chain of a human light chain (hLC). For the scFv-Fc, the scFv encoding sequence was fused to hinge, CH_2 and CH_3 sequences of a human IgG1 (hFc). CaMV 35S, Cauliflower Mosaic Virus 35 S promoter; **Ω**, translational enhancer sequence of Tobacco Mosaic Virus; Nos-ter, Nopaline synthetase terminator sequence; L, signal peptide sequence derived from an embryonic immunoglobulin.

Plant expression, purification and biochemical characterization of the chimeric IgG and scFv-Fc

The engineered genes were cloned in *A. tumefaciens* vector (Figure 1), and transient expression of the Abs was obtained in *N. benthamiana* by the vacuum-infiltration technique (Villani *et al.*, 2009). To assemble the complete chimeric IgG molecule, plants were co-infiltrated with *Agrobacterium* harbouring a light chain (LC) or a heavy chain (HC) constructs (Figure 1). In addition, to enhance the expression levels, an *Agrobacterium* harbouring a gene silencing suppressor (p19 from TBSV) (Voinnet *et al.*, 2003) was co-infiltrated simultaneously. Western blot analysis of extracts from agroinfiltrated leaves revealed high expression levels of both the IgG and the scFv-Fc (Figure 2a). The correct assembly and functionality of the chimeric IgG was evaluated by nonreducing immunoblot and ELISA (data not shown).

The recombinant IgG and the scFv-Fc were then purified from leaf extracts harvested 6–7 days postinfiltration by a single-step Protein A affinity chromatography. For both Ab formats, high yields (in the range of 40 mg for the chimeric IgG and 60 mg for the scFv-Fc per kg of infiltrated plant tissues) were obtained. The correct assembly and integrity of the purified Abs were evaluated by nonreducing SDS–PAGE and gel filtration analysis (Figure 2b). Nonreducing SDS–PAGE showed the expected band of 150 kDa for the chimeric IgG and 110 kDa for the scFv-Fc. As expected, in the reducing SDS–PAGE, two bands at 50 and 25 kDa were separated in the IgG sample, corresponding to the IgG HC and LC, while a single band at 55 kDa was revealed for the scFv-Fc (Figure 2b).

Size-exclusion chromatography on a Superdex™ S-200 (GE Healthcare, Milan, Italy) confirmed that both purified Abs were fully assembled and intact (Figure 2c). Gel filtration analysis revealed a major peak eluting at around 1.62 mL for the chimeric IgG (Figure 2c) and at 1.72 mL for the scFv-Fc (Figure 3c), corresponding to the expected molecular masses of the two Ab formats. Moreover, no peaks corresponding to proteolytic fragments were detected, indicating the substantial absence of degradation in the plant-produced anti-β-glucan Abs.

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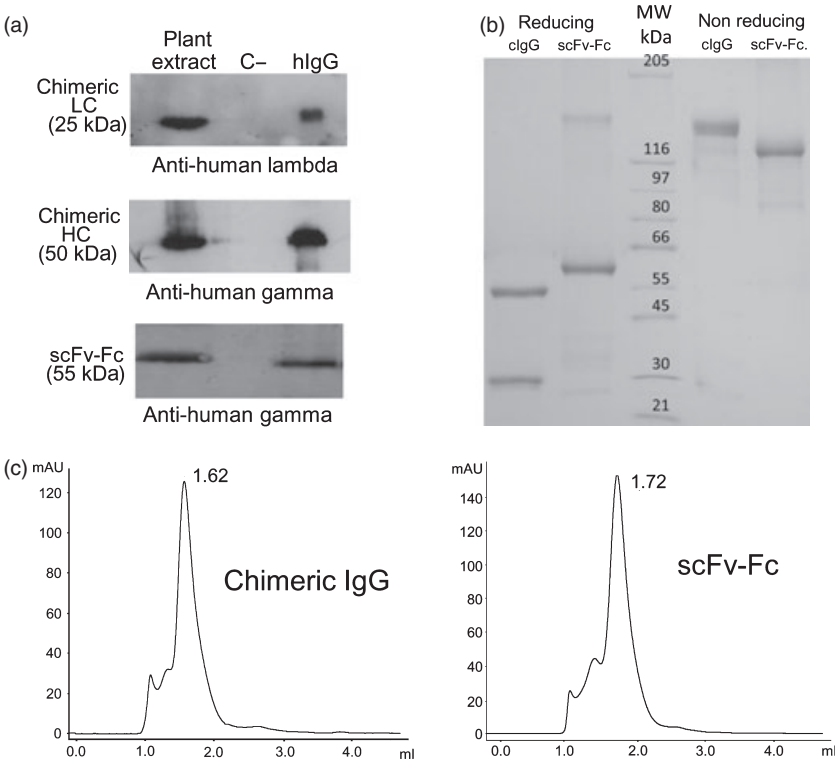

Figure 2 Plant expression, purification and characterization of chimeric IgG and scFv-Fc. (a) Expression of recombinant Abs was evaluated by Western blot analysis of crude extracts of *Nicotiana benthamiana* plants infiltrated with *Agrobacterium tumefaciens* harbouring heavy chain (HC) and light chain (LC) or scFv-Fc construct. Blots were detected with anti-human HC or anti-human LC antibodies conjugated to HRP. C-, protein extract from *N. benthamiana* plants infiltrated with *A. tumefaciens* harbouring an empty vector. hIgG, human serum IgGs. (b) SDS–PAGE (in reducing and non-reducing conditions) analysis of purified chimeric IgG (cIgG) and scFv-Fc. (c) Chromatograms obtained by size-exclusion chromatography on a Superdex™ 200 5/150 GL column of chimeric IgG (left) and scFv-Fc (right). The retention volumes (mL) of the major peaks obtained are reported.

Beta-glucan binding by the recombinant Abs

In previous work, we found that high-grade antifungal protection by the 2G8 mAb was associated with restricted recognition of β1,3-linked glucan sequences (Torosantucci *et al.*, 2009). Therefore, the chimeric IgG and the scFv-Fc were carefully assessed for their binding to differently linked β-glucan sequences, in comparison with the murine mAb 2G8.

We first evaluated the Abs in ELISA for dose–response binding to a panel of β-glucans of different molecular structure, including laminarin [β1,3-linked linear glucan with occasional β1,6-linked branches of a single glucose unit (Read *et al.*, 1996)], pustulan [linear β1,6-linked glucan (de la Cruz *et al.*, 1995)] and soluble β-glucan purified from *C. albicans* [highly branched glucan with intermixed β1,3- and β1,6-linked components (Iorio *et al.*, 2008)]. In these experiments, as reported in Figure 3a, both recombinant Abs showed the same hierarchy of reactivity with the different β-glucans as mAb 2G8: strong binding to laminarin, weak to pustulan and intermediate to *Candida* β-glucan, consistently with the presence of both β-1,3 and β-1,6 sequences in this glucan (Iorio *et al.*, 2008).

