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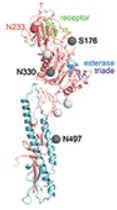
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RESEARCH PAPER



Treatment of Cyclosporin A retains host defense against invasive pulmonary aspergillosis in a non-immunosuppressive murine model by preserving the myeloid cell population

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ABSTRACT

Cyclosporin A (CsA) is widely used as an immunosuppressive agent for organ transplant recipients. CsA inhibits calcineurin, which is highly conserved in mammals and fungi, and thus affects both types of organism. In mammals, the immunosuppressive effect of CsA is via hampering T cell activation. In fungi, the growth inhibitory effect of CsA is via interference with hyphal growth. The aim of this study was to determine whether CsA renders mice susceptible to invasive pulmonary aspergillosis (IPA) and whether it can protect immunosuppressed mice from infection. We therefore examined both the antifungal and the immunosuppressive activity of CsA in immunosuppressed and in immunocompetent mice infected with *Aspergillus fumigatus* to model IPA. We found that daily injections of CsA could not produce an antifungal effect sufficient to rescue immunosuppressed mice from lethal IPA. However, a 100% survival rate was obtained in non-immunosuppressed mice receiving daily CsA, indicating that CsA did not render the mice vulnerable to IPA. The lymphocyte subset was significantly suppressed by CsA, while the myeloid subset was not. Therefore, we speculate that CsA does not impair the host defense against IPA since the myeloid cells are preserved.

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Introduction

Cyclosporin A (CsA) is administered to organ transplant patients to prevent rejection by inhibiting the activation of T-cells through the inhibition of calcineurin.¹ By binding to cyclophilin A, the cyclosporin A-cyclophilin A complex binds to calcineurin, which is required for the dephosphorylation and activation of the nuclear factor of activated T-cells, which increases the transcription of interleukin 2.^{1,2}

Immunosuppression given to organ-transplant recipients also increases patients' susceptibility to opportunistic infections.³ Invasive pulmonary aspergillosis (IPA) is among the major opportunistic invasive fungal infections in these populations, and the mortality rate of organ transplant recipients with invasive aspergillosis is high.^{4–7} The risk factors of post-transplantation invasive fungal infections vary between different studies. For instance, John et al. showed that kidney transplant recipients receiving CsA were associated with higher risk of

systemic mycoses within the first 6 months following kidney transplantation as compared with those receiving prednisolone and azathioprine therapy.⁸ Paya et al. concluded that CsA did not significantly alter the prevalence or severity of fungal infections in solid organ transplantation recipients.⁹ In contrast, Dummer et al. argued that invasive fungal infections developed more as a result of the type of transplantation than of the type of immunosuppression.^{8–10} Collectively, the specific and mechanistic role of CsA treatment as a risk factor for opportunistic fungal infections among organ-transplant recipients, besides increasing the overall net immunosuppressive state, is uncertain.

The drug target of CsA, calcineurin, is conserved in mammals and other eukaryotes.¹¹ In yeasts and molds, calcineurin plays a role in growth and pathogenicity *in vitro*.¹² Although the gene encoding for calcineurin A (*cnaA*) is not essential in *A. fumigatus*, the $\Delta cnaA$ mutant displayed defective hyphal growth.¹³ In humans,

apart from causing T cell dysfunction, calcineurin inhibitors were also recently found to affect the host antimicrobial innate immunity. For example, the antifungal activity of neutrophils from allogeneic haematopoietic stem cell transplant (HSCT) recipients, who received calcineurin inhibitors for the prevention of both graft reject and graft versus host disease, was impaired in one study.¹⁴ The distinct functions of calcineurin in mammals and other eukaryotes provide calcineurin inhibitors with dual antifungal and immunosuppressive activities. Several animal and *ex vivo* studies have been previously conducted to investigate the effect of CsA against invasive fungal infection,^{15–22} which could not produce a consistent conclusion. Moreover, these animal studies generally focus on one of the dual actions (antifungal and immunosuppression) of CsA.

