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Alveolar macrophages from patients with tuberculosis exhibit reduced capacity of restricting growth of *Mycobacterium tuberculosis*: a pilot study of vitamin D stimulation *in vitro*

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Abstract

Background: The role of vitamin D supplementation as adjuvant treatment of tuberculosis (TB) has lately attracted increasing interest. Our aim was to investigate the capacity of alveolar macrophages (AMs) from patients with or without exposure to TB to control intracellular growth of virulent *Mycobacterium tuberculosis* (Mtb).

Methods: AMs were freshly harvested from the bronchoalveolar lavage fluid of 7 patients with a history of TB (4 patients with previous TB and 3 patients with current TB) and 4 non-TB subjects. The H37Rv strain, genetically modified to express *Vibrio harveyi* luciferase, was used to determine the growth of Mtb by luminometry in the AMs from study subjects. Cytokine levels in culture supernatants were determined using a flow cytometry-based bead array technique.

Results: AMs from patients with a TB history were less efficient in restricting Mtb growth. Stimulation with 100 nM 1, 25-dihydroxyvitamin D (1,25D₃) did not significantly influence the capacity of AMs from any study subjects to control the infection. Out of the cytokines evaluated (TNF- α , IL-1 β , IL-10 and IL-12p40) only TNF- α demonstrated detectable levels in culture supernatants, but did not respond to stimulation with 1,25D₃.

Conclusions: We conclude that AMs of TB-patients show reduced ability to control mycobacterial growth *in vitro*, and, that AMs in this pilot study do not respond to 1, 25D₃-stimulation. The former observation supports the concept that innate immunity is crucial for the control of TB infection.

Keywords: Alveolar macrophages, bronchoalveolar lavage, cytokines, H37Rv, tuberculosis

Introduction

Infection with *Mycobacterium tuberculosis* (Mtb), the causative agent of tuberculosis (TB) has several outcomes in the host [1,2]. During the last decades, it has become increasingly clear that some individuals have a highly effective innate immune response against Mtb, which will kill the bacteria upon exposure even before it disseminates and induces cell-mediated immunity [1,3]. While the vast majority among those who develop infection exhibits latent disease, small children or immune-compromised individuals may develop progressive infection [2]. Vitamin D is known to induce anti-mycobacterial effects through an up-regulation of the anti-microbial peptide cathelicidin in experimental models including mononuclear cells from healthy donors [4-7]. However, the relative importance of vitamin D and the mechanisms at the site of infection has not been confirmed in clinical trials [8], other than in subgroups

of patients with polymorphisms in the vitamin D receptor [9].

Much of the knowledge of the innate and adaptive immune mechanisms that are crucial for the control of TB is derived from animal models or from *in vitro* models based on cell lines or monocyte-derived macrophages obtained from the blood of healthy volunteers [1,2]. Although it is known from epidemiological studies that the innate response to Mtb may clear the infection in some individuals, there are few, if any, studies investigating this effect at the cellular level of human alveolar macrophages (AMs) freshly harvested from bronchoalveolar lavage fluid (BALF) from TB patients.

In the present study we investigated the capacity of AMs, obtained from patients with previous or current TB, to inhibit growth of virulent Mtb using a newly developed luminescence-based model for detection of intracellular Mtb growth. In addition, the study evaluated the effects of exogenous

Table 1. Characteristics of the population studied. Data are presented as means \pm SD. BALF: bronchoalveolar lavage fluid. Significant difference from “non-TB” subjects is indicated; * $p < 0.05$ (Students t-test, unpaired analysis).