Epitopes recognized by the chimeric IgG and the scFv-Fc were examined in further detail using a neoglycolipid (NGL)-based oligosaccharide microarray system (Figure 3b). The microarrays comprised NGL probes of linear glucan oligosaccharides of different glucose linkages, anomeric configurations and degree of polymerization (dp), up to 13 glucose residues (Liu *et al.*, 2007). These analyses showed that the binding profiles of the two recombinant Abs were very similar to those observed for the original 2G8 mAb: strong binding to β1,3-linked laminarin-like oligosaccharides and little or no binding to gluco-oligosaccharides with other linkages, i.e. those derived from maltodextrins (α1,4), dextran (α1,6), cellulose (β1,4) and pustulan (β1,6). At the Ab dose of 0.5 μg/mL, tested in the present analyses, binding of all the three Abs to the β1,3-linked oligosaccharide probes was detectable at dp 3 and the strength of binding increased with increasing oligosaccharide chain length, near maximum at dp 7.

Finally, a fine comparison among the chimeric IgG, the scFv-Fc and the original mAb 2G8 for their binding to laminarin was performed by Surface Plasmon Resonance (SPR, Figure 3c). This analysis, in which the recombinant Abs and the murine mAb were injected at different concentrations on immobilized laminarin-CRM conjugate, revealed that the plant-derived recombinant Abs had kinetic properties similar to those of the murine mAb 2G8 (Figure 3c).

Overall, β-glucan-binding pattern and apparent affinity of the chimeric IgG and scFv-Fc almost perfectly matched those observed for the original mAb.

Binding to major fungal pathogens and *in vitro* antifungal activities

The recombinant Abs were then assayed by immunofluorescence against different pathogenic fungal species known to be targeted and inhibited in various infection models by the mAb 2G8 (Torosantucci *et al.*, 2005, 2009; Luther *et al.*, 2007; Rachini *et al.*, 2007). We found (Figures 4 and S1) that both Ab formats bound very efficiently *C. albicans* germ-tubes (hyphal precursors) and *A. fumigatus* hyphae (Figure 4a,b), with a similar, diffuse staining pattern. Positive staining was also evident on *C. albicans* yeast cells (Figure 4c) and poorly encapsulated *C. neoformans* cells (Figure 4d). However, as observed previously (Rachini *et al.*, 2007; Torosantucci *et al.*, 2009), these cells showed either large cell to cell variations in labelling, particularly evident in *C. neoformans* (Figure 4d), or partial reactivity in particular regions, as in *C. albicans* (Figure 4c).

We also assayed the recombinant Abs for some, protection-related biological activities typical of mAb 2G8, namely the

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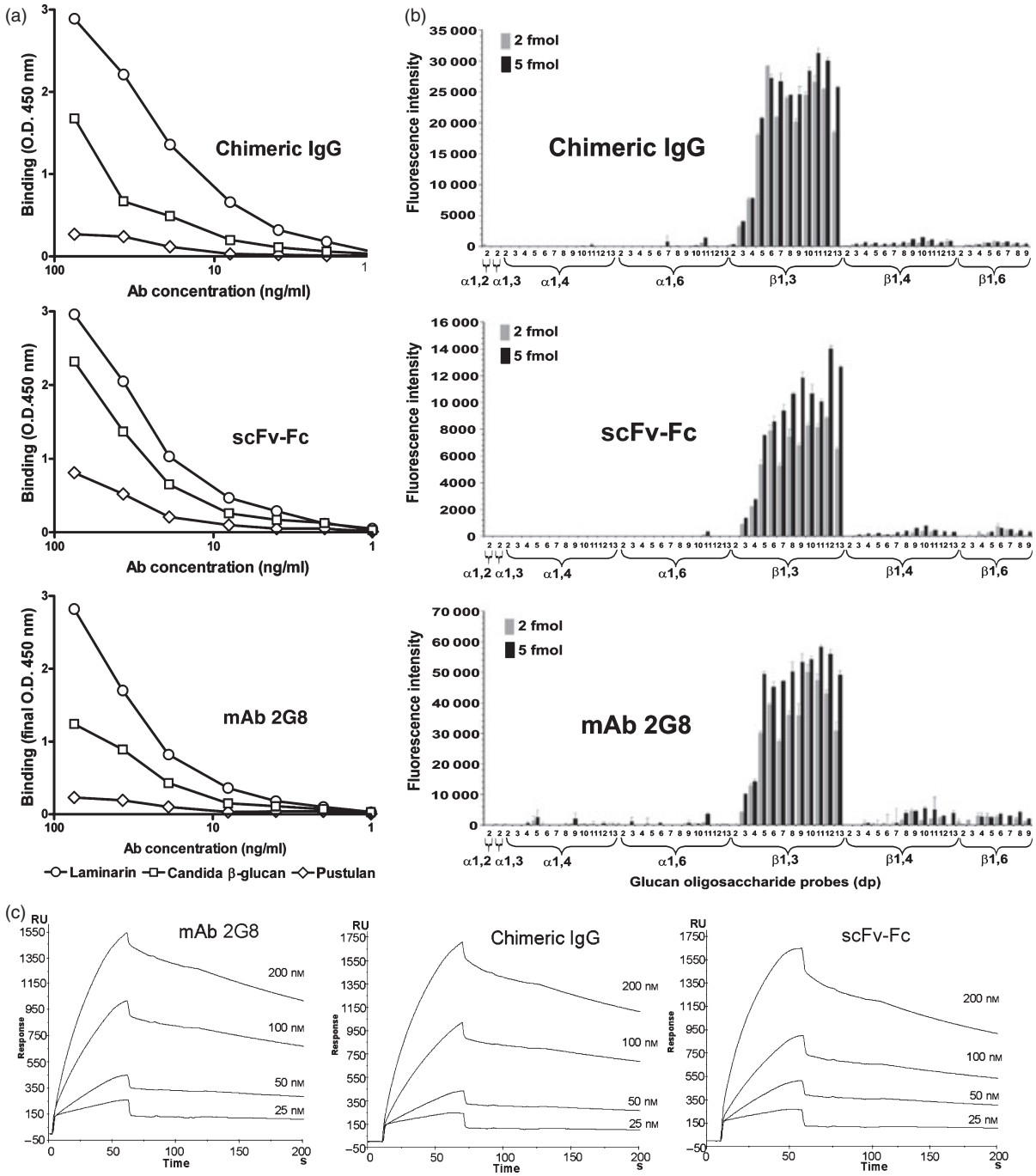