Due to the unclear effect of CsA and conflicting reports on its activity, this study aimed to examine the effect of CsA on the antifungal activity vs. immunity in a murine model of invasive pulmonary aspergillosis. To our knowledge, this study is the first to explore the effect of CsA on immune cellularity, which would certainly be useful in elucidating its role in post-transplantation invasive fungal infections.

Results

In vitro effect of CsA against *A. fumigatus*

CsA inhibited growth and hyphal elongation of *A. fumigatus* (Fig. 1). After 10-h incubation with CsA (6.25 to 400 $\mu\text{g/mL}$), the growth of *A. fumigatus* resting conidia was inhibited by 18% to 31% as compared with the untreated control (Fig. 1A). CsA exerted a profound effect on *A. fumigatus* hyphal elongation by inhibiting 48% to 66% of the mean hyphal length in the same range of concentration (Fig. 1B). Voriconazole, a drug used to

invasive fungal infections, was used as a control. This drug was able to achieve almost complete inhibition of growth and hyphal elongation at a concentration of 0.35 $\mu\text{g/mL}$ (Fig. 1).

In vivo effect of CsA and cyclophosphamide (cyclo) in murine model of invasive pulmonary aspergillosis

The mean weight of all the mice receiving cyclophosphamide, with or without CsA (cyclo group and cyclo/CsA group), showed a similar constant decrease from D-4 until the end of the experimental period (Fig. 2A). The weight drop in the pre-infection period (D-4 to D0) in these mice was due to the effect of cyclophosphamide, which was given to the mice on D-4 and D-1. The weight drop in the post-infection period (from D1 onwards) however was attributed to the infection. In contrast, the mean weight of the mice of the CsA and the control group remained largely constant throughout the experimental period (Fig. 2A). The survival rate of the CsA and the control group mice was 100%, while the survival rates of the cyclo group mice and the cyclo/CsA group mice were both 0% (Fig. 2B). The median survival of cyclo group mice and cyclo/CsA group mice was 4 d for both groups. The similar pattern of weight loss and survival rate between the cyclo group and the cyclo-CsA group indicated that CsA did not rescue the cyclophosphamide-immunosuppressed mice from lethal IPA. On the other hand, the administration of CsA to the non-immunosuppressed mice did not render the animals susceptible to IPA.

The bioluminescence emission acquired from the 4 groups (on D1, D2 and D3) further validated our findings. The images acquired on D3 are shown in Fig. 2C. Low levels of bioluminescence were detected in all mice on D1 (Fig. 2D). On D2, the bioluminescence from cyclo group and cyclo/CsA group mice started to increase and

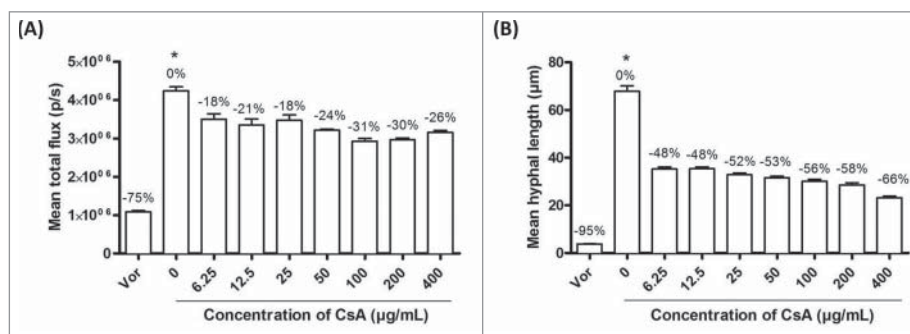


Figure 1. Inhibition of *A. fumigatus* (A) growth and (B) hyphal length by Cyclosporin A (CsA) *in vitro* (6.25 – 400 $\mu\text{g/mL}$). Voriconazole (Vor) (0.35 $\mu\text{g/mL}$) was used as a positive control. The percentages of inhibition compared with the negative control (without CsA) are presented in the graph above the columns. * - The inhibition of CsA and voriconazole were significantly different from that of the negative control across all concentrations tested ($p < 0.05$). No significant differences were found between different concentrations of CsA. It should be noted that even the highest concentration of CsA tested did not result in a complete inhibition of growth in either (A) or (B), suggesting that the antifungal activity of CsA is limited.

