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Full Length Research Paper

Progression of disease in a more clinically relevant mouse model of respiratory melioidosis

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In acute and lethal infections, antibiotics reduce mortality and therefore extend the duration of the immune response. The progression of the immune response to acute, *Burkholderia pseudomallei* K96243 infection, via the aerosol route, in BALB/c mice treated with doxycycline (7 days, daily, orally) was explored. The therapy extended survival and limited bacterial growth in the lungs. After cessation of antibiotic therapy, bacterial numbers resurged. The numbers of phagocytes continued to increase with secondary bacterial growth, while inflammatory cytokine concentrations declined. We propose that this model may be more pertinent for the study of human melioidosis.

Key words: *Burkholderia pseudomallei*, respiratory, pulmonary, innate immunity, cytokines, bacteria, leukocytes, neutrophils, macrophages, monocytes, antibiotics, doxycycline.

INTRODUCTION

The Gram-negative pathogenic bacterium *Burkholderia pseudomallei* is the causative agent of melioidosis. *B. pseudomallei* is endemic to regions of Thailand, Indonesia and Australia, where it can cause a variety of infections (Cheng et al., 2005; Wiersinga et al., 2006). *B. pseudomallei* is generally regarded as suitable for use as a biological weapon for many reasons including its infectious nature as well as a lack of effective therapeutic treatments (Ip et al., 1995). In addition, presently there are no vaccines or truly effective treatment regimes for melioidosis (Cheng and Currie, 2005; Wiersinga et al., 2006).

The mouse model is commonly used to understand melioidosis. Different mouse strains demonstrate varying susceptibilities to strains of *B. pseudomallei* inoculated by various routes (Gan, 2005) and infection is universally more lethal when given via the respiratory route (Tan et al., 2008; Liu et al., 2002). Furthermore, infection by any route is generally more acute in the BALB/c mouse than in the C57BL/6 mouse (Tan et al., 2008). Using these models, a greater understanding of the immune response towards infection with *B. pseudomallei* has been developed. Interferon-γ (IFN-γ) production is vital in controlling the early stages of infection since antibody ablation and gene deletion studies have rendered C57BL/6 mice substantially more susceptible to infection than control antibody-treated or wild type mice (Santanirand et al., 1999; Haque et al., 2006). The IFN-γ is thought to be produced by NK and $CD8^+$ T-cells (Lertmemongkolchai et al., 2001) although these cells are functionally redundant in their production of IFN-γ, in that both cell types must be blocked before the effect of interferon-γ ablation can be seen on pathogenesis. It is also known that macrophages incubated with IFN-γ are able to kill *B. pseudomallei* (Miyagi et al., 1997). Additionally, neutrophils are believed to play a key role in host defense against *B. pseudomallei* infection since antibody ablation causes mice to rapidly succumb to infection (Easton et al., 2007).

Archetypical of *B. pseudomallei* research, where mice have been used, the effect of the bacteria has been tested in isolation. This is true of the research described earlier. In humans, disease will not usually be left untreated. Fundamentally, this research has made the assumption that antibiotic intervention will not impact on the factors investigated. Here we take a step in the

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direction of addressing this potential confounding issue. The main form of intervention is antibiotic therapy. This fact has not escaped researchers in the area and one group have published work where recombinant IFN-γ has been shown to be effective against "*B. pseudomallei*" infection, in mice, only when used in parallel with ceftazidime (Propst et al., 2010). We describe in detail the progression of respiratory melioidosis when treated sub-optimally with the antibiotic doxycycline.

METHODOLOGY

Animal infection

B. pseudomallei strain K96243 was cultured in Luria Bertani broth at 37°C, overnight on a rotary shaker. The challenge dose was estimated by reading optical density against a calibration curve. Six-eight week old BALB/c mice (Charles River) were housed within an ACDP containment level-3 animal facility and were held within a rigid-walled isolator, where they were given unlimited access to food and water. Mice were challenged by aerosol as previously described (Laws et al., 2011). Following challenge mice either received 7 daily, oral doses of 1 mg doxycycline in 25 µl (50 mg/kg (assuming a 20 g mouse), doxycycline hyclate, sigma) or a PBS sham, starting 6 h after infection. One group of mice was used for survival studies and additional mice were culled at 2, 7, 14 and 21 days post-challenge. Blood was collected by cardiac puncture following terminal anaesthesia and lungs, spleen and liver were removed. All procedures and housing were in accordance with the Animal (Scientific Procedures) Act (1986).