| | Non-TB Subjects | Previous TB Infection | Current TB Infection |
|---|--------------------------|--|----------------------------|
| Subjects (n) | 4 | 4 | 3 |
| Mean age (yr) | 52 \pm 16 | 51 \pm 18 | 40 \pm 7 |
| Gender (females; n) | 2 | 2 | 3 |
| Ethnicity (n) | Caucasians 3 Asians 1 | Caucasians 2 Africans 2 | Caucasians 2 Africans 1 |
| Current smokers (n) | 0 | 0 | 1 |
| Scar after BCG (n) | 3 | 2 | 0 |
| IGRA (% positive) | 0 | 75 | 100 |
| TB skin test (mm) | 8 \pm 5 | 16 \pm 5 | 17 \pm 3* |
| BMI (kg/m ²) | 26 \pm 3 | 28 \pm 3 | 22 \pm 4 |
| Serum-albumin (g/L) | 43 \pm 4 | 39 \pm 3 | 40 \pm 6 |
| Serum 25(OH)D (nmol/L) | 43.0 \pm 33.4 | 26.4 \pm 30.0 | 17.4 \pm 15.6 |
| Radiologic findings | Normal 4 | Possibly inactive 3 Possibly active 1 | Possibly active 3 |
| Subjects with | | | |
| Cough (n) | 4 | 2 | 1 |
| Haemoptysis (n) | 0 | 0 | 1 |
| Night sweating (n) | 0 | 1 | 0 |
| Heart rate > 100 (n) | 0 | 0 | 1 |
| Fever (> 38 °C, n) | 0 | 0 | 1 |
| Anemia, Hb < 120 (n) | 0 | 2 | 0 |
| BALF cell count (10 ⁶ cells/L) | 82 \pm 10 | 128 \pm 62 | 505 \pm 235 |
| BALF-macrophages (%) | 93 \pm 5 | 90 \pm 2 | 86 \pm 2 |

active vitamin D (hereafter referred to as 1,25D₃) on anti-mycobacterial properties of AMs, *i.e.* inhibition of intracellular Mtb growth and the production of immunoregulatory cytokines involved in TB immunity. Our hypothesis was that AMs of healthy humans would restrict Mtb growth *in vitro* better than AMs of individuals with a history of TB (previous or current), and that 1,25D₃ stimulation *in vitro* would reinforce the host control of TB exercised by the infected AMs.

Patients and methods

Study population

Patients, planned for diagnostic bronchoscopy on the suspicion of active TB at the Department of Pulmonary Medicine, Linköping University Hospital, were consecutively included in the study during a 2-yrs-period after informed and written consent. Only patients that were not diagnosed by positive sputum smear for acid fast rods were included. Non-TB subjects were patients investigated by bronchoscopy because of a history of persistent cough. The purpose of bronchoscopy was in the case of the non-TB patient inspection mainly, since there was no suspicion of serious lung disease. The study protocol was approved by the Ethical Committee of Linköping University, Sweden. Patients were classified by a senior pulmonary physician and a senior thoracic radiologist as “non-TB”, “previous TB” or “current TB” according to published guidelines [10]. The classification was performed without knowledge of the experimental results. Altogether, 24 patients were included; 8 healthy non-TB subjects (C1-8), 8

patients with previous TB (PTB1-8) and 8 patients with active TB (TB1-8). “Non-TB” were patients with negative TB-history/PPD/IGRA-test, normal chest X-ray and/or high resolution computer tomography (HRCT), and bronchoscopy with negative mycobacterial cultures (4 patients were eligible; C2, C3, C6, C8). “Previous TB” were patients with a medical history of culture-positive TB and radiological findings on chest X-ray and/or HRCT consistent with previous TB, supported by a positive TST and/or IGRA-test but negative cytology and negative mycobacterial cultures/PCR by bronchoscopy (4 patients were eligible; PTB1, PTB2, PTB4, PTB6). “Current TB” were patients that after bronchoscopy were treated for TB because of a positive mycobacterial culture and/or clinical and radiological findings suggestive of active TB (3 patients were eligible; TB2, TB3, TB8). The main reason behind non-eligibility was problems associated with cell culturing, such as inadequate number of cells and unexpected cell death, which caused a lack of laboratory data in these cases. None of the patients investigated was treated with TB drugs at the time when blood samples and BALF were collected. All TB-patients included were HIV-negative, while the healthy non-TB-subjects were not tested for HIV. The patient characteristics are presented in (Table 1).

