

Invasive aspergillosis promotes tumor growth and severity in a tumor-bearing mouse model

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Abstract: Invasive aspergillosis increases in chronic immunosuppressive diseases such as cancer. There is little information about the mechanisms by which *Aspergillus* infection affects the immune regulation and microenvironment of cancer cells. Hence, this study was aimed at investigating the effect of invasive aspergillosis on immunosurveillance, metastasis, and prognosis of cancer in tumor-bearing mice. After implantation of mouse mammary tumor in BALB/c mice, they were infected with *Aspergillus* conidia intravenously. For comparison, groups of mice were experimentally infected with *Aspergillus* conidia or implanted with tumor cells separately. Seven days after *Aspergillus* infection, the serum levels of tissue inhibitor of metalloproteinase-1 (TIMP-1) were measured by ELISA, and subsequently regulatory T lymphocytes were analyzed by flow cytometry. The survival of animals and mean tumor size were then determined. Our results indicated that tumor sizes in mice increased significantly after infection with *Aspergillus* conidia. Moreover, invasive aspergillosis enhanced the population of regulatory lymphocytes and level of TIMP-1. This study supports the idea that massive *Aspergillus* infection could stimulate tumor growth and increases the possibility of a bad prognosis. As a result, treatment of *Aspergillus* infection could be considered an important issue for efficient cancer therapy.

Key words: invasive aspergillosis, tumor progression, TIMP-1, regulatory lymphocytes.

Résumé : Les maladies immunosuppressives chroniques comme le cancer augmentent l'incidence d'aspergillose invasive. Il n'y a que peu d'informations disponibles sur les mécanismes par lesquels l'infection à *Aspergillus* affecte la régulation immunitaire et le microenvironnement des cellules cancéreuses. Ainsi, cette étude visait à examiner l'effet de l'aspergillose invasive sur l'immunosurveillance, la métastase et le pronostic du cancer chez des souris porteuses de tumeurs. Après l'implantation de tumeurs mammaires chez la souris BALB/c, celles-ci ont été infectées par des conidies d'*Aspergillus* de façon intraveineuse. Pour fins de comparaison, des groupes de souris ont été séparément infectés par des conidies d'*Aspergillus* ou implantés avec des cellules tumorales. Sept jours après l'infection à *Aspergillus*, les niveaux sériques de TIMP-1 (*tissue inhibitor of metalloproteinase-1*) ont été mesurés par ELISA et les lymphocytes T régulateurs (Treg) ont été analysés subséquemment par cytométrie de flux. La survie des animaux et la taille moyenne des tumeurs ont ensuite été déterminées. Nos résultats indiquent que la taille des tumeurs des souris augmentait significativement à la suite de l'infection par les conidies d'*Aspergillus*. Parallèlement, l'aspergillose invasive augmentait la population de lymphocytes régulateurs et le niveau de TIMP-1. Cette étude appuie l'idée que l'infection massive par *Aspergillus* puisse stimuler la croissance tumorale et détériorer le pronostic. Conséquemment, le traitement de l'infection à *Aspergillus* devrait être considéré comme un problème important à prendre en compte pour l'efficacité de la thérapie anticancéreuse chez les patients.

Mots-clés : aspergillose invasive, progression tumorale, TIMP-1, lymphocytes régulateurs.

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Introduction

Invasive *Aspergillus fumigatus* infections remain a leading cause of infectious mortality in immunocompromised hosts (Segal and Walsh 2006; Segal 2007). The risk of developing invasive aspergillosis is directly proportional to any deficiencies in immune responses as a result of prolonged neutropenia, therapy with high doses of corticosteroids, cancer, advanced AIDS, allogeneic hematopoietic stem cell transplant, and solid organ transplant recipients (Gustafson et al. 1983; Marr et al. 2002; Bhatti et al. 2006).

Cancer, as a chronic disease, displays multiple immunosuppressive mechanisms to evade T cell responses, either to avoid immune recognition or to disable effector T cells (Zou 2005; Rabinovich et al. 2007). Among several mechanisms that contribute to tumor-related immunosuppression, regulatory T (Treg) cells have been regarded as key elements (Zou 2005; Beyer and Schultze 2006). These cells can inhibit antitumor immune response and encourage tumor development by influencing the activity of effector cell types, including T helper and cytotoxic T lymphocytes (Sakaguchi 2004; Beyer and Schultze 2006; Rabinovich et al. 2007). Recent studies have demonstrated that elevated proportions of Treg cells are present in various types of cancers (Curiel et al. 2004; Taams et al. 2006).