Sample analysis

Samples were taken and analyzed as previously described (Laws et al., 2011). Organs were processed at less than 1 h post-mortem. Blood was diluted 1:10 in PBS. All organs collected (lungs, liver and spleen) were placed into 6-well trays containing 40 µm cell sieves with 2 ml of PBS. Organs were disrupted through the cell sieve using the plunger of a 2 ml syringe and cell suspensions were collected. Subsequently, 100 µl aliquots of the cell suspension or blood were used for enumeration of bacteria on agar plates following serial dilution in PBS. For flow cytometry, 200 µl aliquots of cell suspension or blood were spun down in a micro centrifuge for 5 min approximately 700 rpm. Supernatants were removed for cytokine analysis and stored in flat bottom 96-well trays at -80°C. The supernatants were subsequently investigated using Cytometric Bead Array flex sets (Becton Dickinson™), performed according to manufacturer's instructions, with the additional step of fixing the samples in 4% paraformaldehyde in PBS for at least 48 h at 4°C. Cells were resuspended in 100 µl red cell lysis buffer (Sigma™) and incubated at room temperature for 5 min. Red cell lysis buffer was quenched using 800 µl of PBS and micro-centrifuged at 700 rpm for 5 min. Supernatants were aspirated and resuspended in 100 µl of blocking solution (2 µl Becton Dickinson™ anti CD16/32 and PBS) and incubated for 20 to 30 min at room temperature. Flow cytometry antibodies were added and incubated for 15 to 20 min at room temperature. Cells were washed with 800 µl PBS and micro-centrifuged at 700 rpm for 5 min, then supernatants were removed and resuspended in 4% paraformaldehyde in PBS. Samples were subsequently stored for at least 48 h at 4°C, for the paraformaldehyde to render the B. pseudomallei inactive. All flow cytometry was performed using a 6 colour FACScanto II (Becton Dickinson™) and the analysis / acquisition program FACS Diva (Becton Dickinson™). The standard sample stain for professional phagocytes comprised 2 µl of the following antibody mixture: α-Ly6G-PE (Becton Dickinson™ [clone 1A8]), α-CD11b-PerCP-cy5.5 (Becton Dickinson™ [clone M1/70]), α-CD11c-PE-cy7 (Becton Dickinson™ [clone HL3]), α-F4/80-APC (Insight™ [clone CI:A3-1]) and α-CD45-APC-cy7 (Becton Dickinson™ [clone 30-F11]). The selection criteria for leukocytes comprised the following: (1) Cell-sized events selected on forward vs. side scatter. (2) Doublet reduction was performed using forward scatter height vs. time. (3) Leukocytes were selected on the basis of CD45 expression on a side scatter vs. CD45 plot. (4) Neutrophils ($Ly6G^+$, $CD11b^+$) and non-neutrophils were selected on the basis of Ly6G and CD11b expression. (5) From non-neutrophils, macrophages (F4/80+ sidescatter high) and non-macrophages were selected on a sidescatter vs. F4/80 plot. (6) Monocytes ($CD11b^+$) and "others" ($CD11b^-$) on a CD11b. The macrophage population was further characterized using a CD11c vs. CD11b plot. The cytometric bead arrays were run to determine cytokine concentration on the flow cytometer as described in manufacturers' instructions.

Statistical analysis

All graphs were generated using Graphpad™ PRISM V4.0 and all statistical analysis was performed using PASW (SPSS™ release 18.0), with the exception of non-linear regression, which was performed using Graphpad™ PRISM V4.0. All data were transformed to the logarithm 10 for better fit to the normal distribution (verified probability plots). Data from cytometric bead arrays were analyzed using PRISM, by fitting a quadratic regression to the standard curves and reading the samples as unknown.