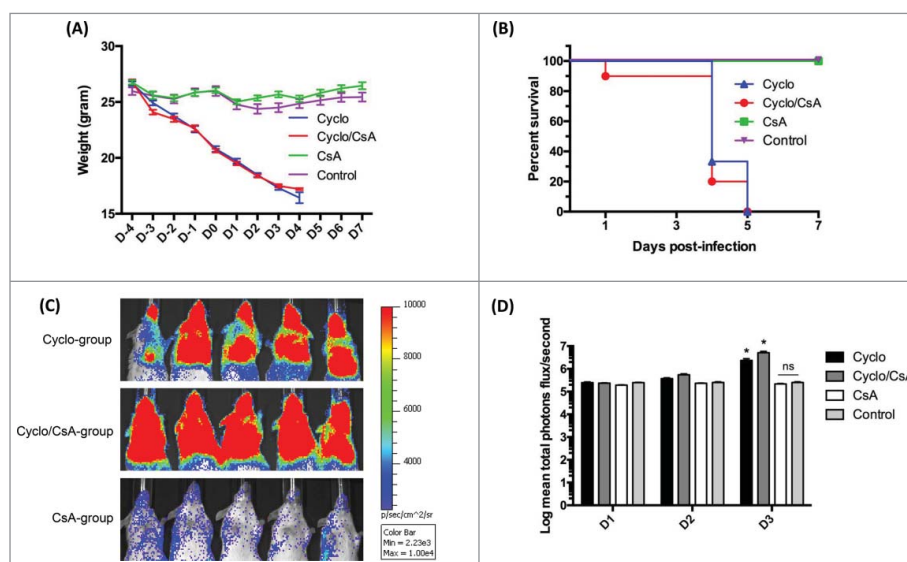


Figure 2. (A) Mean weight of mice. No significant weight loss was observed in the control group and the group receiving only CsA, suggesting an absence of infection. However, the mean weight of all mice receiving cyclophosphamide decreased from D-4 onwards. (B) Survival rate. The median survival for mice in the cyclo group and cyclo/CsA group was 4 d. The survival of the control group and the CsA group mice was 100% and was significantly higher than that of the other 2 groups. (C) Bioluminescence emission from representative mice on D3 after 5-min exposure. (D) Mean total photons flux (expressed in log scale). There was no significant difference in bioluminescence between the groups on D1 and D2. On D3, there was no significant difference in bioluminescence between the control group and the CsA group. The values of both the cyclo group and cyclo/CsA group were significantly higher than those of the CsA group and the control group. In addition, the bioluminescence of the cyclo/CsA group was significantly higher than that of the cyclo group. Each group contained 10 mice. * $p < 0.05$.

peaked on D3 (Fig. 2D). The mean total flux of bioluminescence of the mice from each group was significantly different from each other on D3 ($p < 0.05$) (Fig. 2D). It is noteworthy that the mean total flux of bioluminescence of the mice from cyclo/CsA group was significantly greater than that of the cyclo group. This suggests that CsA worsened the IPA. The level of bioluminescence from CsA and the control group mice remained consistently low from D1 to D3, indicating the absence of infection (Fig. 2D). The bioluminescence acquisition ended on D3, as most of the immunosuppressed mice died on the following day.

