Interferon-gamma as adjunctive immunotherapy for invasive fungal infections: a case series

Running title: IFN-γ treatment in fungal infections

Keywords: Immunotherapy; Interferon-gamma; Candidemia; Aspergillosis;

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Abstract

Background: Invasive fungal infections are very severe infections associated with high mortality rates, despite the availability of new classes of antifungal agents. Based on pathophysiological mechanisms and limited pre-clinical and clinical data, adjunctive immune-stimulatory therapy with interferon (IFN)-γ may represent a promising candidate to improve outcome of patients with invasive fungal infections by enhancing host defense mechanisms.

Methods: In this open-label, prospective case series, we describe eight patients with invasive Candida and/or Aspergillus infections who were treated with recombinant IFN-γ (rIFN-γ, 100 µg s.c., thrice a week) for 2 weeks in addition to standard antimycotic therapy.

Results: Recombinant IFN-γ treatment in patients with invasive Candida and/or Aspergillus infections partially restored immune function, as characterized by an increased HLA-DR expression in those patients with a baseline expression below 50%, and an enhanced capacity of leukocytes from treated patients to produce proinflammatory cytokines involved in antifungal defense.

Conclusions: The present study provides evidence that adjunctive immunotherapy with rIFN-γ can restore immune function in fungal sepsis patients, warranting future clinical studies to assess its potential clinical benefit.

Trial registration: ClinicalTrials.gov - NCT01270490
Introduction

The incidence of fungal infections is steadily increasing in the last years due to invasive medical diagnosis and treatment, as well as an increasing number of immunocompromised patients. Despite development of new classes of antifungal agents (47), invasive fungal infections remain associated with unacceptable high mortality rates and represent a major cause of death worldwide (18, 19, 53, 55, 57). The emergence of significant resistance to the current antifungal treatment further emphasizes the need for novel approaches to treat invasive fungal infections (20, 41). Because invasive fungal infection are most commonly observed in individuals with immune defects, and the number of immunocompromised patients is steadily increasing (24), adjunctive immunotherapy to improve host defense is an attractive strategy to improve the outcome of patients with disseminated fungal infections.

In the past decade, major progress has been made in the understanding of anti-fungal host responses, which has enabled the development of a number of novel molecular and cell-based immunotherapeutic approaches for invasive fungal infections (42). Although invasive candidiasis and aspergillosis are rather different in their pathogenesis, the major protective host response is for both these two fungal infections the effective induction of Th1 and IFN-γ responses (5-8, 33). Th1 cytokine response results for example in activation of effector phagocytic cells that kill the fungus (21). Interestingly, Th1 immunity against A. fumigatus was demonstrated to be cross-protective against C. albicans (50).

Interferon-gamma (IFN-γ), the prototype Th1 cytokine, promotes Th1 differentiation and thus skews the immune response towards a protective Th1 phenotype (46). As such, it has been implicated as a treatment option in (invasive) fungal infections (27, 49). Moreover, limited evidence suggests that recombinant IFN-γ (rIFN-γ) has a beneficial effect on the outcome of
fungal infections in patients with chronic granulomatous disease (CGD) (17), HIV (3, 22, 40) and leukemia (11, 39) as well as in organ transplant patients (1). However, it has not been investigated whether rIFN-γ actually enhanced the immune response in these patients to explain these beneficial clinical effects.

In this report we describe a series of patients with invasive Candida and/or Aspergillus infections in whom we investigated the effects of treatment with rIFN-γ on the host innate and adaptive immune responses.