RESULTS AND DISCUSSION

In this study, untreated BALB/c mice challenged by the aerosol route with approximately 146 CFU (low challenge) or 213 CFU (high) of aerosolized *B. pseudomallei* strain K96243 were all dead by day 8 or 3 post-challenge, respectively with median times to death of 3 and 2.5 days respectively. During the 7 day oral doxycycline treatment regime, only one mouse in the higher challenge dose group succumbed to infection (Day 5). Following the removal of the antibiotic, mice gradually succumbed to infection although there was still a significant survival on Day 21 for both challenge doses ($P < 0.001$, logrank test; 0 of 6 mice in the low challenge untreated group; 7 of 16 low challenge treated mice; 0 of 12 mice in the high challenge, untreated group; and 8 of 12 high challenge, treated mice). This indicates that, although doxycycline offered protection against aerosolized *B. pseudomallei*, the treatment was sub-optimal.

Bacterial numbers within liver and spleen increased steadily during the 21 day period (Figure 1). Numbers of bacteria significantly declined in the lung ($P = 0.016$ Bonferroni's post test), and did not differ in the liver and spleen ($P > 0.05$ Bonferroni's post test) over the course of treatment (2 to 7 days). After completion of treatment, there was a secondary increase in the number of bacteria in the lungs by day 14 post-challenge ($P = 0.008$