Changes in leukocyte counts in peripheral blood of mice treated with immunosuppressants

The cell populations from the peripheral blood samples were analyzed by flow cytometry. The gating strategy is shown in Fig. 3A. The number of leukocytes including myeloid and lymphoid cells from the mice that received cyclophosphamide only decreased significantly on D0 and D1 when compared with D-4 (baseline) (Fig. 3B). The number of leukocytes in the peripheral blood of the mice receiving both cyclophosphamide and CsA also decreased significantly on all days of sampling when compared with D-4 (baseline) indicating that cyclophosphamide suppressed all lymphoid and myeloid subsets. In contrast,

CsA treatment alone did not significantly lower the number of total leukocytes. Since the number of leukocytes was too low, in the cyclophosphamide-treated groups, these 2 groups of mice were excluded from further analysis shown in Fig. 3C, only the blood samples of the CsA-treated mice were analyzed. CsA treatment significantly decreased the number of T- and B-lymphocytes as well as natural killer (NK) cells but not the myeloid population (Fig. 3C). Interestingly, although daily CsA injection induced a decrease in the myeloid population during the pre-infection period (from D-4 to D0), no statistical significance was obtained. In addition, during the post-infection period (from D1 onwards), the myeloid population rose to reach the level of the baseline (D-4).

Discussion

CsA, an antifungal antibiotic extracted from the fungus *Tolypocladium inflatum*, was originally found to have a narrow spectrum of antifungal activity.²³ However, CsA was later discovered to be a potent immunosuppressant, and has since been used to prevent acute rejection in organ transplantation and also graft-vs.-host disease in organ transplantation in allogeneic stem cell transplant recipient.²⁴

The antifungal activity of CsA was originally thought to be limited as no growth inhibition was observed on