Figure 3 Fine β-glucan specificity and antigen affinity of the recombinant Abs when compared to the original murine mAb 2G8. (a) Binding to plastic-adsorbed laminarin (mostly linear β1,3 glucan with occasional β 1,6-linked branches of a single glucose unit), pustulan (linear β1,6 glucan) and *Candida albicans* β-glucan (highly branched, mixed β1,3/β1,6-glucan). Binding is expressed as mean, O.D. 405 nm readings from triplicate wells after subtraction of O.D. from the negative controls (wells with irrelevant Abs). SEM values were always <15% and are not shown. For all the three Abs, $P < 0.0001$ and $P < 0.05$, respectively, comparing binding to laminarin *versus* pustulan or *Candida* β-glucan and $P < 0.05$ comparing *Candida* β-glucan to pustulan. Data are from a typical ELISA experiment out of three performed with similar results. (b) Carbohydrate microarray analyses of the chimeric mouse–human IgG and scFv-Fc Abs and of the 2G8 mAb with various glucose oligosaccharide probes. The lipid-linked probes were printed as duplicate spots at two levels (2 and 5 fmol/spot), and binding was assayed with the antibodies at 0.5 μg/mL. Binding signals are expressed as numerical scores, means of fluorescence intensities of duplicate values recorded at 2 and 5 fmol oligosaccharide probe/spot (grey and black bars, respectively). The error bars represent half of the difference between the two values. The glucose oligosaccharide sequences tested included α1,2 and α1,3 disaccharides and oligosaccharides from maltodextrins (α1,4), dextran (α1,6); curdlan (β1,3); cellulose (β1,4); and pustulan (β1,6). Numbers on the X-axis indicate degree of polymerization (dp). (c) Surface plasmon resonance analysis of the recombinant Abs and mAb 2G8. The Abs were injected at different concentrations ranging from 25 to 400 nM on a CM5 sensor chip immobilized with β-glucan (laminarin) conjugated to the protein carrier CRM197. A reference flow cell was used and the sensorgrams shown are blank subtracted.

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ability to restrict directly fungal growth *in vitro*, without the contribution of host effector cells, and to prevent fungus adhesion to host epithelia (Torosantucci *et al.*, 2005, 2009). *C. albicans*, which is particularly sensitive to the inhibition by anti-β-glucan Abs, was used as the target in these experiments.

As reported in Figure 5a, both recombinant mAbs efficaciously reduced fungal growth, with no difference of inhibition degree with respect to the mAb 2G8 at the highest dose tested (125 μg/mL). At lower doses, however, only the scFv-Fc was comparable in activity with the 2G8 mAb, while the chimeric IgG caused only an approximate 10% reduction of the fungal CFU (Figure 5a). In adhesion assays, both recombinant Abs significantly decreased the adhesion of *C. albicans* to monolayers of Hep-2 cells in a dose-dependent manner, with no statistically significant differences between the chimeric IgG, the scFv-Fc and the 2G8 mAb (Figure 5b).

Finally, we addressed the issue of whether the binding of the recombinant Abs on *C. albicans* surface could facilitate the antifungal activities of human polymorphonuclear neutrophils (PMN) that are crucial defensive effectors in fungal diseases. Pretreatment of *C. albicans* cells with the recombinant anti-β-glucan Abs resulted in a much more efficient fungal killing by neutrophils when compared to the irrelevant Ab-treated *Candida* cells, and this effect was similar to that observed with a human serum containing high levels of opsonizing anti-*Candida* mannoprotein Abs (Figure 5c). No significant differences in ability to enhance neutrophil candidacidal activity were noted between the chimeric IgG and the scFv-Fc formats (Figure 5c).

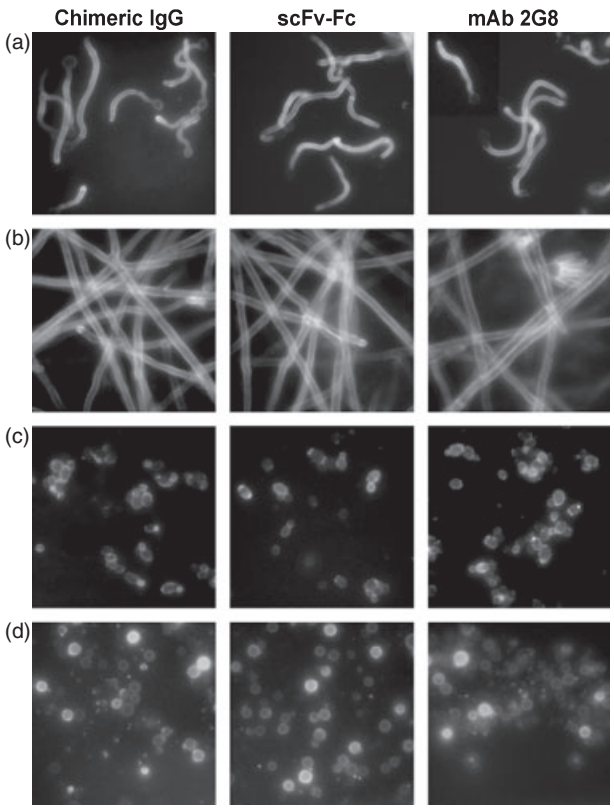

Figure 4 Immunofluorescence staining of major pathogenic fungi by the recombinant anti-β-glucan Abs. *Candida albicans* blastospores (a) and germ-tubes (b), *Aspergillus fumigatus* hyphae (c) and cells of an encapsulated strain of *Cryptococcus neoformans* (d) were stained with the indicated monoclonal reagents at 50 μg/mL. Parallel staining with the secondary antibody reagents only or with the irrelevant, control chimeric IgG or scFv-Fc showed a complete absence of reaction (shown in Figure S1).

Antifungal protection in experimental models of *C. albicans* infection

We have previously demonstrated that mAb 2G8 is able to control fungal infections in different animal models (Rachini *et al.*, 2007; Torosantucci *et al.*, 2005). In this work, we particularly focused upon the main agent of fungal diseases, *C. albicans,* using both a mucosal and a systemic infection model of candidiasis.