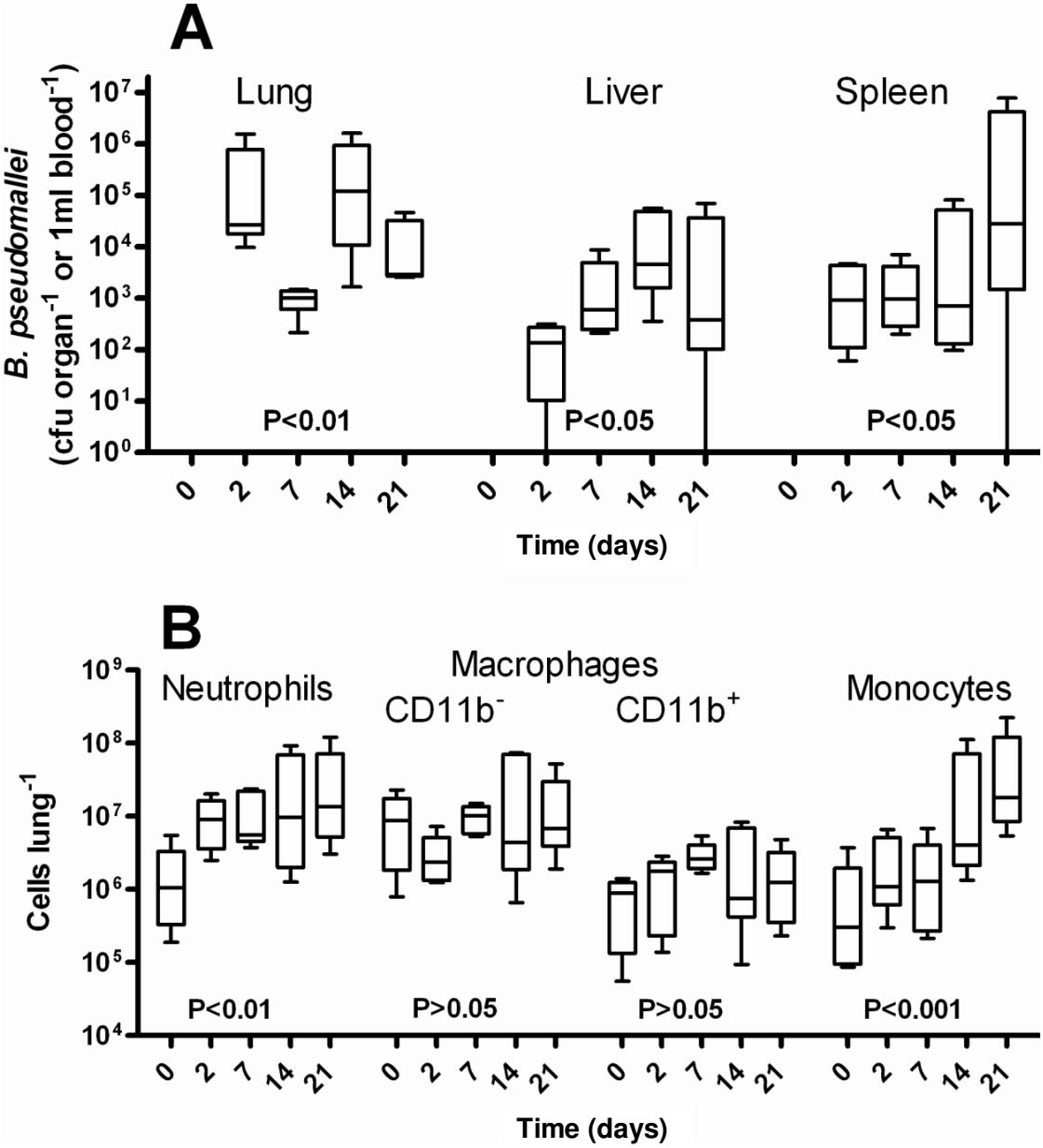

Figure 1. Bacterial colonization and numbers of professional phagocytes in the lungs of BALB/c mice after aerosol exposure to *B. pseudomallei* strain K96243. Following challenge with aerosolized *B. pseudomallei* K96243, lungs, blood, liver and spleen were harvested for bacterial enumeration (Panel A). Further, certain leukocytes were enumerated in the lung by flow-cytometry (Panel B). Data displayed are mice that received a calculated dose of 146 CFU *B. pseudomallei* and were subsequently treated with a sub-optimal regime of doxycycline (1 mg orally, daily for 7 days, initiated 6 h after infection) where 5 mice were culled at 2, 7, 14 and 21 days post-infection. Each point on the graph represents the mean of 5-6 mice and the error bars are indicative of the standard error. Data is representative of two experiments. The P values cited on the figure are indicative of multivariate analysis of the numbers of cells or Univariate analysis of bacteria as the infection develops (performed using PASW, SPSS V18.0).

Bonferroni's post test). A longer treatment schedule may have continued to control the bacterial numbers in the lungs and may have lead to a better prognosis for the mice. Few bacteria were isolated from the blood (one mouse, at day 2 only). The sub-optimal treatment led to incomplete bacterial clearance with the secondary bacterial re-growth in the lungs, leading to morbidity and mortality.

The numbers of different leukocytes in BALB/c mouse lungs following infection with aerosolized *B. pseudomalle*

K96243 are shown in Figure 1. The leukocytes were enumerated as follows, neutrophils ($Ly6G^+$, $CD11b^+$), macrophages ($F4/80^+$, $sidescatter^{high}$), monocytes ($F4/80^-$, $Ly6G^-$, and $CD11b^+$). Macrophages were subdivided into $CD11c^+$, $CD11b^{low}$ macrophages and $CD11c^-$ $CD11b^{high}$ macrophages. Within the resting lung the predominant macrophage (> 90%) was $CD11c^+$ and $CD11b^{low}$ and this was consistent with previous data (Kirby et al., 2006). During the infection, macrophage numbers remained constant ($P = 0.480$ for $CD11b^-$, $P = 0.320$ for $CD11b^+$, multivariate analysis) while numbers of monocytes ($P = 0.001$, multivariate analysis) and neutrophils steadily increased with time post-challenge ($P = 0.010$, multivariate analysis).

Neutrophil recruitment is believed to be a major cellular response following *B. pseudomallei* infection and the importance of neutrophil cells has been demonstrated in ablation studies (Easton et al., 2007). As major effector cells in bacterial clearance the role of these cells is evident. The observed increase in monocyte cells is consistant to previous observation (Laws, et al., 2011). These cells serve diverse functions during infection (Ziegler-Heitbrock, 2007) and their role here certainly warrants further examination.