Materials and Methods

Patients and treatment

To assess the feasibility and preliminary efficacy of IFN-γ in combination with anidulafungin for the treatment of candidemia, a single-centre, prospective, randomized open-label pilot (Phase IIIb) study was conducted. This study was registered at ClinicalTrials.gov (NCT01270490) and approved by the local ethics committee of the Radboud University Nijmegen Medical Centre. Due to slower than anticipated enrollment rates (from August 2010 until March 2013, only 12 patients could be screened, of which 6 were eligible and provided informed consent [Figure 1]), the study was terminated early. However, during the period this study was carried out, several other patients presented with invasive fungal infections that had an insufficient response to standard antifungal therapy. Although these patients did not meet the inclusion criteria (i.e. presenting with one or more positive cultures of blood or normally sterile tissue growing Candida spp.), they were deemed to benefit from adjunctive immunotherapy as “therapy of last resort” as decided by attended physician. Within parameters of standard clinical care these patients were treated according to the same protocol as the patients enrolled in the study, and are therefore included in the present case series. All
patients with a history of documented epileptic seizures, pre-existent severe renal impairment (creatinin clearance <30/mL/min) or severe liver failure (an increased prothrombin time) were excluded. After obtaining informed consent, eight patients (3 study patients, 5 last resort patients) were treated with rIFNγ (Immukine, Boehringer Ingelheim, 50 µg/m² body surface, subcutaneously, three times a week) in addition to standard antifungal therapy as recommended by national and international treatment guidelines (35, 36). Three patients that were included in the Phase IIIb Candida pilot-study were assigned to the control group and did not receive rIFN-γ.

Blood sampling

Plasma, serum and whole blood specimens were collected at baseline (BL) and serially after the start of antifungal therapy (days 1, 2, 3, 7, 14 and 28). Blood cultures were performed as part of routine care.

Leukocyte populations and surface HLA-DR expression

Heparin anticoagulated blood was stored at 4°C immediately after withdrawal and analyzed by flow cytometry (see online supplement for details). To determine the extent of immune suppression, HLA-DR expression was determined by calculating % HLA-DR-positive cells and HLA-DR mean fluorescence intensity (MFI) within CD14+ cells and various lymphocyte subsets within CD45+ leukocytes+. Lymphocyte subsets were defined as: T-cells (CD45+CD3+), T-helper cells (Th, CD45+CD3+CD4+), cytotoxic T-cells (Tc, CD45+CD3+CD8+), B-cells (CD45+CD19+), and NK-cells (CD45+CD3+CD56+). Subset counts were calculated by multiplying the percentage of gated cells by the total lymphocyte count. Patients with <50% HLA-DR positive monocytes at baseline were considered to present immune paralysis (30).
**Cytokine assays**

Venous blood was drawn into 10 mL EDTA tubes, after which peripheral blood mononuclear cells (PBMCs) were isolated as described previously (34). In short, blood was diluted in phosphate buffered saline (PBS) (1:1) and fractions were separated by Ficoll (Ficoll-Paque Plus, GE healthcare, Zeist, The Netherlands) density gradient centrifugation. Cells were washed twice with PBS and resuspended in RPMI-1640+ (RPMI-1640 Dutch modification supplemented with 10µg/mL gentamicin, 10mM L-glutamine, and 10mM pyruvate) (Gibco, Invitrogen, Breda, The Netherlands). The PBMCs were counted using a particle counter (Beckmann Coulter, Woerden, The Netherlands) and were plated in 96 well round-bottom plates (Corning, NY, USA) at a final concentration of 2,5x10^6/mL, in a total volume of 200 µL. The PBMCs were stimulated for 24 hours, 48 hours, and 7 days with medium alone, or medium containing *E. coli* lipopolysaccharide (LPS; 10 ng/mL), phytohaemaglutinin (PHA; 10µg/ml), heat-inactivated *Candida albicans* blastoconidia (1x10^6/ml) or heat-inactivated *Candida albicans* hyphae (derived from 1x10^6/m conidia). After stimulation, cell culture supernatant was collected and stored at -20°C. When all samples were collected, cytokines were measured using commercially available ELISAs (R&D Systems, MN, USA and Sanquin, Amsterdam, The Netherlands) according to the protocols supplied by the manufacturer. Interleukin (IL)-1β, tumor necrosis factor (TNF)α, and IL-6 were measured in culture supernatants of 24 hour cultures, IFN-γ and IL-10 were measured in culture supernatants of 48 hour cultures, and IL-17 and IL-22 were measured in culture supernatants of 7 day cultures.