In the systemic infection model, a single dose of each Ab was administered to mice followed by an intravenous challenge with *C. albicans*, and protection was evaluated by measuring fungal invasion of kidneys at day 2 postinfection. In these experiments, either the chimeric IgG or the scFv-Fc resulted clearly protective, because both induced a significant reduction in fungal load in kidneys when compared to controls (Figure 6a). Apparently, the highest degree of protection was provided by the original mAb, followed by the scFv-Fc and the IgG formats (Figure 6a).

The two plant-produced Abs were also tested for protection in a rat model of experimental vaginitis in which animals received a 'therapeutic' Ab treatment at 1, 24 and 48 h postintravaginal infection with *C. albicans*, and the protective effect was assessed by following the kinetics of fungal clearance from the vagina. Rats treated with the chimeric IgG or the scFv-Fc exhibited faster *C. albicans* elimination from the vagina when compared to control groups, and the CFU values were lower in vaginal fluids at all time points tested (Figure 6b). Differently from that observed in the systemic infection model, mAb 2G8 and the recombinant Ab formats were comparably effective in ameliorating the course of the experimental vaginal infection (Figure 6b).

Discussion

Murine monoclonal Abs have proven excellent biotherapeutics for a wide spectrum of human diseases but their efficacy is limited because of immunogenicity, short serum half-lives and lack of efficient interaction with human immune effector cells. The advent of chimeric, recombinant or fully human Abs, through protein engineering or direct clonal selection from the human antibody repertoire, paves the way to overcome such limitations. In parallel, the persistently high morbidity and mortality of fungal infections and their increasing resistance to chemotherapy have prompted novel approaches to control these diseases through antifungal vaccines or Abs. In this work, we devised and expressed in *N. benthamiana* plants recombinant Abs derived from an anti-β-glucan, murine monoclonal IgG2b (mAb 2G8) that proved protective in animal models of different fungal diseases (Torosantucci *et al.*, 2005; Rachini *et al.*, 2007). Namely, we fused the 2G8 antigen-binding site on the constant human IgG1 region yielding a chimeric IgG and a scFv-Fc recombinant version. This latter format is particularly interesting in that, being encoded by a unique gene, the recombinant molecule retains the antigen-binding avidity and the Fc domain effector functions, mimicking the complete IgG. In recent articles, scFv-Fc Abs directed against different targets (i.e. cancer, virus infections and autoimmune disorders) have been shown to

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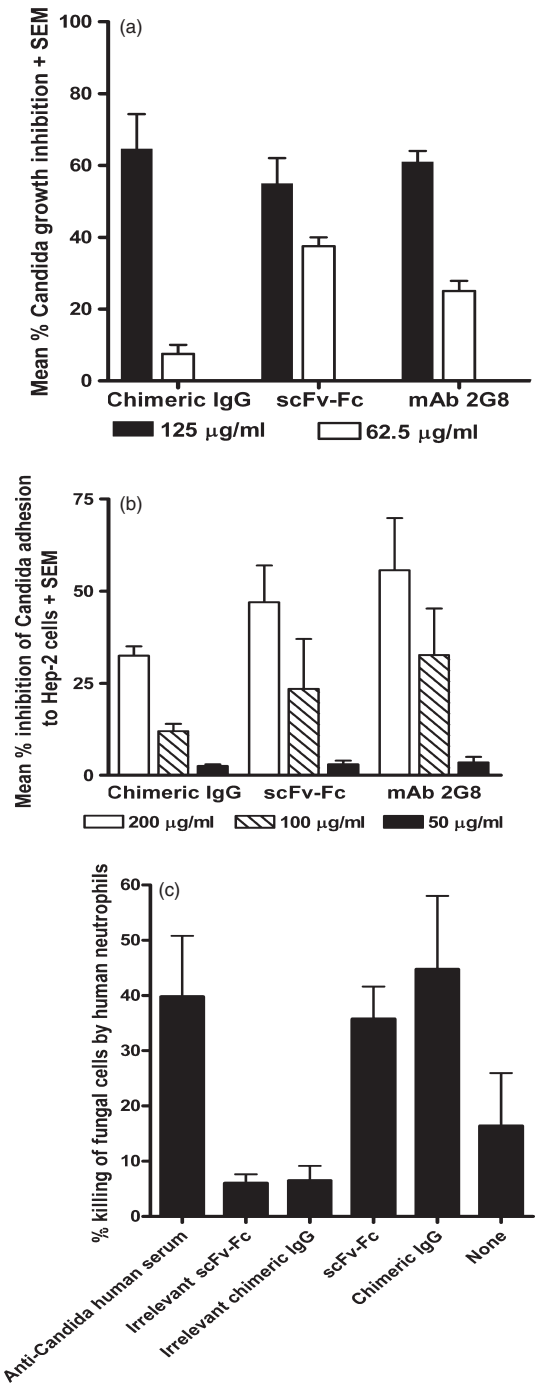

Figure 5 *In vitro* anti-*Candida* activities of the recombinant Abs. (a) Inhibition of fungal growth. The figure shows mean per cent growth inhibition values measured in three independent experiments and calculated by comparing CFU number in fungal cultures (18 h at 37 °C) supplemented with the recombinant Abs to those with equal doses of an irrelevant murine IgG2b mAb, chimeric IgG or scFv-Fc. Statistically significant differences between growth inhibitory activities were only measured at the lowest Ab dose (62.5 μg/mL) and are as follows: chimeric IgG *vs* mAb 2G8 and chimeric IgG *vs* ScFvSc, both $P < 0.05$; mAb 2G8 *vs* ScFv-Fc, not significant. (b) Ability by the Abs to prevent the adherence of *Candida* to human epithelial cells. Values in the graph are mean per cent reduction of the number of adherent fungal cells (recombinant anti-β-glucan Abs *versus* irrelevant Ab of the corresponding format) measured in three independent experiments, each performed in triplicate. Differences in activity between the different Abs are not statistically significant. (c) The recombinant Abs enhance the killing of *Candida albicans* cells by isolated, human polymorphonuclear neutrophils. Killing was measured by fungal CFU counts after a 2-h co-incubation of neutrophils with *Candida* cells treated with the indicated Abs at a 1 : 1 ratio. The anti-*Candida* human serum (positive control) was a serum containing high levels of opsonizing, anti-*Candida* mannoprotein Abs. None = untreated *Candida* cells. Mean per cent killing values are from three independent experiments with neutrophils from different donors and were calculated by comparing CFU values in fungus-neutrophil cultures with those measured in control, *C. albicans* cultures in the absence of neutrophils.

be promising in therapeutic applications (Gould *et al.*, 2005; Kenanova *et al.*, 2007; Cao *et al.*, 2009).