Interferon gamma (IFN-γ), interleukin-6 (IL-6), interleukin-10 (IL-10), tumour necrosis factor (TNF) and monocyte chemo-attractant protein 1 (MCP-1 or CCL2) concentrations were measured in the lungs, spleen and blood (Figure 2). Significantly elevated levels of all cytokines were detected in all tissues at day 2 post-infection ($P < 0.05$), with the exception of MCP-1. Levels of several cytokines dropped between day 2 and 21 post-infection. This data (time points 2, 7, 14 and 21) was interrogated using a multivariate analysis and it was noted that cytokine level changed over time for all of the cytokines (IFN-γ $P < 0.001$; IL-6 $P = 0.001$; MCP-1 $P < 0.001$; TNF-α $P = 0.013$ and IL-10 $P < 0.001$, multivariate analysis). Individual, Bonferroni's analyses further demonstrated the decay of the immune cytokine response (Table 1).

The rapid induction of cytokines has been observed previously in models of melioidosis (Barnes et al., 2001; Ulett et al., 2000a; Ulett et al., 2000b). Further to this, it is known that doxycycline directly reduces TNF, IL-8 and IL-1β in human blood with a defined LPS challenge (Cazalis et al., 2008) and nitric oxide in epithelial cells (Hoyt et al., 2006). However, the observation of immune 'decay' occurs after therapy has been halted. This indicates that the direct immuno-modulatory effects are likely not playing a role here. This could be tested further by the use of an additional antibiotic. These data support previous data (Griffin et al., 2009) indicating that, despite the overwhelming benefit of antibiotic treatment, they can have a detrimental effect on the inflammatory response. In the current climate of rising number of antibiotic resistant infections, therapies that bolster the immune response may have real tangible benefits. It is unclear why this is occurring. It is becoming clear that after many strong inflammatory events, periods of anti-inflammation (or 'immune paralysis') occur (Gustot, 2011). It is possible that in our model early 'immune paralysis' is occurring. Further experiments are required to explore this phenomenon. This might be tested by measuring the response of leukocytes, *ex-vivo*, to positive control antigens (that is, Concanavalin A, unrelated LPS, etc.), where responses of naïve mice and mice at time points post infection and treatment can be compared.

Infectious diseases are still a major cause of morbidity and mortality worldwide. Aside from antibiotics and critical care, the only other truly effective way that bacterial disease can be managed is by the action of a healthy immune system. Despite this fact, very little is known about how the antibacterial properties of antibiotics can affect the development and maturation of the immune response. It is known that macrolides can have a direct effect on the immune response (Giamarellos-Bourboulis, 2008) and doxycycline can reduce certain immune inflammation markers (Cazalis et al., 2008; Hoyt et al., 2006). However, there is little other evidence of direct immuno-regulatory effects of antibiotics. Previous studies have demonstrated that ciprofloxacin can limit the generation of an adaptive immune response to *Salmonella enterica* (Griffin et al., 2009) and that it may also cause a suboptimal immune response following anthrax infection (Klinman et al., 2000). It is unclear whether this is a direct effect or due to the anti-bacterial action of the antibiotic. Further work is required to verify how the doxycycline in this model has contributed to the decline in cytokines observed.

A sub-optimal treatment model of *B. pseudomallei* has been developed which delays lethality and may be exploited to enhance other partially protective post-exposure therapies. In this model, a decrease in the bacterial load in the lungs follows increased neutrophil recruitment and increased cytokine response at day 2 post-challenge. These conditions considerably extend the lifespan of the mouse. During this prolonged survival we observed a decaying cytokine response that may indicate that 'immune paralysis' is occurring. Further research is needed to explore the therapeutic potential for immune stimulation late in infection where bacteria have not been cleared. Ultimately, this model is more clinically relevant and previous work implicating roles for IFN-γ and neutrophils may need to be repeated using this model to verify whether they are important later in the extended pathogenesis.