**Statistical analysis**
In view of the small sample size, normality of distribution was not assumed. Comparisons of baseline with follow up time points were made using Wilcoxon’s signed rank test (within-group comparisons, 2 groups). A p-value of <0.05 was considered statistically significant. Data are expressed as means and standard error of the mean. Calculations and statistical analyses were performed using GraphPad Prism v 5.0 (GraphPad Software, San Diego, CA, USA).
Results

Patient characteristics

The patients treated with rIFN-γ (5 men, 3 women) had a median age of 49.5 [IQR 28.5-68.8] years. The three female patients in the control group were 36, 51 and 73 years old. Clinical characteristics of the participants are listed in Table 1. Of the 6 patients included in the pilot study, three patients had a positive blood culture for *C. albicans*, two patients for *C. glabrata*, and one patient for *C. tropicalis*. During randomization, the three patients with *C. albicans* cultures were assigned to rIFN-γ treatment, whereas the two patients with *C. glabrata* and one with *C. tropicalis* cultures were assigned to the control group. However, no pathophysiological evidence currently exists to suggest that rIFNγ therapy would have a different effect on the immune system in case of albicans vs. non-albicans *Candida* infections.

Of the other 5 patients treated with rIFN-γ as therapy of last resort, three patients had positive cultures for *A. fumigatus* in bronchoalveolar lavage (BAL) fluid, one patient had a positive blood culture for *C. tropicalis* associated with an osteomyelitis who developed under antifungal treatment new suspected lesions on positron emission tomography-computed tomography (PET-CT), and in one patient the CT-scan revealed progression of suspected hepatic *Candida* lesions under antifungal treatment. All patients included suffered some degree of immunosuppression: the 6 patients with positive blood cultures for *Candida spp.* had impaired physical barriers by the presence of indwelling venous catheters (for the need of recurrent blood sampling or total parental nutrition), or an implantable cardioverter-defibrillator (ICD) lead. The patient with progression of suspected hepatic *Candida* lesions on the CT-scan, and the patient with multiple pulmonary cavities and *A. fumigatus* in BAL fluid were immunocompromised because of (therapy for) acute myeloid leukemia. Another patient with *A. fumigatus* in the BAL received immunosuppressive therapy (prednisone and azathioprin) for sarcoidosis and suffered from a co-infection with *Mycobacterium genavense*.
localized in his bone marrow. A second patient with *A. fumigatus* in BAL fluid had a persistent pulmonary cavity after radiotherapy for a T1N0M0 lung carcinoma. Only two patients, both with *A. fumigatus* in the BAL fluid, were admitted to the Intensive Care Unit to receive organ supportive therapy (mechanical ventilation and hemodynamic support).

**Clinical outcome**

The three patients in the control group and five out of eight patients treated with rIFN-γ recovered uneventfully from the fungal infection (Table 1). Two patients with invasive aspergillosis that were already admitted to the ICU at the time of treatment died due to infectious complications of severe pulmonary aspergillosis, despite rIFN-γ treatment. The patient with a *Candida* endocarditis, who despite rIFN-γ treatment developed intracerebral mycotic aneurysm, could be discharged from the hospital 93 days after onset.

In all patients treated, rIFNγ was tolerated well. Five patients reported moderate fever upon administration of rIFNγ, which responded well to paracetamol. Two patients developed liver enzyme abnormalities for which tuberculostatic antibiotics and voriconazol were temporarily discontinued, resulting in recovery of the liver enzyme abnormalities while rIFNγ treatment was continued. No other significant adverse events were observed.

*Effect of rIFN-γ on ex-vivo IL-1β and TNFα production*

To assess the effect of rIFN-γ on the capacity of PBMCs to produce pro-inflammatory cytokines, cells were isolated and stimulated before, during, and after treatment. We monitored the fold change in cytokine production compared with baseline (before start of treatment). IL-1β and TNFα are pro-inflammatory cytokines of the innate immune system crucial in the induction and maintenance of the anti-fungal immune response (2, 14, 16, 43, 44, 54). Before IFN-γ treatment, inter-patient variability in cytokine production was high (e.g.
TNFα median [IQR] concentration after stimulation with LPS was 792 pg/mL [314-2005]). Nevertheless, in all patients an increase in the capacity to induce different cytokines was observed after initiation of IFN-γ treatment, independent of their baseline values (group data shown in figure 2). After stimulation with LPS, PHA and Candida blastoconidia, IL-1β was increased in the 1 to 7 days after initiation of rIFN-γ therapy, and TNFα production was increased in response to PHA and Candida blastoconidia. In contrast, the placebo-treated patients IL-1β and TNFα responses remained similar. The response against hyphae of C. albicans was highly variable between patients. Some rIFN-γ-treated patients demonstrated a profound increase of TNFα production after treatment (up to 70 fold), whereas other patients showed no relevant change in TNFα production. Cytokine production remained similar in patients in the control group.