Preparation of alveolar macrophages

The BALF was taken care of immediately and centrifuged. The cells were counted, re-suspended in Dulbecco’s modified Eagle’s medium (DMEM; Gibco) supplemented with 10 % normal human serum, 100 U/ml penicillin, 100 µg/ml streptomycin (complete DMEM), and seeded in 96-well plates (Sarstedt; 100 000 cells/well). Attached AMs were cultured for 5-7 days and the medium was changed to antibiotic-free DMEM at least one day prior to infection.

Bacteria

For details about the properties of the bacteria used see previous description [11,12]. Briefly, the virulent Mtb strain H37Rv (American Type Culture Collection) harbouring a pSMT1-plasmid encoding *Vibrio harveyi* luciferase were grown in Middlebrook 7H9 broth supplemented with Tween 80 and oleic acid-albumin-dextrose-catalase (OADC) (BD) for 2 to 3 weeks at 37°C with 100 µg/ml hygromycin for selection before being re-inoculated in fresh broth and incubated for 7 days to reach early log phase. Before infection, the bacterial suspension was washed and re-suspended in DMEM and a single-bacillus suspension was obtained by passage through a sterile syringe equipped with a 27-gauge needle. The concentration was determined by optical density at 600 nm (OD₆₀₀) as a function of colony-forming units (CFU)/ml.

Infection procedure

AMs were infected by a pulse-chase approach, where bacteria were added to the AMs in serum-free medium at a multiplicity of infection (MOI) of 10 and incubated at 37°C

for 1 h. The infection was followed by a change of medium to serum-containing antibiotic-free DMEM with 100 nM 1,25D₃ (Sigma-Aldrich). Control wells were given medium without added 1,25D₃.

Measurement of bacterial growth

The number of viable bacteria was acquired through measurement of flash-luminescence with a GloMax® Multi-Detection System (Promega) as previously described [13]. This method, based on flash-luminescence emitted from the luciferase-expressing bacteria, when given the luciferase substrate *n*-decanal, correlates well to CFUs, and display less variation than classic CFU assays. The phagocytic capacity of AMs [viable bacteria correspond to the flash-luminescence expressed as arbitrary luminescence units, (ALU)] was assessed at 1 h after infection. The capacity of AMs to control Mtb growth was assessed at 2 days of infection (expressed as fold change day 2 compared to day 0).

Measurement of cytokine release

The concentrations of cytokines (TNF- α , IL-1 β , IL-10 and IL-12p40), produced by Mtb-infected AMs, were quantified by a BD™ Cytometric Bead Array Flex Set (BD) according to the manufacturer's instructions. Thus, culture supernatants from infected AMs were collected at 24 hrs post-infection, aliquoted and kept at -70°C. For analysis, samples were centrifuged at 10 000 x *g* for 10 min prior to dilution and mixing with cytokine-specific beads. The supernatant-bead suspensions were incubated for 1 h at room temperature, followed by 2 h of incubation in the presence of cytokine-specific PE-conjugated detection antibodies. Samples were sterilized by adding 4% paraformaldehyde (PFA) for 1 h before the final wash step. Flow cytometry (Gallios Flow Cytometer, Beckman Coulter) was used to quantitate each cytokine. Thus, each bead population was separated by a unique ratio of APC and APC-Cy7 and the fluorescence intensity of PE corresponded to the concentration of the specific cytokine, derived from a standard curve of known cytokine concentrations.

Quantiferon assay

Blood samples were collected in tubes provided by the manufacturer for the QuantiFERON®-TB Gold In-Tube assay (Cellestis, Australia). Samples were shaken and incubated at 37°C overnight and then analyzed by ELISA for IFN- γ (IU/ml) according to the manufacturer's instructions.

Measurement of 25-hydroxyvitamin D in serum

Using ELISA (Immundiagnostik, Germany) according to the manufacturer's instructions, we measured the major circulating metabolite of vitamin D, 25-hydroxyvitamin D (serum-25(OH)D).

Statistics

To evaluate the effects of 1,25D₃ stimulation within each

group and comparison of the total non-stimulated with stimulated samples, a paired Student's t-test was used. The means between different study groups were compared using ANOVA with Bonferroni's post-hoc test. Differences between groups are shown as * ($p < 0.05$) or ** ($p < 0.01$) or *** ($p < 0.001$).