One of the critical steps for tumor invasion and metastasis is destruction of the extracellular matrix, which is mainly catalyzed by the matrix metalloproteinases (MMPs) (Hornbeck and Maquart 2003; Hojilla et al. 2003). It was previously reported that *Aspergillus* conidia can bind to extracellular matrix proteins, such as fibronectin, laminin, and collagen type IV (Tronchin et al. 1993; Bromley and Donaldson 1996; Gil et al. 1996).

Tissue inhibitor of metalloproteinase-1 (TIMP-1) is a glycoprotein that exhibits several biological functions, including stimulation of proliferation in various cell types (Lambert et al. 2004), inhibition of MMP activity (Luparello et al. 1999), and inhibition of apoptosis (Holten-Andersen et al. 2000). Recent studies have confirmed that the plasma level of TIMP-1 increased in various types of cancers such as colorectal, breast, and non-small-cell lung cancer (Ree et al. 1997; Würtz et al. 2005). Based on the findings of these studies, at times the elevated serum level of TIMP-1 has been considered a biomarker for tumor development or invasion and has been related to poor clinical outcome (Wu et al. 2008; Würtz et al. 2008).

Several studies have indicated the role of immune responses in *A. fumigatus* infection (Cenci et al. 1997; Hebart et al. 2002), but few or no reports have addressed the dual effect of *Aspergillus* infection and tumor development. Therefore, this study was aimed at developing an experimental model system for the evaluation of host – fungal pathogen interplay in *Aspergillus*-infected tumor-bearing mice (TBM) and describing the theory of how invasive aspergillosis could impair the immunosurveillance of and subsequently augment tumor aggression.

Materials and methods

Mice and experimental design

A total of 57 8- to 10-week-old female inbred BALB/c mice were obtained from the Pasteur Institute of Iran and

randomly divided into 4 separate groups (A–D) as indicated below, with each group containing 12–15 animals:

- (1) Group A: mice engrafted with tumor and infected with *A. fumigatus* conidia;
- (2) Group B: mice engrafted with tumor and received normal saline;
- (3) Group C: mice infected with *A. fumigatus* intravenously and considered infection control mice; and
- (4) Group D: mice only administered with normal saline and considered normal, healthy mice.

The mice were reared, before and after treatment, under similar environmental conditions, with the same availability of food and drinking water, with separate ventilation systems, and were offered food and drinking water ad libitum at the animal research unit of Tehran University. Before the experiment, rooms and cages were vigorously washed and fumigated using formaldehyde.

Aspergillus fumigatus strain, culture conditions, and infection

A clinical isolate of *A. fumigatus* (strain 36607) was maintained on Sabouraud dextrose agar slants (Difco Laboratories, Detroit, Mich.) supplemented with chloramphenicol for 4 days at room temperature. Conidia were recovered from cultures by washing the surface of the slant culture with washing solution (sterile phosphate-buffered saline with 0.05% Tween 20) and gently scraping. After further washing and centrifugation (3 min at 1200g), conidia were resuspended in a washing solution and filtered through 40 µm nylon filters to remove clumps and hyphal debris. Finally, the absorbance at 620 nm was adjusted to 0.6, and conidia were counted using a hemacytometer.

For experimental infections, mice were injected intravenously via the lateral tail vein with 5×10^6 conidia of *A. fumigatus* conidia in 0.5 mL of sterile saline.