We expressed both recombinant molecules in plants, an increasingly used and consolidated production platform for biological therapeutics. In fact, plants represent cost-effective, scalable and animal pathogen-free systems in comparison with current mammalian cell-based systems, thus potentially allowing the rapid implementation of candidate therapeutics in preclinical trials, especially for topic applications. The choice of this technology and, in particular, an optimized *Agrobacterium*-mediated transient expression system allowed to maximize expression levels and purification yields of the engineered Abs. SDS–PAGE and gel filtration analyses demonstrated that both Abs were correctly assembled, showing the expected molecular masses.

It has been reported that plant-expressed Abs may undergo proteolytic fragmentation (Ramessar *et al.*, 2008; Villani *et al.*, 2009; De Muynck *et al.*, 2010). In the present study, there was no evidence of degradation of the two purified proteins. This could be because of an intrinsic low susceptibility of our recombinant Abs to plant proteases present in the extracellular space (Delannoy *et al.*, 2008) and/or to the use of a transient expression system that, as suggested by Villani *et al.* (2009), minimizes Ab proteolysis. In this system, in fact, the peak of protein expression occurs in few days, at variance with other expression systems, such as transgenic plants, in which substantial protein production requires much longer accumulation times.

Different techniques were used to investigate the binding of the recombinant Abs to various β-glucan antigens and to evaluate carefully their ability to exert the biological activities associated with antifungal protection by the original murine mAb (Torosantucci *et al.*, 2009). These investigations were necessary because we had previously found that a mAb with the same variable regions of mAb 2G8 but different constant regions (IgM isotype) differed from the IgG mAb 2G8 in pattern of binding to β-glucan sequences and fungal cell wall molecules and, more importantly, completely lacked protective properties *in vitro* and *in vivo* (Torosantucci *et al.*, 2009). In the present study, both ELISA and glycan array experiments demonstrated that the molecular engineering and expression of mAb 2G8 in a plant did not alter its antigen recognition pattern and epitope specificity. Moreover, the SPR analysis using a β-glucan (laminarin) substrate confirmed the similar binding kinetics of mAb 2G8 and its recombinant derivatives. Ability to inhibit the growth and the adherence of *C. albicans in vitro*, two biological properties strongly associated with both binding specificity and protective antifungal activity (Torosantucci *et al.*, 2009), were also well preserved in both recombinant Ab formats.

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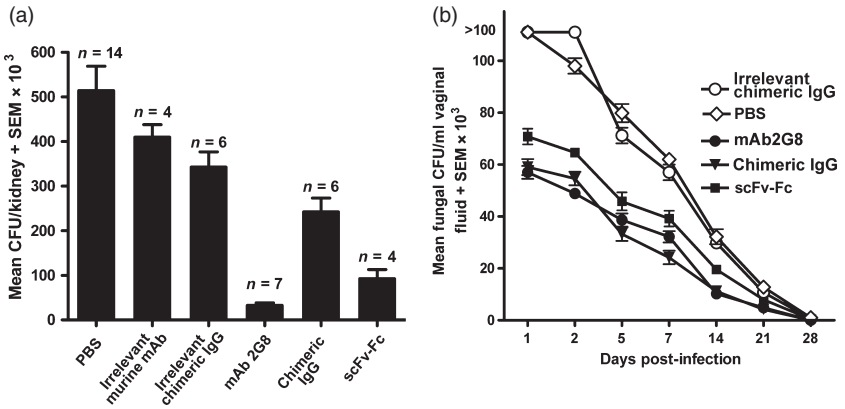

Figure 6 Anti-*Candida* protective activity of the recombinant Abs in animal models. (a) Kidney invasion following a systemic infection with *Candida albicans* in Ab-treated mice. Data presented in Figure are cumulative, mean CFU values from five independent experiments; *n*, number of animal examined for each experimental condition. Untreated or control animals *vs* mice treated with any mAb, $P < 0.001$; mAb 2G8 *vs* chimeric IgG or ScFv-Fc, $P < 0.0001$; chimeric IgG *vs* scFv-Fc, $P < 0.05$; any difference among negative controls is statistically not significant. (b) Protection by the anti-β-glucan, recombinant Abs in a rat model of vulvovaginal candidiasis. The figure shows the kinetics of fungal clearance from the vagina (expressed as mean ± SE values of *C. albicans* CFU in vaginal fluids at the indicated times postinfection) in oophorectomized, oestrogen-treated rats (five per group) that had been intravaginally infected with *C. albicans* and treated with the various Abs or with a control Ab (50 μg/200 μL at 1, 24 and 48 h postinfection) or with PBS alone (200 μL, same schedule). $P < 0.001$ for any comparison between recombinant Ab-treated and control rats. Differences among the various Abs or between PBS- and irrelevant Ab-treated animals are not significant. The experiment depicted in Figure was repeated twice with similar results.

The antifungal potential of the recombinant Abs has been validated *in vivo* exploiting two well-established models (systemic or vaginal) of *Candida* infection that differ profoundly one from the other as for pathogenesis and mechanisms of protection. Both the chimeric IgG and the scFv-Fc format were clearly protective in that they enhanced significantly the elimination of the fungus from target organs in disseminated *Candida* infections and facilitated *Candida* clearance from the vagina in rat vulvovaginal infection.

We found slight differences among the original, murine and the recombinant Abs, as well as between the two Ab formats in *in vitro* and *in vivo* antifungal efficacy assays. Several factors can combine to determine these differences. For instance, non-cognate interaction with murine phagocytes could account for the less potent protection observed *in vivo* by the recombinant Abs, when compared to the murine mAb 2G8. This hypothesis is supported by the finding that a lower protective activity by the recombinant Abs was manifest exclusively in the invasive infection model [where phagocyte action plays a pivotal role for protection (Richardson and Rautemaa, 2009)] and not in the mucosal infection model [where the role of phagocytes is subsidiary, if not aggravating the severity of the infection (Fidel, 2007)]. The different pattern of Ab glycosylation in plants when compared to animal cells (Gomord *et al.*, 2010) could also contribute to the above differences. In fact, it has been recently demonstrated that Fc glycans can significantly influence the Ab affinity for the C1q fraction of complement and the different Fc receptors on host effector cells, thus impacting on complement- and cell-mediated antimicrobial Ab activities (Jefferis, 2009; Strasser *et al.*, 2009). Fc glycan-induced modulation of direct, non host-cell-mediated anti-infectious activities, such as virus neutralization, has also been reported, although mechanisms underlying this effect are still unknown (Forthal *et al.*, 2010). Notably, the scFv-Fc format showed a very efficient antifungal activity that resulted at least as good as that of the chimeric IgG in both *in vitro* and *in vivo* assays.