Results

All eligible patients were thoroughly characterized and as shown in (Table 1), clinical symptoms, radiological findings as well as laboratory results rendered the classification into "non-TB", "previous TB" and "current TB". Patients with "current TB" tended to be younger, slimmer (lower BMI) and deficient of vitamin D (assessed with serum-25(OH)D). Their BALF contained more inflammatory cells, but compared to the other groups there were no statistical differences which was most likely because of few subjects. In one case of "previous TB", radiologic findings suggested "active TB", but bronchoscopy including mycobacterial culture and clinic-radiologic follow-up ruled out this possibility.

AMs isolated from patients with previous TB differed from those harvested from "non-TB" and "current TB" patients, when challenged with virulent Mtb. AMs isolated from "previous TB" patients exhibited a tendency towards a reduced phagocytic capacity of Mtb (Figure 1A,1C) and, importantly, significantly reduced efficiency to restrict Mtb growth ($p < 0.05$ vs. non-TB; (Figure 1B,1D). AMs harvested from patients with "previous" or "current TB" were clearly inferior to "non-TB" AMs to control Mtb infection *in vitro* (Figure 1B,1D).

To further investigate factors that may explain the variable capacity to control intracellular growth in AMs, we focused on vitamin D stimulation *in vitro* and production of cytokines linked to innate immunity. No significant effects of 1,25D₃ stimulation of AMs on Mtb growth were observed (Figure 1E). Most cytokines were not detectable in the supernatants. Indeed, measurable levels were observed only for TNF- α , which remained at the same level when Mtb-infected AMs were stimulated with 1,25D₃ (data not shown).

Discussion

Experimental data on mycobacterial killing in freshly harvested BALF macrophages from patients with active or previous exposure to TB is scarce. In that perspective, we believe the results of our study is of importance, although it is small pilot study. As BALF is routinely collected in many centers, BALF studies may, indeed, become an excellent way to describe cellular effects of host defense to Mtb. Therefore, and despite the weaknesses of the present studies, we believe that the observation that AMs isolated from "previous TB" patients are less able to restrict Mtb growth, when challenged with virulent Mtb, have opened a window towards a better understanding how the immune system operates to restrict Mtb growth. This observation indicates that the status (genotypic or phenotypic) of AMs could influence the ability of Mtb to establish infection, which supports the epidemiological observation that not

