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Dendritic Cells Pulsed with Fungal RNA Induce Protective Immunity to Candida albicans in Hematopoietic Transplantation

Angela Bacci,* Claudia Montagnoli,* Katia Perruccio,† Silvia Bozza,* Roberta Gaziano,‡ Lucia Pitzurra,* Andrea Velardi,† Cristiana Fe' d'Ostiani,* Jim E. Cutler,§ and Luigina Romani‡,*

Immature myeloid dendritic cells (DC) phagocytose yeasts and hyphae of the fungus Candida albicans and induce different Th cell responses to the fungus. Ingestion of yeasts activates DC for production of IL-12 and Th1 priming, while ingestion of hyphae induces IL-4 production and Th2 priming. In vivo, generation of antifungal protective immunity is induced upon injection of DC ex vivo pulsed with Candida yeasts but not hyphae. In the present study we sought to determine the functional activity of DC transfected with yeast or hyphal RNA. It was found that DC, from either spleens or bone marrow, transfected with yeast, but not hyphal, RNA 1) express fungal mannoproteins on their surface; 2) undergo functional maturation, as revealed by the up-regulated expression of MHC class II Ags and costimulatory molecules; 3) produce IL-12 but no IL-4; 4) are capable of inducing Th1-dependent antifungal resistance when delivered s.c. in vivo in nontransplanted mice; and 5) provide protection against the fungus in allogeneic bone marrow-transplanted mice, by accelerating the functional recovery of Candida-specific IFN-γ-producing CD4+ donor lymphocytes. These results indicate the efficacy of DC pulsed with Candida yeasts or yeast RNA as fungal vaccines and point to the potential use of RNA-transfected DC as anti-infective vaccines in conditions that negate the use of attenuated microorganisms or in the case of poor availability of protective Ags. The Journal of Immunology, 2002, 168: 2904–2913.

In humans, Candida albicans is the most frequently isolated fungal pathogen (1). The delicate balance between the host and this otherwise harmless commensal fungus turn into a parasitic relationship, resulting in the development of severe infections. Invasive candidiasis is a significant cause of morbidity and mortality in differently immunocompromised patients, including those undergoing bone marrow transplantation (BMT). It is recognized that the nature and extent of the impairment of host defense influence the manifestation and severity of infection (1).

Clinical evidence and experimental data indicate that both the innate and the adaptive immune systems regulate resistance to Candida infections (3–5). In murine experimental models of infection, it has been demonstrated that Th cell reactivity plays a central role in regulating immune responses to the fungus, Th1 reactivity being responsible for resistance and Th2 reactivity being associated with susceptibility (6). The development of protective anticanidial Th1 responses requires the concerted actions of several cytokines, including IFN-γ and IL-12, in the relative absence of Th2 cytokines, such as IL-4 and IL-10, which inhibit development of Th1 responses (6).

However, the fungus is not a mere passive participant in the infectious process, and a hypothetical set of virulence factors has been attributed to it (7). Among these, the ability to reversibly switch between budding yeast to the filamentous growth form or hypha, all of which can be found in infected tissues (1), is thought to be important for virulence (8–10).

Recent evidence indicates that dendritic cells (DC) are uniquely capable of decoding the fungus-associated information required to elicit the qualitative nature of the adaptive immune response (11). DC finely discriminated between the two forms of C. albicans in terms of type of immune responses elicited. By the production of IL-12 and IL-4 in response to the nonviral and virulent forms of the fungus, DC were uniquely capable of Th1 priming and education in vitro and in vivo (11). This finding is particularly relevant in candidiasis, because the fungus behaves as a commensal as well as a true pathogen of skin and mucosal surfaces (1), known to be highly enriched for DC.

DC are uniquely specialized to initiate T cell immunity in vitro and in vivo (12). When delivered in vivo, DC charged with peptide Ags, tumor lysates, or viral vectors that encode the relevant Ags induce immune responses, which include protective and therapeutic immunity to tumors and pathogens in animals (11, 13–18) and clinical antitumor responses in humans (19–22).

Studies have shown that DC transfected with tumor RNA could also serve as potent cancer vaccines (23–25). DC transfected with chicken OVA RNA stimulate primary anti-OVA CTL responses in vitro. Moreover, vaccination of mice with OVA RNA-transfected DC has been used to treat OVA-positive tumor metastases to lung.
(23). These findings are particularly relevant, as they would extend vaccination to conditions of limited availability of protective Ags. However, whether or not induction of antimalarial immunity with DC transfected with pathogen-derived RNA could represent a strategy of DC-based therapy in infections remains to be determined.