**Effect of rIFN-γ on ex-vivo IL-17 and IL-22 production**

Both IL-17 and IL-22 are cytokines that are thought to be protective in the host defense against invasive fungal infections (10, 14, 15, 28, 31, 48). PHA-induced IL-17 and IL-22 production was increased 1 day after initiation of rIFN-γ treatment (figure 3). However, at subsequent time points only a trend towards increased IL-17 and IL-22 production was observed, which reverted to baseline levels at day 28. Production of IL-17 and IL-22 upon stimulation with Candida blastoconidia was elevated after rIFN-γ treatment in 6 of 8 patients and for stimulation with hyphae 4 of 8 and 5 of 8 demonstrated elevated IL-17 and IL-22 production respectively. Patients who received placebo therapy did not display any trend towards increased IL-17 or IL-22 production during the course of treatment (group data shown in figure 3).

**Effect of rIFN-γ on ex-vivo IL-10 production**
In addition to pro-inflammatory cytokines, the capacity to produce anti-inflammatory cytokines can also influence disease outcome. In particular, the anti-inflammatory cytokine IL-10 has been associated with protection against immunopathology during severe infections. IL-10 production in response to stimulation with LPS, PHA and *Candida* was highly variable between patients and did not show a distinct pattern following rIFN-γ treatment (figure 4). No relevant differences compared to the placebo-treated patients were observed.

**Cell populations**

There were no significant changes in the total leukocyte and granulocyte numbers in rIFN-γ-treated patients (figure 5A). Monocyte counts significantly increased one week after initiation of rIFN-γ therapy (figure 5C) and lymphocyte numbers significantly increased at 2 and 7 days after initiation of rIFN-γ therapy (figure 5D), which could be attributed to slight changes in CD4 lymphocytes (figure 5E), B-lymphocyte (figure 5F) and NK-cell numbers (figure 5G) and a significant increase of CD8 lymphocytes (figure 5H). No clear changes in leukocyte (subset) counts were observed in placebo-treated patients.

**HLA-DR expression**

The numbers of HLA-DR-positive monocytes, a marker of immunosuppression, varied substantially at admission between patients (39.05 % [27.5-61.6] vs. 90.6 [88.7-92.5] in IFN-γ-treated patients and placebo-treated patients, respectively). Baseline mHLA-DR levels tended to inversely correlate with severity of underlying illness and tissue involvement (Table 1). In five out of eight IFN-γ treated patients HLA-DR positive monocyte levels below the “immunoparalysis threshold” of 50% (30) were found and in these patients, an increase of HLA-DR-positive monocytes after IFN-γ treatment between 10% and 44% was observed which persisted throughout the study period (Figure 6). Patients with a baseline HLA-DR
expression higher than 50% did not show a change in expression. The patient with a HLA-
DR-expression <50% who did not show increased levels of HLA-DR positive monocyte
numbers at any timepoint, was one of the two patients who died due to infectious
complications. No correlation was found between the level of mHLA-DR expression and
TNFα production of LPS-stimulated PBMCs (Table 1).
Discussion

While several small clinical trials illustrated the beneficial clinical effects of adjuvant treatment with IFNy, the proposed immunostimulating effect of IFN\(\gamma\) as the mechanism of action has not been investigated. In this case series we demonstrate for the first time that adjunctive immunotherapy with rIFN-\(\gamma\) improves the leukocyte immune responses in patients with severe invasive fungal infections. This was primarily reflected by an increase in HLA-DR expression in those patients with a low cellular expression as a measure of their immune suppression, increased ex-vivo responses of pro-inflammatory cytokines of the innate immune system such as IL-1\(\beta\) or TNF\(\alpha\), as well as an increased production of the T-cell cytokines IL-17 and IL-22, which are known to play an important role in the anti-fungal host defense (10, 14, 15, 28, 31, 48).