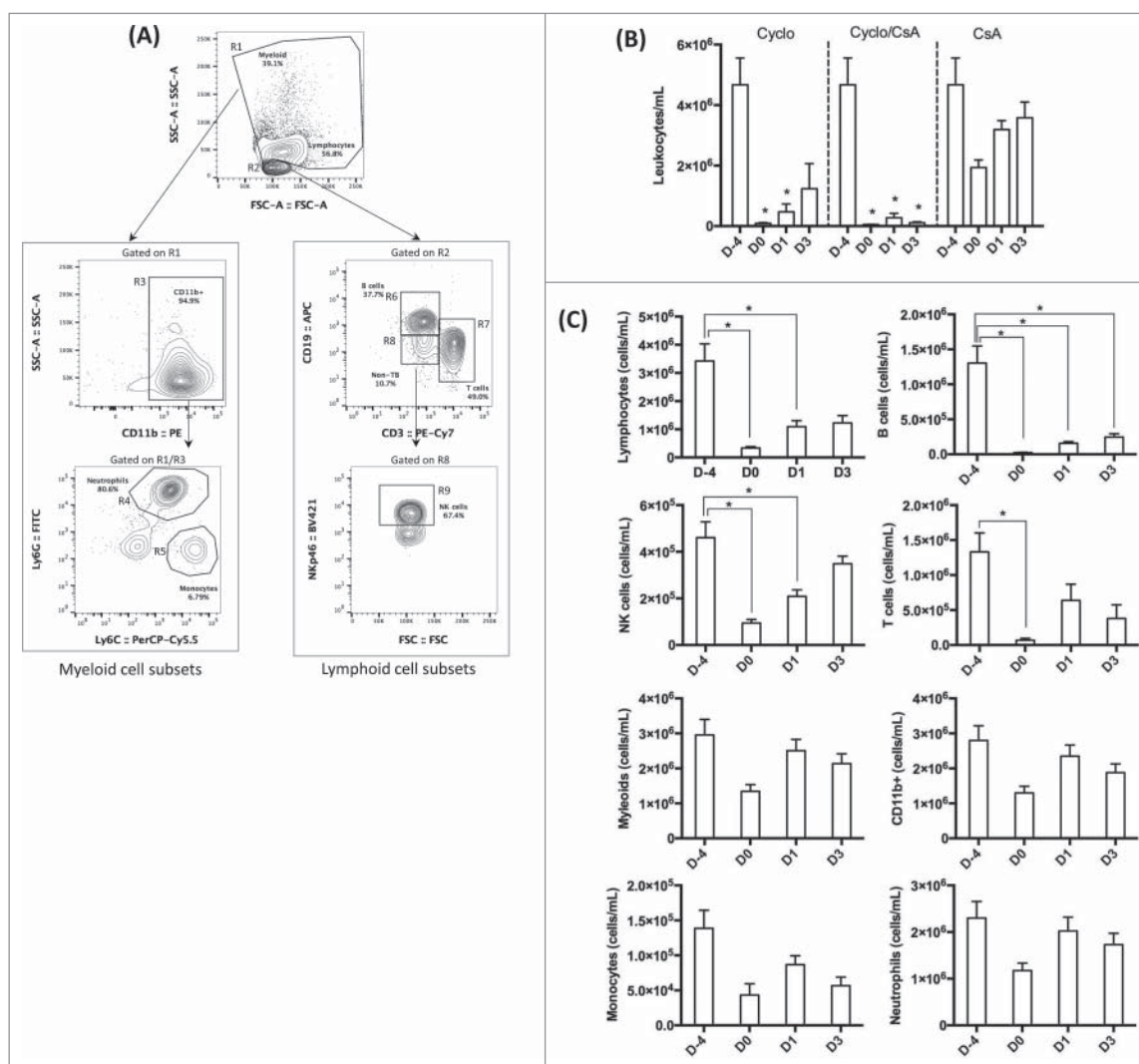


Figure 3. Mean leukocyte count from peripheral blood. (A) Gating strategy of the lymphocyte and myeloid subsets. (B) The average total number of leukocytes per milliliter of peripheral blood from the mice. Cyclo – cyclophosphamide-treated mice; Cyclo/CsA – mice received cyclophosphamide and cyclosporin A; CsA – mice received only cyclosporin A. The bars of D-4 are the same for the 3 groups, as they represented the baseline number of leukocytes of all mice ($n = 30$) before the start of drug administration. (C) Leukocyte population of peripheral blood obtained from mice received CsA only. * - significant ($p < 0.05$).

various fungi, including *Candida albicans* and *Saccharomyces cerevisiae*.²³ However, contrary to this original finding, the fungal calcineurin pathway was found to be involved in growth and pathogenicity in fungi, suggesting the great potential of calcineurin inhibitors, such as CsA and tacrolimus (FK506), as novel antifungal agents.¹² Calcineurin is essential for growth and virulence in a variety of fungi, including *A. fumigatus*,¹³ *C. albicans*²⁵ and *Cryptococcus neoformans*.²⁶

Invasive aspergillosis (IA) is the leading major mold infection among HSCT recipients as well as solid organ-transplant recipients.²⁷ The mortality rate of IA in organ transplant recipients ranges from 63% to 100%.^{6,28} There exists a discrepancy among epidemiological studies regarding the association of CsA and fungal infections. A study by

John et al. concluded that CsA is associated with a 4-fold risk of systemic mycoses within the first 6 months of kidney transplantation as compared with prednisolone and azathioprine therapy.⁸ In contrast, Dummer et al. concluded that fungal infections are only found in the group of patients who received a liver transplant with CsA treatment and argued that fungal infections is associated with the type of transplantation rather than the type of immunosuppression.¹⁰ In addition, it has been found that CsA treatment did not significantly reduce the incidence of invasive fungal infections but rather of bacterial and viral infections among organ transplant recipients compared with other immunosuppressants.²⁹ Such a conflict also exists among various animal studies of the effect of CsA on fungal infections. Some studies have demonstrated that CsA-treated mice

were susceptible to systemic fungal infections,^{15–18} while others have shown otherwise.^{19–22} The association between CsA treatment and fungal infections is therefore uncertain.