We have already shown that DC pulsed with viable Candida yeasts, but not hyphae, were capable of inducing protective Th1 immune responses when adoptively transferred in vivo (11). In the current study we tested whether transfecting DC with Candida yeast RNA is an effective way to induce antifungal protective immunity in vivo. Ex vivo transfected DC were adoptively transferred into recipient mice, and parameters of infection and immunity to it were evaluated upon the i.v. challenge with the fungus. We also used a very stringent experimental system to assess the priming capacity of transfected DC, which includes the infusion of DC in an allogeneic T-depleted BMT experimental model (26). In this model, autologous reconstitution of host stem cells is greatly reduced to the benefit of a long-term, donor-type chimerism in >95% of the mice and low incidence of graft-vs-host disease (27–29). We found here that DC transfected with yeast, but not hyphal RNA, were capable of stimulating Candida-specific Th1 immunity that conferred antifungal resistance to susceptible mice, either untransplanted or after allogeneic hematopoietic transplantation.

Materials and Methods

Animals

Female, 8- to 10-wk-old, inbred C3H/HeJ and hybrid (BALB/c x DBA/2F1) CDF2mice were obtained from Charles River Breeding Laboratories (Calco, Italy). Breeding pairs of homozygous IFN-γ−/− mice raised on BALB/c background (kindly provided by Dr. M. Kopt, Basel Institute for Immunology, Basel, Switzerland) were bred under specific pathogen-free conditions at the breeding facilities of the University of Perugia (Perugia, Italy). Mice of both sexes, 8–10 wk old, were used. Hybrid CDF2 mice were used as wild-type mice. Bone marrow (BM)-transplanted mice were kept in small sterile cages (five animals in each cage) and fed with sterile food and water. Procedures involving animals and their care were conducted in conformity with national and international laws and policies.

Irradiation

C3H/HeJ mice were exposed to a single, lethal dose of 9 Gy from a gamma beam 150A, 40Co source (Clinac 600/C Varian; Cernusco, Milan, Italy) with focus to skin distance of 75 cm and 0.7 Gy/min dose rate. For irradiation, 150-mm polystyrene tubes at room temperature were used at the RNA/DOPAT ratio of 1:2. The complex was added to DC in a total volume of 2 ml and incubated at 37°C in a water bath with occasional agitation for 2 h. After pulsing, the cells were washed and left for an additional 24 h in culture before FACS analysis, measurement of cytokine in the supernatants, and adoptive transfer. Some RNAs were treated with 400 μg/ml proteinase K (Sigma-Aldrich) or 100 U/ml RNase A (Sigma-Aldrich) for at least 2 h. Consistent with previous reports (31, 32), greater than 95% of the BM population did not react with the 2.43 anti-CD80 antibody (34-5-3), anti-I-A db (34-5-3), anti-CD80 (1G10), and anti-CD86 (GL1) (all from Brie y, after overnight plastic adherence to remove macrophages, 108 cells (containing >95% of contaminating T cells on FACS analysis) were purified from spleens (SP-DC) by magnetic cell sorting with CD11c MicroBeads before magnetic separation. BM-derived DC (BM-DC) were prepared as described (31). Briefly, BM-DC, obtained from femurs of mice, were depleted of red cells and seeded (2 × 106/ml) in six-well plates (Falcon; BD Labware, Lincoln Park, NJ) in 3 ml IMDM (Life Technologies, Milan, Italy) with 10% FCS, 20 mM 2-ME, 50 μg/ml gentamicin sulfate, 2000 U/ml GM-CSF (Sigma-Aldrich), and 1 × 104 U/ml IL-4 (BD Pharmingen, San Diego, CA) for 6 days. One day of culture before the assay 3 of culture medium was exchanged, and fresh medium containing GM-CSF and IL-4 was added. On day 6 of culture, DC were isolated by transferring nonadherent cells to new culture plates and incubating at 37°C for at least 2 h. Consistent with previous reports (31, 32), >90% SP-DC (at ~10% yield recovery) routinely express high levels of CD11c integrin, while BM-DC (at ~10% of the BM population) uniformly showed low level of CD11c staining. More than 90% of BM-DC did not react with the 2.43 anti-CD80 antibody, while 25–30% of SP-DC expressed CD80 (data not shown). For pulsing, 2 × 106 DC were exposed to live yeasts or hyphae (at DC:yeast ratio of 1:1 and 1:10, respectively) for 2 h after addition of 2.5 μg/ml amphotericin B (Sigma-Aldrich) to prevent Candida overgrowth, as described (11). Pulsing of DC with RNA was performed in serum Opti-MEM medium (Life Technologies), as described (33). DC were washed twice in Opti-MEM and reseeded in Opti-MEM medium at 2–5 × 104 cells/ml and added to 15-ml polypropylene tubes (Falcon). The cationic lipid, N-[1,2-diolooxypropyl]-N,N,N’,N’-trimethylammonium methylsulfate (DOTAP; Boehringer Mannheim, Mannheim, Germany) was used to deliver RNA into cells (33). RNA (25 μg in 250 μl Opti-MEM medium) and DOTAP (50 μg in 250 μl Opti-MEM medium) was mixed in 12 × 75-mm poly styrene tubes at room temperature for 20 min at the RNA:DOTAP ratio of 1:2. The complex was added to DC in a total volume of 2 ml and incubated at 37°C in a water bath with occasional agitation for 2 h. After pulsing, the cells were washed and left for an additional 24 h in culture before FACS analysis, measurement of cytokine in the supernatants, and adoptive transfer. Some RNAs were treated with 400 μg/ml proteinase K (Sigma-Aldrich) or 100 U/ml RNase A (Sigma- Aldrich) or RNase-free DNase I (Life Technologies, Gaithersburg, MD) for 30 min at 37°C. The enzymes were inactivated by incubating samples at 65°C for 15 min.