In addition to enhanced ex-vivo responses, subtle changes in the leukocyte differentiation were observed following IFN\(\gamma\) treatment. Although there were no significant differences in total leukocyte numbers after treatment with rIFN-\(\gamma\), shifts in leukocyte subpopulations such as increased monocyte and lymphocyte counts were apparent. While lymphocyte numbers increased after rIFN\(\gamma\) therapy, it could not directly be attributed to a specific subset as all of them showed increased values. The most significant increase was that of CD8 cells one week after initiation of rIFN-\(\gamma\) therapy. Monocytes and lymphocytes are known to be crucial cells in the host defense against fungal infections. However, the increase of monocytes and lymphocytes during rIFN-\(\gamma\) therapy went at a cost of slightly decreased circulating granulocyte numbers. It is not known whether this reduction is due to activation and migration into the infected tissue, or whether a true decrease in granulocyte generation was induced by the treatment. Although the decrease in granulocyte numbers was slight, the fact that granulocytes, and especially neutrophils, are crucial in the antifungal host defense
warrant that this decrease of granulocytes should be carefully monitored during IFNγ treatment.

Several clinical studies or case reports have previously demonstrated beneficial effects of rIFN-γ in combination with antifungal therapy on outcome of fungal infections (for example in patients with CGD (n=130) (17, 38, 45), HIV (n=173) (3, 22, 40), leukemia (n=5) (11, 39), and transplant patients (n=7) (1), in a patient with S. aureus liver abscess and invasive C. albicans infection (29), in a patient with intracerebral aspergillosis (13), in two patients with progressive chronic pulmonary aspergillosis (23), and in two patients with idiopathic CD4 lymphopenia and cryptococcal meningitis (32)). However, in contrast with our study, ex-vivo immune responses in these patients were not investigated. Due to the limited number of patients and the very heterogeneous population, we could not assess clinical endpoints, although a mean mortality of 25% in the IFN-γ treated patients lies below the mean 40% estimated in patients with invasive fungal infections (24, 37).

To the best of our knowledge, we are the first to describe mHLA-DR expression, a widely used marker of immunosuppression in (bacterial) sepsis patients ((25)), and in patients with invasive fungal infections. In all IFN-γ treated patients that showed baseline mHLA-DR levels below the immunoparalysis threshold of 50% (30) and survived, IFN-γ mediated upregulation of mHLA-DR expression was observed. In agreement with the data presented in this case series, rIFN-γ has been shown to significantly increase numbers of HLA-DR-positive monocytes both in a human preclinical bacterial sepsis model and in septic patients (12, 26).

Reduced production of TNFα by leukocytes ex vivo stimulated with LPS has also been shown to be marker of immunoparalysis in sepsis patients. In contrast to our study, mHLA-DR
expression and *ex-vivo* TNF-α production were found to be highly correlated in bacterial sepsis patients (4, 12). A possible explanation for this discrepancy is that, in contrast with the emerging consensus that immunoparalysis renders patients more vulnerable to opportunistic infections in general (25), different defects in immune defenses may be responsible for enhanced susceptibility towards different pathogens.

Based on the apparent inverse correlation of baseline mHLA-DR levels with severity of underlying illness and tissue involvement (Table 1), mHLA-DR levels seem to reflect disease severity and general immune status, and not specific immune defects per se. Hence, patients with invasive fungal infections and associated impaired anti-fungal immune responses will probably benefit most from immunostimulatory treatment compared to patients with only impaired physical barriers, e.g. due to indwelling catheters and apparent intact anti-fungal immune responses. However, biomarkers reflecting the capacity of specific anti-fungal immune defenses are lacking. Our data suggest that it is important to identify patients who suffer from invasive fungal infections due to impaired cell-mediated immunity, and to attempt a tailored approach to immunotherapy depending on the actual level and type of immunoparalysis of that specific patient.