We also addressed the issue of whether the presence of recombinant, anti-β-glucan Abs could facilitate the antifungal activities of human neutrophils. These phagocytic cells are crucial players in defence against invasive fungal infections and Ab-mediated opsonization is a key cooperative mechanism for a successful clearance of infectious fungi from host tissues. In addition, *C. albicans* can oppose to the neutrophil candidacidal activity through the expression of a cell wall–associated protein, the Hyr1p (Luo *et al.*, 2010). Intriguingly, this protein is one of the best recognized by the mAb 2G8 in *C. albicans* secretions, because, as most other GPI-anchored proteins, it contains a β-glucan moiety (Torosantucci; Cassone *et al.*, 2010). Thus, binding of Hyr1p by anti-β-glucan Abs might result in an increased neutrophil anti-*Candida* activity independently of opsonization, as recently described for an anti-Hyr1p Ab (Luo *et al.*, 2010).

We report in this study that the binding of both chimeric Abs to *Candida* cells is able to enhance significantly their killing by human neutrophils in *ex vivo* models of infections. Considering that Ab-reactive β-glucan epitopes (including those associated with the Hyr1p) are strongly expressed by *Candida* germ-tubes and hyphal filaments during host tissue invasion (Wheeler *et al.*, 2008), anti-β-glucan Abs are likely to potentiate neutrophil activity also *in vivo*. This activity might complement other phagocyte-independent, protective actions of the recombinant Abs (such as the direct inhibition of fungal growth and adherence to epithelia), at least in those subjects not affected by profound neutropenia.

In conclusion, we clearly show here the potential of plant-produced anti-β1,3-glucan recombinant Abs as therapeutic/prophylactic agents against human candidiasis. These antibodies, like the original murine mAb 2G8, recognize a highly conserved β-glucan motif common to most infectious fungi. Using new approaches to humanizing their glycosyl moiety, such as those proposed by Strasser *et al.* (2009) or Vézina *et al.* (2009), these plant antibodies hold promise as a 'universal' therapeutic tool to confer protection against a broad range of fungal pathogens.

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Experimental procedures

Gene engineering

Total RNA was isolated from the 2G8 mAb-expressing hybridoma (Torosantucci *et al.*, 2005, 2009), and the corresponding cDNA was obtained by reverse transcription using M-MuLV reverse transcriptase (Biolabs, Hitchin, UK). The V_H and V_L genes of 2G8 mAb were amplified by PCR from cDNA with primers containing flanking *NcoI/BstE*II (VHNcoI-CGGCCATGGCCCAGGTCCAAC-TGCAGCAGTCTGG and VHBstE:-AGACGGTGACCGTGGTCCCT-GCGC) and *KpnI/Not*I (VLKpnI-ACTCGGTACCTCGGATATTGTGA TGACCCAG and VL NotI-ATCTGCGGCCGCCCGTTTTATTTCCA GCTTGG) restriction sites. The V_H gene was cloned in pDN332 phagemide vector by NcoI/BstEII digestion, and the V_L gene was cloned by KpnI/NotI digestion in pBSsk. To obtain the chimeric 2G8 light chain, the V_L gene was fused by *Splicing* by *Overlap* Extension (SOE) with a human λ C_L gene, the resulting gene fusion was amplified by PCR with primers containing *Kpn*I and *Sac*I restriction sites and cloned in pBI-SP vector to obtain pBIL2G8. For the chimeric HC, the 2G8 V_H gene was amplified using primers containing flanking *KpnI/Sal*I (VHKpnI-ACT-CGGTACCTCGCAGGTCCAACTGCAGCAGTCTGG and VH SalI - ACTCGTCGAC TGAGGAGACGGTGACCGTGGTCC) restriction sites and cloned in a pBI vector containing a mouse SP and the constant region of a human IgG1 to obtain pBIH2G8. For the scFvFc construct, the single chain encoding sequence was obtained fusing by SOE the V_L sequence to V_H through a linker $(Gly_4Ser)_3$ encoding sequence, using overlapping primers (linkerFOR-CTGGCGGTGGCGGATCGGATATTGTGATGACCCAGTC and linker-REV-GACTGGGTCATCACAATATCCGATCCGCCACCGCCAG). The resulting scFv gene was amplified with primers VHKpn and VLNot and cloned by digestion *KpnI/Not*I in pBSsk to obtain pBS2G8. Then, the human sequence encoding IgG1 Fc domain (hinge, CH_2 and CH_3) was amplified by PCR using primers containing flanking *NotI/Sac*I, the DNA fragment was digested *NotI/Sac*I and ligated in pBS 2G8. The resulting scFvFc2G8 gene was excised from pBS by *KpnI/Sac*I digestion and cloned in pBI-SP vector to obtain pBIM2G8. PCR was performed using *Pfu* turbo DNA polymerase (Stratagene). The sequences of all constructs were verified by sequencing (CRIBI).

Transient gene expression

Agrobacterium tumefaciens strain LBA 4404 (Invitrogen, Carlsbad, CA) was transformed with pBIH2G8, pBIL2G8 and pBIM2G8. *Agrobacterium* cultures were grown o.n. at 28 °C and suspended in 10 mM MES, pH 5.5 (each at $OD_{600\ nm}$ = 0.6). To obtain the chimeric IgG, equal volumes of the *Agrobacterium* cells were mixed and used for agroinfiltration of *N. benthamiana* leaves. The leaves were harvested 5 days postinfiltration and immediately processed or stored at −80 °C.

Antibody purification

Plant tissue was ground in liquid nitrogen using a mortar and pestle and homogenized in PBS. The homogenate was prefiltered through Miracloth (Calbiochem, San Diego, CA) and centrifuged for 20 min at 10 000 ***g***. The homogenate was then passed through a 0.45-μm filter and loaded on a 1 mL rProtein A F.F. column (GE Healthcare) after equilibration with PBS. After feed injection, the column was washed with PBS buffer to remove unbound material, and the bound material was eluted with 0.1M citrate buffer, pH 3.0. The eluted samples were immediately neutralized using 1 M Tris–HCl (pH 8.0) to minimize degradation and aggregation of the antibody and analysed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE).

Western blot analysis

Ten micrograms of purified protein was resolved by 10% SDS–PAGE and transferred to Immobilon-P (Millipore, Billerica, MA, USA) using a Semi-Dry Transfer Unit (Hoefer TE70, GE Healthcare). Anti-human γ chain (8419, Sigma-Aldrich, St Louis, MO, USA) or anti-human λ chain (A5175, Sigma-Aldrich), HRP-conjugated Abs, diluted 1 : 5000 in 2% (w/v) skim milk in PBS, were used to detect HC or scFv-Fc and LC, respectively, using an enhanced chemiluminescence revelation system (ECL Plus, GE Healthcare). Human IgGs were used as a positive control (Sigma-Aldrich).