Adaptive immunization, fungal challenge, and assessment of protection

DC (3–5 × 104/each injection) were injected either s.c. in 20 μl of PBS or i.v. in 0.5 ml of PBS following different protocols, as described in Results. In BM-transplanted recipients, DC were administered s.c. twice, 1 and 7 days after BMT. For infection, a week after the last DC administration, mice were i.v. infected with 105 C. albicans cells of the live vaccine strain or the virulent strain in 0.5 ml PBS as described (26). Resistance to infection with the virulent strain was done 14 days after the primary infection with the live vaccine strain. Resistance to infection was assessed by quantifying the number of CFU (mean ± SE) per organ and production of Th1 and Th2 cytokines in culture supernatants of Ag-stimulated splenocytes (106 splenocytes stimulated with 103 heat-inactivated C. albicans cells for 48 h), and enumerating the IFN-γ-producing cells, by ELISPOT assay, as described (11). Total and differential white blood cell counts were done by hemocytometry and by staining blood smears from transplanted mice with May-Grünwald Giemsa reagents (Sigma-Aldrich) before analysis.

Flow cytometry

For DC analysis, cell surface phenotype was assessed with the following mAb reagents: FITC-conjugated mAb anti-CD11c (N418), anti-CD8α (2.43), anti-I-Aα (34-5-3), anti-CD80 (1G10), and anti-CD86 (GL1) (all from BD Pharmingen). Unrelated isotype-matched mAbs were used as controls. For assessment of Candida protein expression, DC pulsed with viable yeasts or hyphae or transfected with RNA from yeasts or hyphae were sequentially reacted with the IgM B6.1 mAb, specifically reacting to the phosphomannan component of the fungal cell wall, β-1,2-mannotriose (34, 35) or with polyclonal antiserum from vaccinated mice (36) and the FITC-conjugated goat anti-mouse IgA plus IgG plus IgM (H + L) (Euroclone, Milan). Before all labeling experiments, Fc blocking was performed by incubating cells with 5% normal mouse serum. For chimerism assessment in BMT, the analysis was done as described (26). For lymphocyte analysis, splenocytes were reacted with FITC-conjugated anti-
CD4 (L3T4, clone GK1.5), anti-CD8α (Ly-2, clone 53-6.7), and anti-SE6NK (clone 5E6). Staining with PE-conjugated anti-CD44 (clone GL1) molecules. Unrelated isotype-matched mAbs were used as a control (gray lines). Analysis was performed on a FACScan. Numbers indicate fluorescence values. Data were evaluated both as the percentage of positive cells and as median fluorescence intensity (MFI).