The intracellular mechanism(s) through which the beneficial effects of IFN-γ are mediated remain to be elucidated. Recently it was proposed that IFN-γ exerts its effects at the transcription level (52), while others have demonstrated that IFN-γ reverses tolerance-associated epigenetic modifications (9). Another possible mechanism involved in the IFN-γ mediated reversal of immunoparalysis is the downregulation of negative TLR regulators such as IRAK-M, a protein that negatively regulates LPS-induced inflammatory responses and contributes to the development of immunoparalysis (56).
Administration of rIFNγ was tolerated well. Several patients developed a mild fever upon administration, which responded well to paracetamol treatment. No other side effects were observed. The most important limitation of the present study data is the limited number of patients studied. Because the control group consisted of only three patients, no statistical analysis between the treatment and control groups could be performed. However, despite the small sample size, the increase in HLA-DR expression in immunoparalyzed patients and the increased *ex-vivo* response of several cytokines that are crucial in antifungal host defense is a promising observation that underlines the potential of immunotherapy. The slow enrollment of patients presenting with candidemia was the main factor contributing to the decision to terminate the phase IIIb *Candida* pilot-study early. With a reported incidence of 2.5-11 per 100.000 persons in Europe (51), and based on previous epidemiological data in our hospital this low enrollment was not expected at the time of the initiation of the study. The much lower incidence of candidemia in the last two years in our hospital is most likely due to a new antibiotic stewardship introduced recently in our hospital, which has reduced the incidence of opportunistic infections. Larger studies are required to confirm the data obtained here. To do so, multicentre studies should be facilitated in order to fully explore the potential of IFNγ immunotherapy.

Our data indicate that adjunctive immunotherapy with rIFN-γ in patients with invasive fungal infections partially restores cell-mediated immunity. This suggests that IFN-γ treatment enhances anti-fungal immunity and represents a promising intervention to improve outcome in patients presenting with systemic fungal infections. Larger trials are warranted to confirm these results and extend them with clinical effects. Biomarkers of impaired anti-fungal
immunity should be described in order to identify patients who will benefit most from immunostimulatory therapy.

Acknowledgements

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Competing interests

None of the authors have any competing interests regarding this study. The support for the immunological assessments in this study was provided by an unrestricted grant from BioMérieux.

Authors’ contributions

CD, PP, AP, BJK and MN conceived and designed the study. CD, JL and CBR screened and included patients. MG and FF carried out in-vitro experiments. CD, MG, JL, FV and MK analysed the data. MG, JL, MK, PP, GM, AP and MN participated in the data interpretation. CD, MG, JL wrote the manuscript draft. MG, JL, MK, PP, BK and MN contributed to writing the final manuscript. All authors read and approved the final manuscript.
Figure legends

Figure 1. Screening, Randomization, and Follow-up of the Study Patients. The principal investigator was immediately notified when *Candida spp.* were cultured in blood. With at least one systemic inflammatory response syndrome (SIRS) symptom present in the 24 hours prior to blood culture withdrawal, and administration of systemic antifungal therapy < 72 hours, patients were deemed eligible for the ‘IFN-γ as an adjunctive treatment for candidemia’ pilot-study. In addition, 5 patients not meeting inclusion criteria but who were also treated with rIFN-γ as a therapy of last resort, were included in analysis.

Figure 2. Effect of rIFN-γ on *ex vivo* IL-1β and TNFα production

PBMCs of patients were isolated at baseline and day 1, 2, 7, 14 and 28 after rIFN-γ administration. Isolated PBMCs were stimulated for 24 hours with LPS, PHA, *C. albicans* blastoconidia, or *C. albicans* hyphae. IL-1β(a) and TNFα(b) concentrations were measured in culture supernatants. Baseline concentrations were used as control and set at 1; subsequent measurements are plotted as the mean relative change ±SEM. Significant change from baseline was determined by subjecting the data to Wilcoxon’s signed rank test. (*=p<0.05; **=p<0.01).

Figure 3. Effect of rIFN-γ on *ex vivo* IL-17 and IL-22 production

PBMCs of patients were isolated at baseline and day 1, 2, 7, 14 and 28 after rIFN-γ administration. Isolated PBMCs were stimulated for 7 days with PHA, *C. albicans* blastoconidia, or *C. albicans* hyphae. IL-17(a) and IL-22(b) concentrations were measured in culture supernatants. Baseline concentrations were used as control and set at 1; subsequent
measurements are plotted as the mean relative change ±SEM. Significant change from baseline was determined by subjecting the data to Wilcoxon’s signed rank test. (*=p<0.05).

**Figure 4. Effect of rIFN-γ on ex-vivo IL-10 production**

PBMCs of patients were isolated at baseline and day 1, 2, 7, 14 and 28 after rIFN-γ administration. Isolated PBMCs were stimulated for 48 hours with LPS, PHA, *C. albicans* blastoconidia, or *C. albicans* hyphae. IL-10 concentrations were measured in culture supernatants. Baseline concentrations were used as control and set at 1; subsequent measurements are plotted as the mean relative change ±SEM.