Drugs with a dual antifungal and anti-inflammatory activity have recently been proposed as novel therapeutic strategy for fungal infections.³⁰ As a result, we sought to examine the effect of CsA, which has that dual activity, in murine model of invasive pulmonary aspergillosis, the most common form of IA in organ-transplant recipients.^{4,31}

The growth and hyphal elongation of *A. fumigatus* were significantly inhibited *in vitro* by CsA (6.25 to 400 $\mu\text{g/mL}$) (Fig. 1). This is consistent with previous results, which showed that CsA delays and impairs hyphal growth.^{13,32} Voriconazole, which is a fungicidal antifungal agent for *A. fumigatus*, was used as the positive control, at the concentration of 0.35 $\mu\text{g/mL}$. The inhibition of growth and hyphal elongation by voriconazole was much more pronounced than that of CsA. Moreover, there was no significant difference among the values obtained from different concentrations of CsA. No complete inhibition of fungal growth and hyphal elongation was observed with concentrations up to 400 $\mu\text{g/mL}$ of CsA, suggesting that CsA has limited fungistatic activity against *A. fumigatus*.

Since there are no pharmacodynamic or pharmacokinetic studies of CsA in mice, the maximum concentration of CsA *in vivo* could only be calculated by assuming that the bioavailability of CsA via the intraperitoneal route is 100%, and that CsA is evenly distributed within the mice.³³ Under these assumptions, the *in vivo* dose of 100 mg/kg could be equated to the *in vitro* concentration of 100 mg/L or 100 $\mu\text{g/mL}$. Therefore, 100 $\mu\text{g/mL}$ of CsA *in vitro* is comparable to the amount of CsA administered to our murine model. At this concentration, the amount and mean hyphal length of *A. fumigatus* was reduced by 31% and 56%, respectively, when compared with that of the control (Fig. 1). This suggested that the dose of CsA for the mice, besides the immunosuppressive effect, might have an antifungal effect as well.

Our well-established cyclophosphamide-immunosuppressed murine model of IPA was used to investigate the *in vivo* antifungal activity of CsA. Since cyclophosphamide causes neutropenia and reduces the number of lymphocytes in mice,^{34,35} the influence of host immunity could be eliminated. Mice in 2 of the groups were immunosuppressed by 2 injections of cyclophosphamide on D-4 and D-1, followed by an intranasal inoculation on D0, with a lethal dose of 5×10^5 conidia. CsA was given daily intraperitoneally to the mice (test group) from D-4. The mean weight and survival of the control group and test group were similar throughout the experimental period. All mice, from both cyclophosphamide-containing groups, consistently lost weight and died by D4 (Fig. 2A and B). The similarity between the 2

groups suggested that the antifungal activity of CsA, even in this high dosage, was insufficient to rescue the mice. It is unclear if the dose of CsA was instead too high and therefore contributed to a stronger immunosuppressed state. However, the mice did not die at an accelerated rate compared with the cyclophosphamide-only suppressed mice.

Interestingly, the bioluminescence of the mice receiving both cyclophosphamide and CsA (cyclo/CsA group) was significantly higher than that from of the mice receiving only cyclophosphamide on D3 post-infection (Fig. 2C–D). The significantly higher fungal burden suggested that the infection was not alleviated, but on the contrary, was worsened by CsA. CsA did not display any antifungal activity *in vivo* in our study. In addition, it seems likely that the immunosuppressive – antifungal balance was tipped to favor exaggerated immunosuppression, resulting in a higher fungal burden.

Infected mice receiving daily CsA injections and no cyclophosphamide had a 100% survival rate (Fig. 2B), indicating that CsA-treated mice retained their resistance to IPA. A 100% survival rate was also seen among immunocompetent mice (control group) challenged with the same inoculum size of 5×10^5 . Previous studies of the *in vivo* effect of CsA showed contradictory results and failed to suggest a definitive conclusion.^{15–22} The contradiction could have arisen from a difference in protocols (dose of inoculation, dose of CsA, infection route, etc.). Moreover, some studies that found CsA treatment to be a risk factor of fungal infections also used another immunosuppressant in their animal model,¹⁸ whereas in studies that used CsA alone, the CsA did not exacerbate the fungal infections.^{20,22}