Cytokine assays

The levels of IL-2, IL-4, IL-6, IL-10, and IL-12 p70 in culture supernatants were determined by ELISA (R&D Systems, Space Import-Export, Milan, Italy). Cytokine titers were calculated by reference to standard curves constructed with known amounts of recombinant cytokines (from BD Pharmingen). The detection limits of the assays were <4 pg/ml for IL-2, <3 pg/ml for IL-4, <15 pg/ml for IL-6, <8 pg/ml for IL-10, and <16 pg/ml for IL-12 p70.

ELISPOT assay

IFN-γ-producing cells were enumerated by ELISPOT assay, as described (37). Briefly, freshly isolated splenocytes or purified CD4+ T cells were cultured (1 × 10^5 cells/well) in complete medium (RPMI 1640 containing 10% FCS, 50 mM 2-ME, and 50 μg/ml gentamicin sulfate) for 18 h in 96-well plates previously coated with rat anti-murine R4-6A-2 mAb. Biotinylated AN-18.17.24 mAb was used as the detecting reagent, avidin-biotin-peroxidase complex, and 3,3'-diaminobenzidine tetrahydrochloride (Life Technologies). cDNA was synthesized from 1 μg of total RNA according to the manufacturer’s directions (Superscript Moloney murine leukemia virus reverse transcriptase; Life Technologies). cDNAs were amplified with random primers derived from the M13 phage core sequence (5’-GAGGGTTGGCGGTTCT-3’), known to amplify hypervariable interrepeat DNA sequences from Candida strains (38). Amplifications were performed in volumes of 25 μl containing 10 ng of cDNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM each dATP, dGTP, dTTP, and dGTP, 3 mM magnesium acetate, 10 ng of primer, and 1.5 U of AmpliTag DNA polymerase. The PCR was performed in a PerkinElmer thermal cycler (model 480; PerkinElmer, Wellesley, MA) with an initial denaturation of 97°C for 3 min, followed by 40 cycles of 20 s at 93°C, 60 s at 50°C, and 20 s at 72°C, and a final cycle of 5 min at 72°C. Amplification products were separated by electrophoresis in 1.5% agarose gels in 1× Tris-acetate-EDTA buffer for 13 h at 2 V/cm. A 100-bp ladder (New England Biolabs, Celbio, Milan, Italy) was run in parallel for approximate PCR product band sizing. Amplification products were analyzed by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining.

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.

Results

Maturation and cytokine production by DC upon exposure to Candida yeasts or transfected with yeast or hyphal RNA

To assess whether DC undergo functional maturation upon exposure to viable yeasts or RNA from yeasts or hyphae, the surface expression of MHC class II Ags and of CD80 and CD86 costimulatory molecules was assessed in SP-DC and BM-DC exposed to either type of stimuli. The results showed that the expression of...
and of costimulatory molecules was greatly enhanced in both types of DC upon exposure to yeasts or yeast RNA (Fig. 1). Treatment of yeast RNA with DNase or proteinase did not abolish the induction of costimulatory molecules, as did treatment with RNase (Fig. 2), a finding suggesting that sensitization of DC was mediated by RNA.

In contrast, transfecting with hyphal RNA did not result in up-regulated expression of costimulatory molecules (Fig. 1), a finding suggesting that maturation of DC is a specific effect of yeast RNA and not a consequence of a nonspecific effect of the transfection method itself. The inability of hyphal RNA to sensitize DC was not due to a defective entry of RNA into the cells, as both yeast and hyphal RNA could be detected inside the cells by random amplified polymorphic DNA (RAPD) analysis (Fig. 3). An 867-bp band that was similar in size to that amplified in fungal cDNAs (Fig. 3) or in genomic DNAs from Candida strains (38) was detected in DC transfected with either yeast or hyphal RNA. As higher amounts of hyphal RNA still failed to up-regulate costimulatory molecule expression on DC (data not shown), all together, these data suggest that the induction of DC maturation is a property of yeast RNA.

We have already shown that myeloid DC produce opposite sets of Th cytokines upon exposure to yeasts (IL-12 p70 and IL-6) or hyphae (IL-4) (11). In this study we found that both types of DC produce IL-12 p70 and IL-6, but not IL-4 or IL-10, upon exposure to either yeasts or yeast RNA (Table I). Therefore, these data suggest that both live yeasts and yeast RNA are effective stimuli capable of inducing functional maturation and cytokine production by myeloid DC.