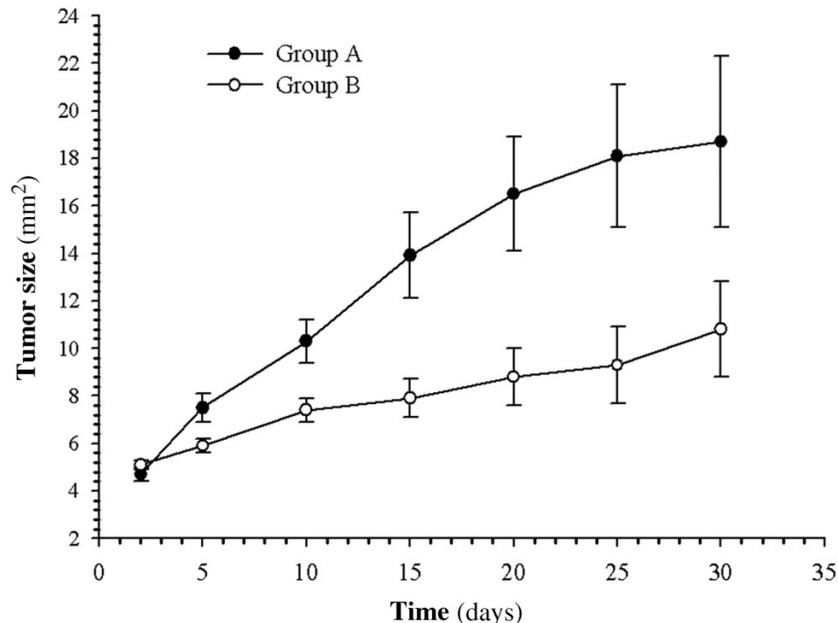
For histological analysis, lungs were excised and embedded in paraffin sections, immersed in 10% buffered formalin, paraffin embedded, and stained with haematoxylin and eosin and Gomori methanamine silver. After injecting normal saline or *A. fumigatus* conidia into the animals, their mortality rate was monitored for 30 days.

Spontaneous mouse mammary tumor (SMMT)

SMMT is an invasive ductal carcinoma. Previous studies have shown that this tumor develops spontaneously in normal BALB/c mice (Hassan et al. 2003). Tumor transplants were surgically removed from a mature breast cancer-bearing BALB/c mouse and cut into pieces of less than 0.5 cm³ with forceps and scalpel. Each piece was transplanted subcutaneously to the syngenic female BALB/c mice. All transplanted animals were daily palpated for tumor formation, and the size of the tumor mass was measured using a digital caliper, starting from the first day until the end of the experiment. Tumor size was calculated by the following formula: $V = 1/2 \times LW^2$, where V represents volume, L is length, and W is width.

One week after the initial surgery, when tumor diameter appeared to be 4–6 mm, the prepared groups of mice were infected with *A. fumigatus* conidia, while the remainder received normal saline. When the tumor's diameter reached

Fig. 1. Effect of invasive aspergillosis on tumor growth. After administration of *Aspergillus fumigatus* conidia, tumor growth of infected and noninfected groups was monitored for 30 days. Invasive aspergillosis enhanced the tumor growth in TBM (group A) compared with that in noninfected TBM animals (group B). Each value is represented by area (in mm²) ± SE (8–9 mice per group). The horizontal axis shows the number of days that elapsed since *Aspergillus* infection.



approximately 12 mm, the mice were prepared for immunological studies. Six to 9 mouse samples were observed for tumor-free survival in each group over a total period of 30 days.

Measurement of TIMP-1 in serum

Serum levels of TIMP-1 were assayed by Quantikine Mouse TIMP-1 Immunoassay (R&D Systems, Wiesbaden-Nordenstadt, Germany), according to the manufacturer's instructions. Blood was collected from orbital veins, clotted at room temperature, and left at 4 °C overnight. Sera were collected by centrifugation at 3000g for 10 min at 4 °C and stored at –20 °C until use. The detection limits of the assays were less than 10 pg/mL.

Flow cytometric analysis of Treg cells

One week after infection, mice were sacrificed and their spleens were removed. Single-cell suspensions were prepared by grinding tissue through a sterile nylon mesh. Erythrocytes were removed by suspending the cells in pH 7.2 lysis buffer (0.15 mol/L NH₄Cl, 1 mol/L KHCO₃, 0.1 mmol/L Na₂EDTA). A total of 10⁶ spleen cells were stained with fluorescein isothiocyanate conjugated anti-CD25 and phycoerythrin-conjugated anti-CD4 monoclonal antibodies (eBioscience, San Diego, Calif.). After staining surface antigens, intracellular FoxP3 staining was performed using phycoerythrin–Cy5 conjugated FoxP3 monoclonal antibodies with a buffer set from eBioscience. For blocking of FcR, cells were incubated with FcR-blocking reagents. Samples were then analyzed by FACScalibur flow cytometer (Becton Dickinson, Mountain View, Calif.).