Size-exclusion chromatography

The purified recombinant Abs were analysed by size-exclusion chromatography at 20 °C in PBS on a Superdex™ 200 5/150 GL column (GE Healthcare) (flow rate, 0.3 mL/min) using a AKTA FPLC P920 instrument (GE Healthcare). Protein absorbance, expressed as absorption units (mAU), was measured at 280 nm. Column calibration was performed using gel filtration calibration kits (low and high molecular weight, GE Healthcare), following the manufacturer's instructions. A purified human IgG1-λ (I 5029, Sigma-Aldrich) was also used as a positive control.

Surface plasmon resonance analysis

Binding assays were performed on a Biacore X instrument, and data were analysed by BIAevaluation version 4.1. Beta-glucan (laminarin)-CRM- conjugate, prepared as described previously (Torosantucci *et al.*, 2005) and kindly provided by Dr Paolo Costantino, Novartis Vaccine and Diagnostics, Siena, Italy, was immobilized on the surface of a CM5 sensor chip using an amine coupling kit (GE Healthcare), by injection at a concentration of 50 nM in 10 mM acetate buffer pH 5.2 at a flow rate of 5 μL/min. After the coupling procedure, the surface was conditioned with two pulses of 100 mM HCl and immobilization level was about 5000 RU. Different concentrations of each recombinant Ab, ranging from 25 to 200 nM, were injected on the chip surface. All binding experiments were performed at 25 °C in PBS at a flow rate of 20 μL/min, and pulses of 10 mM HCl were used to regenerate the sensor chip.

Murine, anti-β-glucan mAb 2G8

The mAb (IgG2b subclass) was generated from mice immunized with a soluble preparation of *C. albicans* β-glucan, conjugated to the recombinant diphtheria toxoid CRM_{197} (Torosantucci *et al.*, 2005). Nucleotide sequences of variable regions (GenBank FJ790243, bankit1189566) were determined as reported elsewhere (Torosantucci *et al.*, 2009). Hybridoma cells were routinely cultured in protein-free CD Hybridoma medium (Gibco, Grand Island, NY), supplemented with 100 U penicillin/mL, 100 μg streptomycin/mL, 1 mM sodium pyruvate and 2 mM L-glutamine (Hyclone, Logan, UT), and the mAb was purified from culture supernatants by affinity chromatography onto Protein A-Sepharose 4B (GE Healthcare), as already described (Torosantucci *et al.*, 2005) and quantified by a commercial protein assay (BioRad, Richmond, VA) following the manufacturer's

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instructions. An irrelavant, control murine mAb (an IgG2b against the unconjugated CRM_{197} protein) was obtained from another hybridoma following an identical procedure (Torosantucci *et al.*, 2005).

Microorganisms

The *C. albicans* strain 3153 (Istituto Superiore di Sanità type collection) was used in the model of disseminated *Candida* infection in mice and in *in vitro* experiments, whereas vaginal infections in rats were induced by the vaginopathic strain SA-40. Fungal cells from stock cultures in Sabouraud-dextrose agar (Difco-BBL, Franklin Lakes, New York) were cultured at 28 °C for 24 h in Winge medium [strain BP (Bromuro *et al.*, 2002)] or in YEPD medium [strain SA-40 (Torosantucci *et al.*, 2005)], then harvested by centrifugation, washed, counted in a hemocytometer and resuspended to working concentration in PBS. *Candida* germ-tubes were obtained by culturing yeast cells at 37 °C for 1 (short germ-tubes) or 4–6 h in Lee's medium at 37 °C, as previously described (Romagnoli *et al.*, 2004).

Aspergillus fumigatus 495 and *C. neoformans* ATCCL, both from the type collection above, were maintained on Sabouraud-dextrose agar slants. *C. neoformans* yeast cells were grown for 18 h at 28 °C in Sabouraud-dextrose broth under slight agitation, washed and resuspended in PBS. Hyphae of *A. fumigatus* were obtained from conidial suspensions cultured in RPMI-2% FCS (Hyclone) at 37 °C for 18 h, as previously described (Torosantucci *et al.*, 2005).

Immunofluorescence staining

Live yeast cells of *C. albicans* or *C. neoformans* were allowed to adhere on immunofluorescence microscope slides, extensively washed with PBS containing 0.1% Tween 80 and blocked (1 h, 37 °C) with 3% bovine serum albumin (BSA, Sigma Aldrich, St Louis Ma) in PBS, essentially as described previously (Torosantucci *et al.*, 2009). *A. fumigatus* and *Candida* germ-tubes were directly cultured in microscope chamber slides (NUNC, Roskilde, Denmark), then washed and blocked as above. Spots were reacted (2 h, 37 °C) with various dilutions of the Ab reagents in PBS-3% BSA, followed by PBS washings and revelation (1 h at 37 °C) with the opportune fluorescein isothiocyanate-conjugated, anti-mouse or -human IgG antibodies (Sigma-Aldrich). After extensive washings, the slides were mounted in glycerol, pH 9.6, and examined under a Leitz Diaplan fluorescence microscope. Parallel staining with negative control mAb reagents or with the secondary antibodies alone was carried out in all the experiments.

ELISA

Polystyrene microtiter plates (MaxiSorp; NUNC) were coated with the β-glucan antigens, namely soluble *C. albicans* β-glucan (GG-Zym) (Iorio *et al.*, 2008), laminarin (Sigma-Aldrich) and pustulan (Calbiochem, La Jolla, CA). After blocking with 3% BSA (Fraction V, Sigma-Aldrich) in PBS, the plates were reacted with twofold dilutions of the various mAb reagents, followed by alkaline phosphatase-conjugated, secondary antibody reagents (anti-mouse or anti-human IgG antibodies, Sigma-Aldrich) and p-nitrophenyl phosphate disodium (Sigma-Aldrich) as the enzyme substrate and, finally, read for absorbance at 405 nm (Bromuro *et al.*, 2002). Values from negative controls (wells reacted with irrelevant Abs) were subtracted from all absorbance values.