On the cellular level, daily administration of CsA significantly suppressed the lymphoid subsets but not the myeloid subsets in the peripheral blood of the mice (Fig. 3C). Both lymphoid and myeloid cells play a role in the immunity against *A. fumigatus* infection, but the myeloid subsets, and especially neutrophils, act as the first line of defense,^{36–38} which explained the resistance against IPA in CsA-treated mice. This is consistent with the previous finding that CsA does not affect the production of neutrophils,³⁹ and that it does not affect the antifungal activity of phagocytes against *A. fumigatus* at relevant therapeutic concentrations.⁴⁰ However, Greenblatt et al. found that CsA-treated neutrophils are unable to kill *C. albicans ex vivo*, although no abnormality was observed in several effector responses, such as phagocytosis.¹⁷ Another *ex vivo* study also showed that the antifungal activity of neutrophils from neutrophil extracellular traps with previous CsA treatment was impaired, which was likely due to the reduced production of neutrophil extracellular traps.¹⁴ Collectively, these findings suggest that CsA moderately impairs the antifungal activity of neutrophils, as shown in

ex vivo studies, but that the effect is not sufficient to render the host susceptible *in vivo*.

Although CsA is not a potent antifungal, it has synergistic effect with echinocandin and azoles *in vitro* against *C. albicans* and *A. fumigatus*.^{32,41} Antifungal prophylaxis and immunosuppressants are commonly administered concomitantly to transplant recipients. Our result, which indicated that CsA does not impair the host immune defense against IPA, further suggested that CsA is an ideal immunosuppressant for populations who are otherwise prone to invasive fungal infections. Future study of the *in vivo* synergism between antifungals and CsA would be of great clinical interest.

Furthermore, it would be of interest to investigate the antifungal activity of CsA using different knockout mice known to be susceptible to *A. fumigatus* infection without immunosuppression. For instance, the gp91/Phox strain (X-CGD mice) and the CXCR2 knockout mice are susceptible to IPA due to defect in phagocyte oxidative killing mechanism and impaired neutrophils recruitment, respectively.^{38,42} In addition, a murine model immunosuppressed by corticosteroids, which impairs the pro-inflammatory response of the phagocytes by inhibiting NF- κ B pathway, could also be used.^{43,44}

In conclusion CsA, despite its *in vitro* antifungal activity, could not rescue the mice from a lethal challenge of IPA under lymphopenia and neutropenia caused by cyclophosphamide. It is possible that the dose of CsA was too high, tipping the balance of action toward additional immunosuppression and therefore limiting the antifungal activity. To clarify this, multiple doses of CsA would have to be used to define the precise balance. On the other hand, this study is the first to discover that, although CsA suppressed the lymphoid subset in mice, the myeloid subset was largely intact for host defense against lethal challenge of IPA.

Materials and methods

A. fumigatus strain

The bioluminescent *A. fumigatus* strain 2/7/1 was used in this study for both *in vitro* and *in vivo* assays. Strain 2/7/1, contains the *Photinus pyralis* luciferase gene *luc_{Opt}*, was generated from the background of wild-type *A. fumigatus* strain CBS144.89.^{45,46}

In vitro effect of CsA against *A. fumigatus*

Each well of a 24-well plate contained 5×10^4 resting conidia of *A. fumigatus* strain 2/7/1 in 500 μ L RPMI containing 10% of fetal bovine serum, 5% penicillin and streptomycin, sodium pyruvate and HEPES (All from Gibco). CsA

(Abcam) of concentrations ranged 6.25 – 400 μ g/mL was added to the wells in triplicate. The control wells contained no CsA. Voriconazole (0.35 μ g/mL) was used as a positive control. The plate was incubated at 37°C for 10 h before bioluminescence acquirement and hyphal length measurement. The fungal growth is represented by the mean total flux detected from the fungi. The mean hyphal length was determined by measuring the length of 100 *A. fumigatus* hyphae in each well using ImageJ. Both assays were repeated for 3 times. The percentage inhibition of growth and hyphal length by CsA was expressed by the ratio of mean growth or hyphal length between the test and the negative control (without CsA). The equation used in this calculation was (test – control)/control $\times$ 100%. The methodology was previously defined as congruent with EUCAST antifungal susceptibility testing.⁴⁷

Mice and ethics statement

Eight-week old male BALB/c mice of approximately 25 g (Janvier, France) were used in this study. All procedures were performed in accordance with Institut Pasteur guidelines in compliance with European guidelines. This study was approved by the ethical committee for animal experimentation CETEA (Comité d'éthique en expérimentation animale, Project license number 2013-0020).