Statistical analysis

Data were analyzed using SPSS for Windows version 15

(SPSS, Inc. 2006). Comparisons of the data for each group were performed to test for significance ($P < 0.05$) by using one-way analyses of variance when variance between groups was homogeneous and distribution of the data was normal or a nonparametric test (Kruskal–Wallis) when the normality test or homogeneity of variance test failed.

Results

Measurement of the tumor size

When tumor masses in transplant recipients were detected, their sizes were monitored every day and their growth levels were then recorded as the mean tumor diameter in millimetres.

The kinetics of tumor growth in mice bearing tumors are shown in Fig. 1.

Approximately 3–4 days after transplantation, the tumor mass appeared. After experimental aspergillosis infection of TBM, the mean tumor size was greatly increased in the *Aspergillus*-infected TBM group (141.7 ± 36.4 mm²) compared with the noninfected TBM control group (81.3 ± 18.1 mm², $P = 0.032$) (Fig. 1).

Measurement of the survival rate

The survival of mice was recorded as the percentage of mice surviving after tumor induction from day 1 up to day 30 (Fig. 2). Mice that appeared moribund were sacrificed. Thirty days after the SMMT challenge, all healthy control and 60% of noninfected TBM survived. In contrast, only 20% of *Aspergillus*-infected TBM and 40% of *Aspergillus*-infected nontumor mice survived.

Percentage of Treg cells

To measure the percentage of Treg cells, 5 mice from

Fig. 2. Lethality induced by *Aspergillus fumigatus* conidia in tumor-bearing mice (TBM) and control mice. The survival curves of mice 30 days after administration of *A. fumigatus* conidia show a significant decrease in *Aspergillus*-infected tumor and nontumor-bearing animals compared to phosphate-buffered saline treated TBM and normal controls ($P < 0.05$ and $P < 0.01$, respectively). Moreover, TBM have a lower survival rate in comparison with that of the normal control group. Results are from 2 independent experiments, and the Kaplan–Meier method was used for comparing significance levels between each specific treatment protocol. (A) TBM with invasive aspergillosis ($n = 10$); (B) TBM ($n = 9$); (C) invasive aspergillosis ($n = 9$); (D) normal control ($n = 6$).

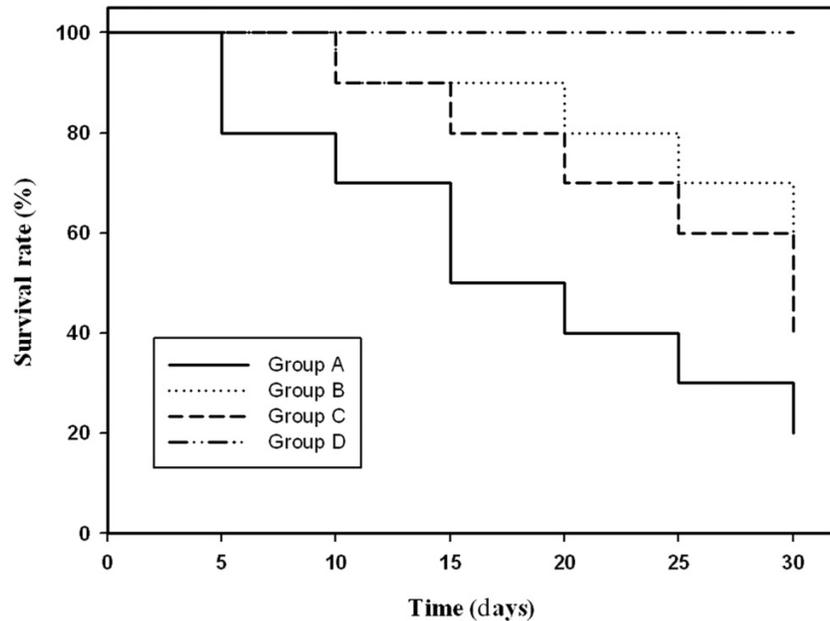
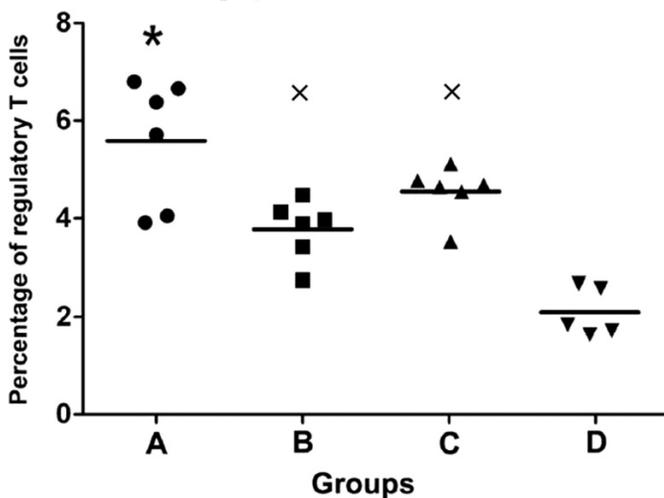