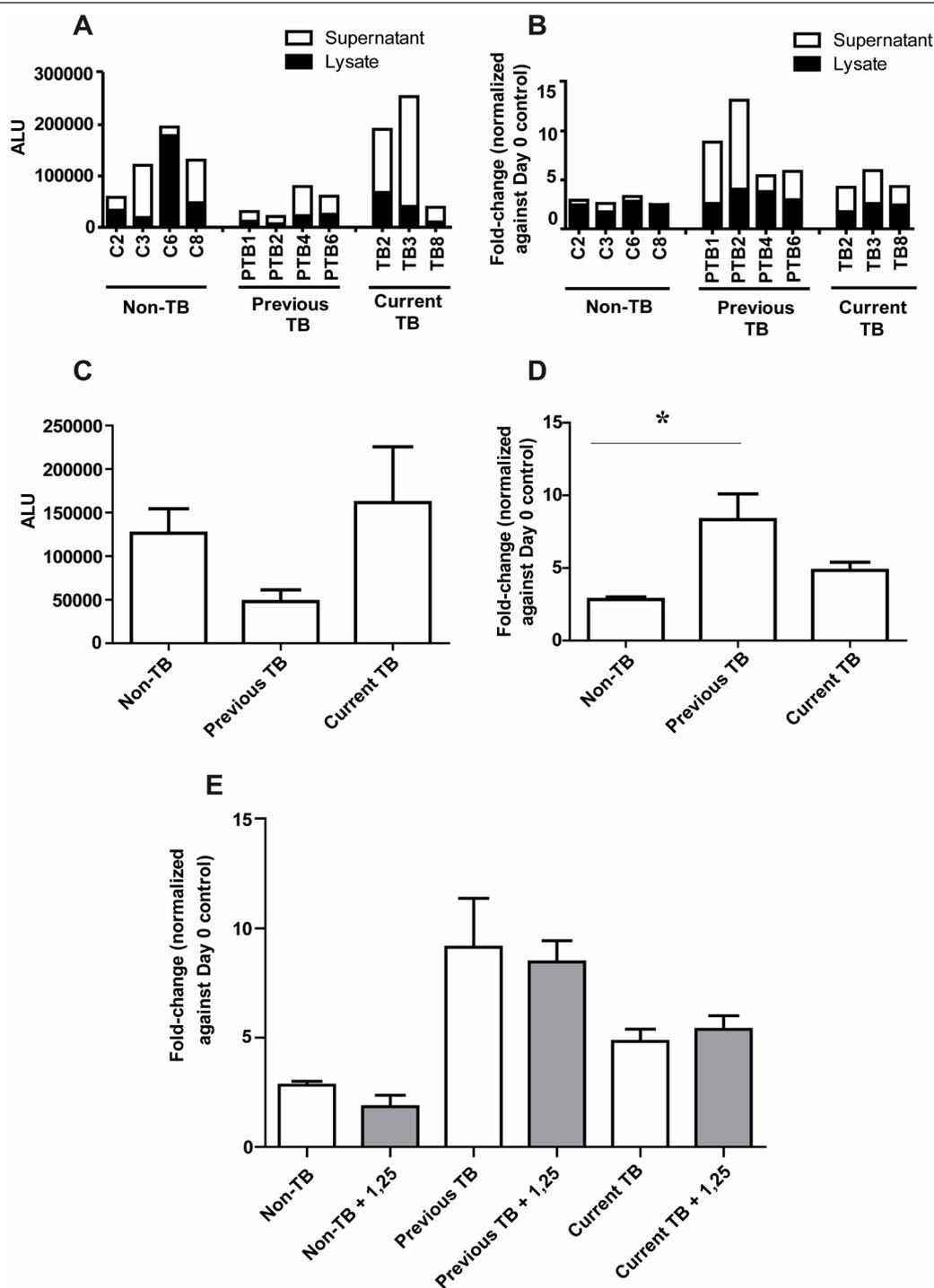


Figure 1. Phagocytosis and growth of H37Rv in cultures of BAL-macrophages.

Alveolar macrophages from the three groups of patients were infected with H37Rv at MOI 10 as described. Luminescence (ALU) was measured 1 h after pulse-chase (D0 value) as a measure of phagocytosis. After another 2 days of incubation, luminescence was measured again and growth was evaluated (D2 value). (A) The figure shows the ALU values from individual patients in each group with the proportion of lysate (*i.e.*, bacteria inside AMs)- and supernatant (extracellular bacteria)-associated bacteria 1 h after pulse-chase. (B) The figure shows the relative growth of H37Rv (D2/D0) in the cells of individual patients, with the proportion of relative growth in the supernatant (extracellular) and lysate (intracellular). (C) The mean ALU (intracellular + extracellular) value of each of the groups 1 h after pulse-chase (phagocytosis). (D) The mean relative growth (intracellular + extracellular) of H37Rv at 2 days post-infection from each group is shown. (E) The relative growth (intracellular + extracellular) of H37Rv (D2/D0) in the three study groups, in the absence or presence of 1,25-dihydroxyvitamin D3. Non-TB (n=4), previous TB (n=4) and current TB (n=3). Bar graphs show the mean and SEM. The means were compared using ANOVA with Bonferroni's post-hoc test. Differences in growth with or without 1,25-dihydroxyvitamin D3 stimulation within each group was tested by a paired Student's t-test.

more than 25-50% of close household contacts exposed to active pulmonary TB will acquire Mtb infection measured as a positive TST [3,14]. Moreover, our finding is well in line with a previous study showing that AMs from household contacts of patients with active TB are less capable of controlling Mtb infection compared to community controls [15]. *In vivo*, it should also be considered that other inflammatory cells may interact with AMs in the human lung during the innate immune response to hamper the growth of Mtb.