FIGURE 2. Effect of different treatments on the ability of yeast RNA to induce DC maturation. Splenic DC were transducted yeast RNA for 24 h at 37°C before the assessment of CD80 (A) and CD86 (B) costimulatory Ag expression by immunofluorescence analysis, as detailed in Fig. 1. Before transfection, some RNAs were treated with 100 U/ml RNase A, 100 U/ml DNase I, or 400 μg/ml proteinase K for 30 min at 37°C. The enzymes were inactivated by incubating samples at 65°C for 15 min. MFI of nontransfected DC was 12 for CD80 and 10 for CD86. *, p < 0.05, RNA-treated vs untreated (none) RNA.

FIGURE 3. Detection of fungal RNA in transfected DC by RAPD-PCR. cDNAs from fungal RNA or from SP-DC, after 30 min of transfection with yeast or hyphal RNA, were amplified with random primers, as detailed in Materials and Methods. Amplification products were analyzed by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining. Lane 1, H20 contamination control; lane 2, SP-DC transfected with DOTAP only; lane 3, SP-DC transfected with yeast RNA; lane 4, SP-DC transfected with hyphal RNA; lane 5, cDNA from yeast RNA; lane 6, cDNA from hyphal RNA. bp, DNA ladder.

Expression of fungal Ags on DC upon exposure to Candida cells or Candida RNA

To assess whether effective transduction of fungal RNA occurs in DC after RNA transfection, we measured the surface expression of fungal proteins by reacting cells with a polyclonal antiserum from vaccinated mice, previously shown to contain Candida-specific Abs (36). We also assessed the levels of expression of mannanproteins, known to induce protective immune responses against disseminated candidiasis (39), with the use of the B6.1 mAb directed to the β-1,2 mannortriose of the phosphomannan protein complex (34, 35). The results show that DC stained positive with either the polyclonal or the mAbs after pulsing with viable yeasts. DC transfected with yeast RNA stained positive with polyclonal Abs and less with the mAb (Fig. 4). In contrast, DC pulsed with hyphae or transfected with hyphal RNA did not show positive staining with either type of Abs. Therefore, these data suggest that the transduction of yeast RNA in DC is responsible for the surface expression of fungal Ags, which may include those endowed with protective efficacy in vivo.

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*SP- and BM-DC were obtained from CDF2 mice as described in Materials and Methods. DC (2 × 10⁵ cells) were pulsed with either live Candida yeasts (at DC: fungus ratio of 1:1) or 25 μg fungal RNA in 250 μl of DOTAP for 2 h at 37°C. After 2 h of pulsing, amphotericin B was added to the cultures of DC pulsed with viable yeasts, and the cells were cultured for an additional 22 h before measurement of cytokine contents in the culture supernatants. <, Below the detection limit of the assays.

* Cytokine levels (measured in picograms per milliliter ± SE) as determined by specific ELISA.
Immunization with yeast RNA-transfected DC induces Th1-mediated antifungal resistance

DC pulsed with *Candida* yeasts, but not hyphae, were capable of inducing Th1 priming and antifungal resistance when adoptively transferred into naive recipients (11). To assess whether DC transfected with yeast RNA also induce antifungal resistance in vivo, SP-DC or BM-DC were exposed to yeasts or to RNA from yeasts or hyphae in vitro and injected into naive recipients following different experimental protocols, which include different routes and timing of DC administration. Mice were subsequently infected i.v. with virulent *C. albicans* and fungal growth in the kidneys was recorded a week after the infection. The results show that the fungal growth was significantly decreased in mice who had received either type of yeast-pulsed DC administered s.c. 14 days before challenge (Fig. 5). However, consistent with previous data (11), the fungal growth was even more restrained when DC were administered twice, 14 and 7 days before i.v. route. Results with yeast or hyphal RNA-transfected DC showed that the adoptive transfer of yeast, but not hyphal, RNA-transfected DC s.c., twice, also resulted in a significant inhibition of fungal growth (Fig. 5). To understand whether, similar to what had been observed with yeast-pulsed DC (11), the antifungal resistance correlates with the priming of Th1 cells, the production of Th1 (IFN-γ) and Th2 (IL-4 and IL-10) cytokines was evaluated in the spleens of mice who had received multiple s.c. injections of RNA-transfected SP- or BM-DC. The results (Fig. 6) show that production of IFN-γ was higher and that of IL-4/IL-10 was lower in mice who had received multiple s.c. injections of yeast RNA-transfected DC as compared with either mice who had received DC transfected with hyphal RNA or control mice not receiving DC. Together, these data suggest that yeast RNA-transfected DC, similar to yeast-pulsed DC, are capable of inducing Th1 priming and antifungal resistance in vivo, and that the priming capacity is strictly dependent on mode of DC delivery in vivo.