Fig. 3. Invasive aspergillosis increased the percentage of regulatory T cells in tumor-bearing mice (TBM). One week following the administration of *A. fumigatus* conidia, the percentage of CD4⁺, CD25⁺, and FoxP3⁺ cells was analyzed by flow cytometry. Each value is the mean of 5 mice. The asterisks indicate that the normal control group is significantly different from the *Aspergillus*-infected TBM group (*, $P < 0.01$) and the *Aspergillus*-infected or TBM group (×, $P < 0.05$). (A) TBM with invasive aspergillosis; (B) TBM; (C) invasive aspergillosis; (D) normal control.



each group were used, and the percentage of CD4⁺, CD25⁺, and FoxP3⁺ was analyzed by flow cytometry. The results in Fig. 3 indicated that in comparison with that of healthy control animals, the Treg cells significantly increased in TBM that were infected with *A. fumigatus* conidia ($P < 0.01$).

Moreover, the percentage of Treg cells in TBM and *Aspergillus*-infected nontumor animals was higher than that of the control group ($P < 0.05$) (Fig. 3).

Plasma level of TIMP-1

To clarify the effects of the invasive aspergillosis and SMMT on serum levels of TIMP-1, their levels were measured by ELISA on the seventh day after *Aspergillus* infection. For this reason, 5 mice from each group were sampled as representative ones. The results in Fig. 4 show that invasive aspergillosis could predominantly increase the serum level of TIMP-1 in tumor- or nontumor-bearing mice (412.3 ± 198.6 ng/mL and 398.4 ± 241.2 ng/mL, respectively).

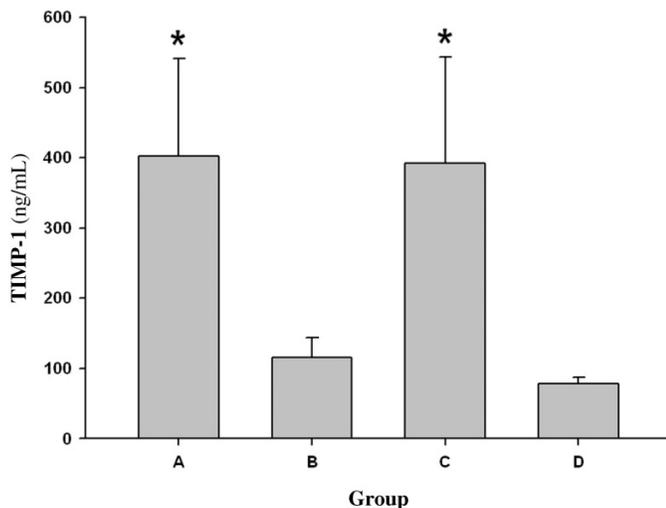
Although the level of TIMP-1 was partially elevated in TBM, the level was not significantly different compared with that of the control group (112.2 ± 42.2 ng/mL, $P = 0.85$).

Discussion

In the present study, we used experimentally disseminated aspergillosis to evaluate the interplay of *Aspergillus* infection and tumor development and prognosis. Our results clearly show that (1) tumor development in *Aspergillus*-infected TBM was predominantly increased; (2) TIMP-1 serum levels increased in both conditions; (3) concerning all above-mentioned findings, invasive aspergillosis generated and increased the Treg cells population in TBM.