Oligosaccharide probes and microarray analyses

The microarrays comprised 58 glucose oligosaccharide sequences with differing linkages and chain lengths, as follows: (i) α1,2-linked disaccharide (kojibiose, Sigma) and α1,3-linked disaccharide (nigerose, Wako Chemicals); α1,4-linked oligosaccharides, dp 2–7 (Sigma) and dp 8–13, separated from a maltodextrins acid hydrolysate (V-labs, Covington, LA, USA); (ii) α1,6-linked oligosaccharides, dp 2–13, generated from dextran (MW 500 kDa, Amersham Biosciences, Little Chalfont, UK) by acid hydrolysis, (iii) β1,3-linked oligosaccharides, dp 2–4 (Dextra, Reading, UK), dp 5–6 (Megazyme, Wicklow, Ireland), dp 7 (Seikagaku America, East Falmouth, MA, USA) and dp 8–13, separated from a curdlan acid hydrolysate (Megazyme); (iv) β1,4-linked oligosaccharides, dp 2 (Sigma) and dp 3–13 separated from a cellulose acid hydrolysate (Megazyme); (v) β1,6-linked oligosaccharides, dp 2–9, generated from pustulan (Calbiochem) by acid hydrolysis. Details of the polysaccharide acid hydrolysis, separation and analysis of oligosaccharide fragments will be described in detail elsewhere (A.S.P, Y.L and T.F *et al.,* manuscript in preparation).

For microarray printing, the reducing oligosaccharides were prepared as oxime-linked NGL probes as described (Liu *et al.*, 2007). The NGLs were arrayed robotically on nitrocellulose-coated microarray slides (Palma *et al.*, 2006), then overlaid with the anti-β-glucan Ab reagents diluted to a concentration of 0.5 μg/mL in 0.02% casein (Pierce, Rockford, IL, USA) containing 1% (w/v) BSA (Sigma-Aldrich) and 5 mM $CaCl_2$ (Palma *et al.*, 2006). Binding of mAbs was revealed with biotinylated anti-mouse-IgG (Sigma) or anti-human IgG (Vector Laboratories, Burlingame, CA, USA) followed by Alexa Fluor-647-labelled streptavidin (1 μg/mL, Molecular Probes). Imaging was as described (Palma *et al.*, 2006), and data analysis and presentation were performed with a dedicated software (Stoll and Feizi, in press). With the newly prepared oligosaccharide probes and microarrays, the unexplained binding signal previously observed by 2G8 mAb to the β1,6 pustulan-derived trisaccharide probe (Torosantucci *et al.*, 2009) was not detected in the present study.

Functional assays *in vitro*

To evaluate the growth inhibitory activity of the Ab reagents, *C. albicans* cells (1.5×10^3/mL in 200 μL of RPMI-FCS) were cultured in the presence of the mAb 2G8, the chimeric IgG or the scFv-Fc or the irrelevant, control mAb reagents, essentially as reported elsewhere (Torosantucci *et al.*, 2005). Each condition was assayed at least in duplicate. Fungal growth was quantified by enumeration of CFU in the fungal cultures after incubation for 18 h at 37 °C, and inhibition by the various anti-β-glucan Abs was evaluated by comparing CFU to those obtained from *Candida* cultures with the respective, irrelevant Ab.

Inhibition of fungal adhesion was assayed in the Hep-2 human epithelial cell line, as described previoulsy (Torosantucci *et al.*, 2009). Hep-2 cells monolayers were incubated with *Candida* cells (8×10^4/well in PBS) with or without the anti-β-glucan or control Ab reagents, in triplicate. After 2 h of incubation at 37 °C, nonadherent fungal cells were removed by gentle washings with PBS, whereas adherent fungi were recovered from cell monolayers with PBS containing 0.2% Triton X-100 and enumerated by CFU counts. Percentage of inhibition

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of fungal adhesion was calculated by comparing the number of adherent fungi in wells containing the anti-β-glucan Abs with that in wells containing equal concentrations of the irrelevant control reagents.

Human PMN were separated from venous blood buffy coats as described previously (Cassone *et al.*, 1997) and resuspended at in RPMI with 5% FCS. Short *C. albicans* germ-tubes were opsonized with the chimeric IgG, the scFv-Fc or the corresponding control Abs (150 μg protein/10^5 fungal cells/mL) for 30 min on ice, washed and cocultured with PMN (5×10^4 or 10^5 cells/mL), at a 1 : 1 or 2 : 1 *Candida*: PMN ratio. Control cultures were Ab-treated fungal cells in the absence of neutrophils. After a 2-h co-incubation at 37 °C, PMN were lysed by addition of 2% Triton X-100, and fungal CFU were evaluated by a standard plate count. Per cent killing values were calculated by comparing CFU in cultures of opsonized *Candida* + PMN with control cultures of opsonized *Candida* without PMN. A human serum with high titres of opsonizing, anti-*Candida* mannoprotein Abs was used as the positive control.

Protection assays *in vivo*

Protective activity of recombinant Abs was assessed in animal models of both systemic and vulvovaginal *C. albicans* infection. In the systemic infection model, female, 4-week-old CD2F1 mice (Harlan-Nossan, Milano, Italy) were given a single i.p. dose of either Abs (100 μg/0.5 mL) and, 2 h later, were infected i.v. with *C. albicans* (5×10^5 cells/0.2 mL). Controls received the same dose of irrelevant Abs. Protection was evaluated 2 d post-infection by measuring fungal load through enumeration of fungal CFU in the left kidney (Torosantucci *et al.*, 2005).

Vulvovaginal candidiasis was induced in oophorectomized Wistar rats (Charles River Breeding Laboratories, Calco, Italy) maintained under pseudoestrus by the s.c. administration of estradiol benzoate (Amsa Farmaceutici srl, Rome, Italy), as described previously (De Bernardis *et al.*, 2007). Rats were infected intravaginally with 10^7 *Candida* cells in 0.1 mL of saline 6 days after the first estradiol dose and then administered by the intravaginal route three doses (50 μg/200 μL each, given at 1, 24 and 48 h postinfection.) of anti-β-glucan or irrelevant Abs or 200 μL of PBS. Protection was evaluated by estimation of fungal CFU in vagina until day 21–28 after infection, as already described (De Bernardis *et al.*, 2007).

All animal studies were approved by the Istituto Superiore di Sanità intramural Institutional Review Committee.

Statistical analysis

Data were analysed by the GraphPad Prism 4 software (GraphPad Inc., La Jolla, CA, USA). ELISA and vaginal infection data were assessed for statistical significance by curve fit and linear regression analysis. Data from CFU counts, both in *in vitro* and in *in vivo* experiments, were analysed by two-tailed Student's *t* test.

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Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1 Control immunofluorescence staining of fungal cells with the murine or chimeric, fungus-irrelevant Abs used in this study.

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