Yeast RNA-transfected DC induce antifungal resistance in allogeneic T-depleted BM-transplanted mice

In a mouse model of allogeneic T-depleted BMT, we have recently shown that susceptibility or resistance to candidiasis correlates with the temporal occurrence of Th2 and Th1 cell responses, with Th2 reactivity accounting for susceptibility to the infection in the early engraftment period (26). To assess whether the infusion of *Candida*-pulsed DC would accelerate Th1 cell recovery and antifungal resistance in transplanted mice, recipients mice received at 1 and 7 days post-BMT donor DC in vitro pulsed with yeasts or yeast RNA. One week after the last DC administration, mice were i.v. infected with the *Candida* live vaccine strain and 14 days later they were infected with the virulent strain. Mice were monitored for resistance to infection with virulent cells and for parameter of Th1-mediated antifungal resistance, such as the frequency of IFN-γ-producing CD4+ T splenocytes. The results (Fig. 7) showed that the infusion of yeast-pulsed or RNA-transfected DC greatly accelerated the recovery of antifungal resistance, as compared with control mice not receiving DC. A reduced fungal burden was observed in the kidneys and was comparable to that seen in donor-vaccinated mice upon reinfection. Interestingly, the fungal growth was also significantly reduced in organs that are sites of fungal disease and pathology in BMT, such as the gastrointestinal tract, the liver, and the lungs (26). The infusion of yeast-pulsed or RNA-transfected DC also increased resistance toward the primary infection with virulent *Candida* cells (data not shown). Total and differential counts of blood leukocytes indicated that the absolute number of circulating lymphocytes significantly increased in DC-infused and transplanted mice and was similar to that of donor-vaccinated mice, as shown in Fig. 8 for mice infused with RNA-transfected DC. The number of monocytes and neutrophils did not show significant variations among groups (data not shown). Cytofluorometric analysis of spleen cells revealed that the numbers of CD4+, CD8+, and NK cells were significantly higher in mice who had...
received RNA-transfected DC as compared with mice not receiving DC and were similar to those of donor-vaccinated mice. Interestingly, CD4+ T cells also stained positive for the CD44 and CD25 activation surface markers (Fig. 8).

On assaying the pattern of cytokine production, we found that the frequency of IFN-γ-producing cells was increased in both freshly isolated T splenocytes and purified CD4+ T cells from mice receiving either yeast-pulsed or RNA-transfected DC as compared with recipients not receiving DC and was similar to that of donor-vaccinated mice (Fig. 9a). Upon Ag stimulation in vitro, splenocytes from DC-infused mice produced levels of IL-4 that were lower than those of mice not receiving DC and were similar to those of vaccinated donor mice (Fig. 9b). Interestingly, however, levels of IL-2 were also lower and those of IL-10 higher in DC-infused mice, as compared with not only untreated recipients but also donor-vaccinated mice. Therefore, the activation of CD4+ lymphocytes producing IFN-γ occurs together with IL-10 but not IL-2 production in hematopoietic BM recipients upon adoptive transfer of Candida-pulsed DC. Experiments in which RNA-transfected DC were infused into recipient mice who had received T-depleted BM cells from IFN-γ−/− mice demonstrated that the efficacy of RNA-transfected DC was mediated by the occurrence of donor IFN-γ-producing cells. Fig. 10 shows that the fungal growth in these mice was comparable to that observed in mice not receiving DC and significantly higher than that of mice transplanted with T-depleted BM cells from IFN-γ-sufficient mice. As a result, similar to what we observed in mice not receiving DC, all the mice succumbed to the infection as opposed to the long-term survival, with no signs of diseases, of mice who had received T-depleted BM cells from IFN-γ−/− mice.

Discussion

The present study shows that, consistent with previous results obtained with myeloid DC pulsed with Candida yeasts (11), DC transfected with yeast RNA are fully competent to induce Th1 priming upon adoptive transfer in vivo. Induction of antitumor immunity with tumor RNA-transfected DC represents a recently described approach for DC-based vaccines (25). In this study we show that this approach could be used to induce antimicrobial immunity as well.