Invasive aspergillosis caused by *A. fumigatus* is one of the most considerable problems in immunocompromised patients (such as those with cancer, neutropenia, and AIDS) and those who have received glucocorticoid drugs for a

Fig. 4. Effect of invasive aspergillosis on serum levels of TIMP-1 in normal and tumor-bearing mice (TBM). Seven days after intravenous administration of *Aspergillus* conidia, the mean TIMP-1 serum concentration was predominantly greater in *Aspergillus*-infected groups (A and C) compared with that of noninfected groups (B and D) (*, $P < 0.01$). Values represent means \pm SE from 5 mice. (A) TBM with invasive aspergillosis; (B) TBM; (C) invasive aspergillosis; (D) normal control.



long period (Wald et al. 1997; Balloy et al. 2005; Segal and Walsh 2006).

Several studies have supported the idea that development of invasive aspergillosis is dependent upon host immune responses (Cenci et al. 1997; Hebart et al. 2002). In immunocompromised patients with weakened immune systems, *Aspergillus* could tend to be aggressive toward systemic infection (Cenci et al. 1997; Asciglu et al. 2002).

Some previous reports in experimental models and humans have suggested that tumor growth is stimulated during infection (Hojilla et al. 2003; Hornebeck et al. 2005). In fungal infection, this may be a result of soluble factor production induced by infection.

Moreover, tumor cells can change the microenvironment and cause altered immune responses (Zou 2005; Whiteside 2006). For example, expansion of CD4⁺, CD25⁺, and FoxP3⁺ Treg cells can be induced in cancer patients and suppresses appropriate immune defense (Zou 2005; Beyer and Schultze 2006; Rabinovich et al. 2007). In this study, we showed that infection with *A. fumigatus* can strongly induce regulatory lymphocytes in TBM. This would be a good condition for reinforcement of tumor cells against antitumor defense in infected hosts.

TIMP-1, as a regulatory enzyme of MMPs, has an important role in the remodeling of the microenvironment and thereby can be implicated in cell invasive strategies (Hojilla et al. 2003). Recent reports considered that TIMP-1 was found to be an essential factor for tumor progression and was associated with a poor prognosis in some types of cancer (Hojilla et al. 2003; Wu et al. 2008). Therefore, this molecule has been implicated in cancer pathogenesis or metastasis and its serum or tissue levels have been suggested as a biomarker for prognosis of some types of cancer (reviewed in Würtz et al. 2008). Talvensaaari-Mattila and

Turpeenniemi-Hujanen (2005) have found that TIMP-1 can be involved in the development and metastasis of tumor cells in breast carcinoma. Also, Wu et al. suggested that MMP-9 and TIMP-1 may be evaluated as biomarkers for predicting progression and prognosis of breast cancer (Wu et al. 2008).

Interestingly, we found that TIMP-1 was not significantly up-regulated in the serum of SMMT-bearing mice. This may be related to the difference in strain-specific susceptibilities or tumor variety. Moreover, it may be a consequence of tumor stage and will be investigated in subsequent studies.

It was surprising that when normal or tumor-bearing animals were infected with *A. fumigatus* conidia, the TIMP-1 levels remarkably increased. To our knowledge, this is the first report showing the importance of TIMP-1 in invasive aspergillosis and suggests that the level of TIMP-1 may further be considered a biomarker for predicting progression of invasive aspergillosis. Furthermore, the increased level of TIMP-1 was associated with the decreased survival and increased mean tumor size. This would be correlated with the aggressive form of invasive aspergillosis, which may be responsible for irregularity of immune responses and microenvironment architecture in *Aspergillus*-infected TBM animals. Interestingly, these data support the notion that invasive aspergillosis could change the matrix proteins and regulatory network of the immune system and consequently facilitate invasion of infection and tumor, whereupon the prognosis would be poor and the mortality of these animals would increase. Accordingly, it was highly recommended for fungal infection control in cancer patients. With this strategy, an early diagnosis and proper antifungal management could be a critical issue in these patients for increasing their survival.

In summary, the results of the present study indicate that invasive aspergillosis in TBM may lead to a poor prognosis of cancer. This would result in tumor development, disarrangement, or degradation of matrix proteins and induction of Treg cells. These data suggest that in an immunocompromised host, treatment of invasive aspergillosis may be useful and is a critical step for improvement of disease outcomes.

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