Immunosuppression of mice by cyclophosphamide and cyclosporin A (CsA) treatment

Four groups of mice ($n = 10$) were used in this study: cyclo group (mice receiving cyclophosphamide only), cyclo/CsA group (mice receiving cyclophosphamide and CsA), CsA group (mice receiving CsA only) and control group (immunocompetent mice that received neither cyclophosphamide nor CsA). Cyclophosphamide (Sigma-Aldrich) was dissolved in sterile distilled water and given to the mice on day -4 (D-4) and day -1 (D-1) by intraperitoneal injection (200 mg/kg).⁴⁵ CsA (Abcam) was dissolved in ethanol in a concentration of 500 mg/mL. An intraperitoneal injection containing 2.5 mg of CsA in emulsion of 15% v/v ethanol/castor oil was given to the mice daily from D-4 onwards.

Establishment of invasive pulmonary aspergillosis in murine model

Invasive pulmonary aspergillosis was established in a murine model, as described previously, in each of the 3 groups of mice on D0.³⁸ Bioluminescent *A. fumigatus* strain 2/7/1 was subcultured on 2% malt agar for 8 d at room temperature. Conidial suspension was prepared in 0.1% Tween 20 and PBS and mycelia were filtered with 40- μ m cell strainer (BD Falcon). First, the mice were anaesthetized

with an intramuscular injection of 150 μ L containing 10 mg/mL ketamine and 10 mg/mL xylazine. The mice were inoculated intranasally with 5×10^5 conidia in a volume of 25 μ L. The control group mice were inoculated with 25 μ L PBS. Weight and survival of the mice were monitored daily. The bioluminescence measurement was started one day post-infection and was repeated every day thereafter. Luciferin was injected intraperitoneally before each measurement. Then, mice were anaesthetized by 2.5% isoflurane for 5 min using the XGI-8 gas anesthesia system.⁴⁶ The bioluminescence was recorded after 5-min exposure by the IVIS 100 system (PerkinElmer, Boston, MA). The mice were killed on D7 using CO₂.

Leukocyte counts in murine peripheral blood by flow cytometry

Blood samples were collected from the submandibular vein on D-4, D0, D1 and D3 for leukocytes quantification by flow cytometry (MACSQuant, Miltenyi Biotec). Blood was collected in 500 μ L Eppendorf tubes with 50 μ L EDTA and incubated with 1 mL of red blood cell lysis buffer for 5 min at 4°C. Cells were transferred to a 15 mL tube, topped up to 10 mL in wash buffer (PBS with 0.5% fetal calf serum and 2 μ M EDTA) and centrifuged for 7 min at 1,400 rpm. The supernatant was removed and the pellet was suspended in 100 μ L wash buffer with fluorescent antibodies at the recommended concentrations (NKp46 – BV421 (BB Bioscience), Ly6G – FITC, CD11b – PE, Ly6C – PerCP-Cy5.5, CD3 – PE-Cy7, CD19 – APC (eBioscience)) and transferred to a 5 mL tube for 15 min at 4°C. The cells were washed in PBS and labeled with live/dead dye (eBioscience). Following another centrifugation in wash buffer the cells were resuspended in 200 μ L of wash buffer for acquisition. Fluorescent labeling and cell counts were performed simultaneously using total events for cell counts and live cells for fluorescent label analysis.

Statistical analysis

Statistical significance of the data was analyzed with Prism 6 (GraphPad Software). Two-way ANOVA with Bonferroni's correction was performed for multiple comparison. A statistical significance is achieved if p-value is less than 0.05. Error bars in graphs denote standard error of the mean.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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