It has been shown that DC transfected with MUC1 RNA expressed MUC1 tumor Ag along with costimulatory and adhesion molecules (24), a finding suggesting that newly synthesized molecules are potential targets for CTL recognition of RNA-transfected cells (24). We found in this study that DC from both BM and spleens underwent functional maturation in vitro upon pulsing with either live yeasts or yeast RNA, but not with hyphal RNA, as revealed by the up-regulated expression of MHC class II Ags and costimulatory molecules. No variation in CD8α expression was observed upon pulsing with either type of stimulus (data not shown), a finding suggesting that, in our system, myeloid DC do not convert into lymphoid DC upon activation, as suggested (40). Moreover, similar to yeast-pulsed DC, DC transfected with yeast but not hyphal RNA produced IL-12 p70 along with IL-6. As the ability of yeast-pulsed DC to induce Th1 priming in vivo was lost in conditions of IL-12 deficiency (11), these data suggest that DC transfected with yeast and not hyphal RNA would be fully competent to induce Th1 activation.

For this to occur, a prerequisite would be the expression of fungal Ags capable of inducing protective immunity on the surface of DC upon transfection with yeast RNA. This appeared to be the case as DC transfected with yeast but not hyphal RNA positively reacted with polyclonal Abs specifically reacting with Candida cells (36) and, partially, with mAb binding to a specific component of the acid-labile portion of the phosphomannan complex, which is known to be endowed with the ability to induce protective immunity in experimental candidiasis (39).

FIGURE 6. Cytokine production in mice with Candida albicans infection upon adoptive transfer of fungal RNA-transfected DC. SP-DC or BM-DC were isolated and pulsed as in Fig. 1 and injected s.c. twice into CD2F1 mice infected as in Fig. 3. Three days after infection, levels of cytokines in culture supernatants of stimulated with inactivated Candida cells were determined by cytokine-specific ELISA.

FIGURE 7. Adoptive transfer of Candida-pulsed DC induces resistance to C. albicans infection in a murine model of allogeneic T-depleted BMT. lethally irradiated C3H/HeJ mice were transplanted with 2 × 10^5 T cell-depleted allogeneic BM cells from CD2F1 mice, infected with 10^5 cells of the live vaccine strain of C. albicans 14 days later, and reincubated with 10^6 virulent C. albicans cells 2 wk after the primary infection. One and 7 days post-BMT, mice received 5 × 10^3 DC pulsed with either Candida yeasts or yeast RNA. Donor CD2F1 mice vaccinated with 10^6 cells of the live vaccine strain of C. albicans and reincubated 14 days later with virulent C. albicans strain; BM-transplanted mice not receiving DC at 3 days after reinfusion, the fungal growth in different organs was evaluated and expressed as CFU per organ. * p < 0.05, mice receiving DC or donor mice vs mice not receiving DC.
Pathways and mechanisms of fungal RNA and Ags processing by DC are largely unknown at the moment and remain to be explored.

Upon adoptive transfer in vivo, yeast RNA-transfected DC were capable of inducing the activation of *Candida*-specific Th1 cells, as revealed by the predominant production of IFN-γ over that of...
IL-4/IL-10. Moreover, the IL-12/IL-10, a marker of CD4+ Th1 cells in candidiasis (5), was expressed on CD4+ T lymphocytes (data not shown). Associated with the occurrence of Candida-specific Th1 reactivity was the induction of a state of antifungal resistance, as revealed by the significant decrease of fungal growth in the target organs, such as the kidneys and the brain (data not shown).

Previous work in vivo in mice and in humans has suggested that, in addition to the maturation state (41), the mode of delivery of DC is important in eliciting T cell responses (42–45). DC given i.v. localized in the reticuloendothelial system and lung, while those given s.c. preferentially home to the T cell areas of the draining lymph nodes (42). In particular, induction of IFN-γ production, but not IL-4, was seen only with DC injected intradermally or intralymphatically but not i.v. (43). We found a similar result, as Th1 cell activation was observed after s.c. but not i.v. administration of DC. Although multiple injections of DC could lead to clonal exhaustion of the responding T cell population (43), the optimal protection against C. albicans was observed after two s.c. injections of Candida-pulsed DC. Labeling studies will clarify whether DC loaded with Candida home to different organs upon administration by different routes and whether the extent to which the ability of DC to migrate to relevant lymphoid organs will regulate the type and magnitude of the anticandidal immune response.