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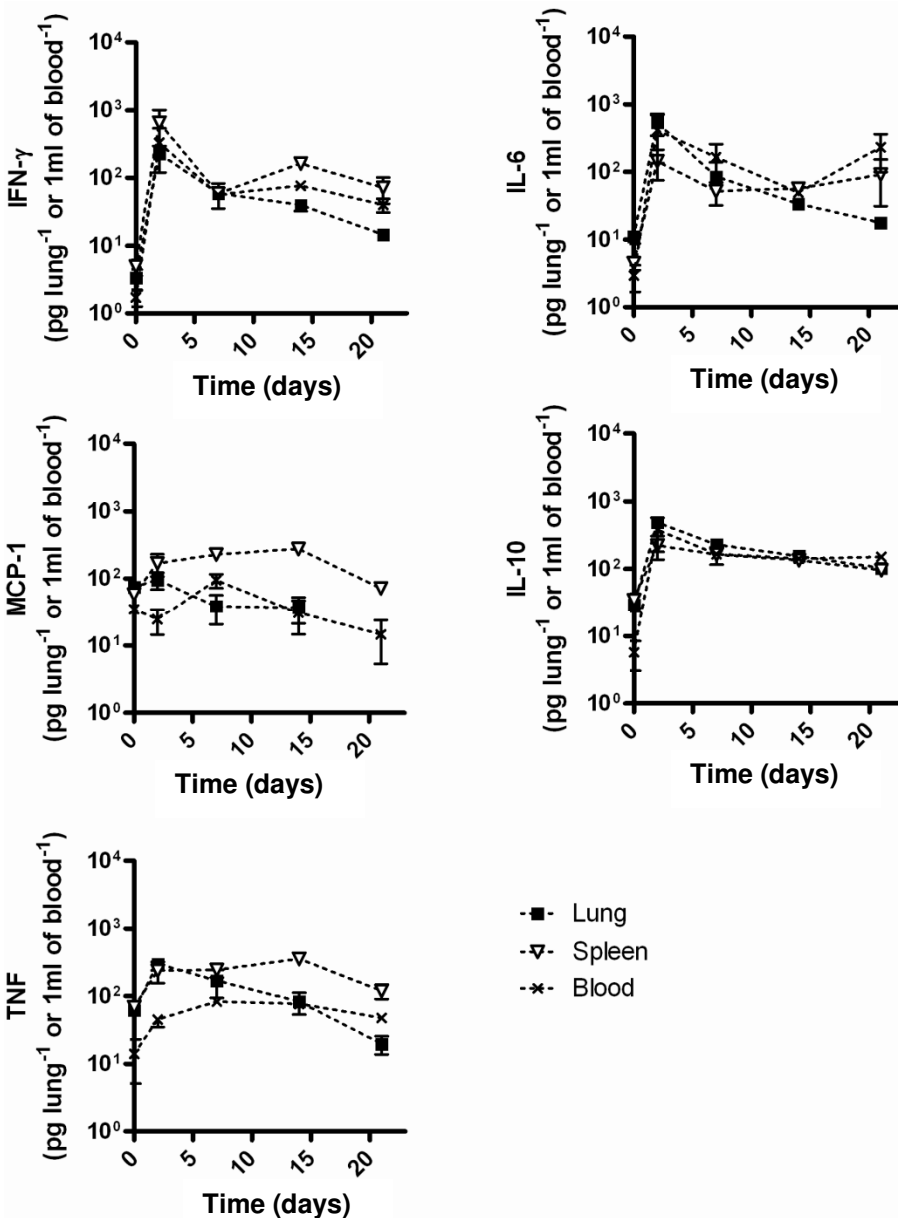

Figure 2. Cytokine production within different organs of BALB/c mice infected by *B. pseudomallei* strain K96243 after aerosol exposure. Concentrations of IFN-γ, MCP-1, TNF, IL-6 and IL-10 were measured in the lungs (filled squares), blood (crosses) and spleens (empty triangles) of BALB/c mice following challenge with aerosolised *B. pseudomallei* K96243 (1.5×10^2 CFU / mouse) and were subsequently treated with a sub-optimal regime of doxycycline (1 mg orally, daily for 7 days, initiated 6 h after infection) where 5 mice were culled at 2 , 7 , 14 and 21 days respectively. Each point on the graph represents the mean of 5-6 mice and the error bars are indicative of the standard error. Data is representative of duplicate experiments. This data (time points 2, 7, 14 and 21) was interrogated using a multivariate analysis and it was noted that cytokine level changed over time for all of the cytokines (IFN-γ $P<0.001$; IL-6 $P=0.001$; MCP-1 $P<0.001$; TNF-α $P=0.013$ and IL-10 $P<0.001$, multivariate analysis).

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