TB is a rare infection in Sweden. Most lung TB patients present with productive cough, and TB diagnosis is therefore often easily made by diagnostic tests for TB performed on sputum. Consequently, there is usually no medical reason to perform bronchoscopy. Hence, all TB-patients in the present study had negative TB-diagnostics of sputum, and most of them few symptoms of TB (Table 1). The main reason for bronchoscopy was radiologic findings indicative of TB infection, and the procedure was therefore performed either to rule out (patients later categorized as "previous TB") or to confirm a current infection (patients later categorized as "current TB"). Studies on BALF-cells are also associated with problems related to cell culturing, of which inadequate number of cells and unexpected cell death are the main issues to handle. Indeed, study patients underwent bronchoscopy to collect BALF for TB-diagnostics, and, subsequently, the portion of BALF decided for research sometimes was too small. Despite these major issues, altogether 11 of 24 cases included during a 2-yrs-period were considered eligible.

A limitation of the present study is the few study subjects in each group resulting in a potentially heterogeneous study population. Thus, a mismatch regarding important variables of the individuals included (sex, age, ethnicity, BMI, smoking habits and the level of vitamin D in blood) may have some influence on the results of the present study. Notably, there was a trend for lower levels of serum-25(OH)D in patients with active TB, but there were too few subjects to make any firm conclusions. Since UVB-light is the main source to active vitamin D, the levels of serum-25(OH)D could show significant seasonal variations in countries, which like Sweden is situated close to the Poles. Although study subjects were included at all four seasons, clearly, all TB-subjects were deficient of vitamin D (defined as serum-25(OH)D \leq 35 nmol/L) [16].

The presence of vitamin D had no influence on Mtb growth in AMs, which is the opposite compared to results from 1,25D₃-stimulated human monocyte-derived macrophages (hMDM) which has been reported by us [17] and others [4,5]. However, it should be noted that monocyte-derived macrophages may be phenotypically different from AMs and are also differentiated *in vitro*. Thus, a different response to vitamin D between these two cell types is reasonable since other important differences such as that production of iNOS is present in AMs but not detectable in hMDMs have been reported [18]. Recently, we reported that 1,25D₃-stimulation of hMDM, collected from the same individuals as in the present

study, results in a hampered Mtb growth in infected cells as well as an increased production of pro-inflammatory cytokines [17]. The lack of cytokine production following vitamin D stimulation observed in the present study might therefore suggest that the reason behind AMs not responding to 1,25D₃ would be on the phenotypic level. Alternatively, the lack of 1,25D₃ influence might be explained by a rather short period for intra-cellular mycobacterial growth (2 days post-infection). This time-point for evaluation was based on pilot studies by our group showing that beyond 2 days of post-infection, less than 10% of monocyte-derived human macrophages are viable, when infected at an MOI of 10 [19]. In future studies, it might be of interest to extend the incubation time to better evaluate differences between 1,25D₃ stimulated and non-stimulated cells in respect to bacterial growth.

Conclusions

In conclusion, the present study indicates that AMs retrieved from patients with a history of TB have a significantly reduced ability to control Mtb infection *in vitro* compared to AMs harvested from "non-TB" patients. This finding supports the concept that innate immunity is crucial for the control of infection. Future studies to verify this observation and evaluate the influence of vitamin D on human AMs are warranted.

List of abbreviations

AMs: alveolar macrophages
ALU: arbitrary luminescence units
BALF: bronchoalveolar lavage fluid
CFU: colony-forming units
1,25D₃, 1,25-dihydroxyvitamin D
DMEM: Dulbecco's modified Eagle's medium
HRCT: high resolution computer tomography
hMDM: human monocyte-derived macrophages
Mtb: *Mycobacterium tuberculosis*
MOI: multiplicity of infection
OADC: oleic acid-albumin-dextrose-catalase
OD: optical density
PFA: paraformaldehyde
TB: tuberculosis

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

All listed authors meet the criteria for authorship outlined by the International Committee for Medical Journal Editors.

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