An interesting observation of the present study was that the infusion of Candida-pulsed DC accelerated the recovery of functional antifungal Th1 responses in a murine model of T cell-depleted allogeneic BMT. Patients receiving T cell-depleted BMT are unable to develop Ag-specific T cell responses soon after transplant (46). It has been demonstrated that T cell depletion of allogeneic BMT is associated with a slow recovery of CD4+ and CD8+ T cells (46). However, functional recovery of the T cell system after T cell-depleted allogeneic BMT has been demonstrated (47, 48), and both donor and recipient DC may contribute to the reconstitution of the T cell repertoire in transplantation through distinct pathways of Ag presentation (49). Interestingly, it has been reported that T cell reactivity to Candida readily recovered after T cell-depleted allogeneic BMT, and the fact that recovery was related to the absence of antifungal prophylactic measures indicates that antifungal reactivity of residual T cells could be maintained through continuous exposure to the fungus (50).

We have recently demonstrated that an unbalanced production of Th1 and Th2 cytokines was responsible for the susceptibility to candidiasis observed in our BMT model. However, readressing the balance between Th1 and Th2 subsets, as by treatment with Th2 cytokine antagonists, accelerated the recovery of Th1-mediated antifungal resistance (26). In this study we show that recovery of functional NK and T cells producing IFN-γ could also be accelerated by the infusion of Candida-pulsed DC. This, along with the decreased IL-4 production, translated in the occurrence of a state of antifungal resistance as revealed by the impaired fungal growth in different target organs, including those most frequently associated with fungal disease and pathology in BMT settings, such as the gut, liver, and lungs. Therefore, although conflicting data exist as to whether donor DC are “friends” or “foes” in transplantation (49), our findings suggest that DC may contribute to the educational program of T cells in BMT during reconstitution, in line with the recent observation of the immunization capacity of tumor-pulsed (51) or Aspergillus-pulsed (18) DC in BM-transplanted mice and humans.

The observation that the occurrence of a protective Th1 reactivity coexisted with the detection of significant levels of IL-10 is intriguing. It is known that high levels of IL-10 are associated with tolerance to HLA-mismatched BM stem cells (52) and IL-10 is required for the induction of regulatory T cells mediating tolerance to alloantigens in vivo (52–55). Different regulatory T cells have been reported and they are known to differentiate upon the encounter with immature DC (53, 55). However, fully competent DC were also found to be inducers of T cell anergy when presenting a self-epitope with altered peptide ligand features (56). Whether IL-10 produced in BM-transplanted mice infused with Candida-pulsed DC may serve to support the growth of regulatory T cells preventing donor Th1 alloreactivity remains a working hypothesis, although it may help to explain the long-term, disease-free survival of the mice. In terms of interference with the development and activity of antifungal Th1 reactivity, it is well established that the effect of IL-10 is dependent upon the dose of the cytokine. At high doses, IL-10 had a negative effect on Th1-mediated antifungal resistance (57), but at low doses IL-10 was required for optimal induction and maintenance of antifungal Th1 immune response (58).

A recent study showed that yeast cells could act as an efficient vaccine and elicited protective cell-mediated immunity by virtue of their ability to induce DC maturation, IL-12 production, and efficient priming for MHC class I- or II-restricted Ag-specific T cell responses (59). We show in this work that not only the yeasts but yeast RNA could be an efficient trigger of DC maturation, IL-12 production, and induction of protective Ag-specific responses to Candida. Interestingly, inactivated yeasts failed to induce DC maturation in vitro, and DC pulsed with inactivated yeasts failed to promote Th1 immunity upon adoptive transfer in
vivo (data not shown). Therefore, these data may account for the long-standing observation of the inability of inactivated *Candida* to induce memory antifungal protective immune responses.

It has been shown that human DC phagocytose and process *C. albicans* (60, 61) as well as other fungi (18, 62), and ex vivo-generated DC pulsed with *Aspergillus* conidia restored antifungal immunity in BM-transplanted patients (18). This work suggests that DC could act as effective vaccines against fungal infections and that RNA-transfected DC could be of vaccinating potential in conditions that negate the use of attenuated microorganisms, such as immunosuppression, or in the case of poor availability of protective Ags.

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**References**


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