**Figure 5. Changes in immune cell populations**

Total leukocyte numbers (a) and numbers of granulocytes (b), monocytes (c) and lymphocytes (d) measured in peripheral blood. Numbers of CD4 lymphocytes (e), B-lymphocytes (f), CD8 lymphocytes (g) and NK cells (h) within the lymphocyte population were quantified using flowcytometry.

**Figure 6. mHLA-DR expression**

Panel A. mHLA-DR expression in rIFN-γ treated patients (solid dots) and in patients in the control group (open squares). Panel B. mHLA-DR expression in rIFN-γ treated patients, divided into immunoparalyzed patients with baseline HLA-DR expression below 50 % (solid dots), and without HLA-DR defined immunoparalysis (open dots). Data are expressed as median [IQR].
References


Flow cytometric analysis of mHLA-DR expression and lymphocyte subset counts

To ascertain that expression levels did not change due to a delay between withdrawal and analysis, we performed separate experiments on 5 different blood samples. Expression was determined immediately after withdrawal and after 24 hours storage at 4°C. When samples were immediately stored at 4°C after withdrawal and analyzed within 24 hours, we did not observe significant differences in % or MFI compared with samples that were immediately analyzed after withdrawal. Therefore, analysis was performed within 24 hours after immediate storage at 4°C. After withdrawal, 100 μl blood was incubated with the following fluorochrome-conjugated monoclonal antibodies, for 15 minutes protected from light at 4°C. After erythrocyte lysis (NH₄Cl: 180 mL + 20 mL lysis stock dilution [BD Pharm-Lyse, BectonDickinson]), cells were washed three times in PBS and monocytes and lymphocytes were identified in a 8-color immunophenotyping (NAVIOS flow cytometer, Beckman Coulter, Miami). Monocytes and lymphocytes were identified by forward and side scatter and by cell-specific binding. The following monoclonal antibodies were used for monocyte HLA-DR analysis: HLA-DR-PE (Immu-357), CD14-ECD (RMO52), CD45-KO (J33). Lymphocyte subpopulations were identified by gating on the lymphocyte population in the CD45/SS plot followed by a gating on CD3-APC (UCHT1), CD4-PECy5.5 (13B8.2) , CD8-APCAlexa700 (B9.11), CD19-APCAlexa750 (HD37) and CD56-PECy7 (N901) to determine the helper T cells, cytotoxic T cells, B cells and NK cells within the lymphocyte gate (all MoAbs were obtained from Beckman Coulter, Marseille, France).
Figure 1.

12 patients were assessed for eligibility

7 patients met one or more exclusion criteria, or did not provide informed consent

6 patients were randomized

3 patients were assigned to receive IFN-γ

3 patients were included in the analysis at day 28

3 patients were assigned to receive placebo

3 patients were included in the analysis at day 28

5 patients who did not meet the inclusion criteria were treated with IFN-γ as “therapy of last resort”

5 patients were included in the analysis at day 28

In total, 11 patients were analyzed: 3 study patients and 5 “last resort” patients received IFN-γ
Figure 2

(A) IL-1β
- IFNγ treated
- Placebo

(B) TNFα
- IFNγ treated

C. albicans blastoconidia

C. albicans hyphae

IFNγ treatment
Figure 3
Figure 5

A. Leucocytes

B. Granulocytes

C. Monocytes

D. Lymphocytes

E. CD4 Lymphocytes

F. B-Lymphocytes

G. CD8 Lymphocytes

H. Natural Killer cells
Figure 6

mHLA-DR expression

- ● Immunoparalyzed IFN patients
- ○ Immunocompetent IFN patients

monocyte HLA-DR expression (%)

Time (days)

0 2 1 14 28
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Table 1: Clinical characteristics of all patients with invasive fungal infections. Patient 1-8 were treated with IFNγ, patient 9 and 10 belong to the control group. Yrs, years; F, female; M, male; HIV, human immunodeficiency virus; L-AMB, liposomal amphotericin B; BAL, bronchoalveolar lavage; *, baseline TNFα production in pg/ml in response to LPS.
Additional files provided with this submission:

Additional file 1: ONLINE SUPPLEMENT.doc, 24K
http://www.biomedcentral.com/imedia/1903225325112458/supp1.doc