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Expression of TNF-α, IL-1β, and IFN-γ in *Staphylococcus epidermidis* slime-positive experimental endophthalmitis is closely related to clinical inflammatory scores

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Abstract *Purpose:* The purpose of this study was to investigate the expression of inflammatory cytokines TNF-α, IL-1β, and IFN-γ in the vitreous after experimentally induced endophthalmitis by a *Staphylococcus epidermidis* slime-producing strain. *Methods:* Seventy-two experimental Lewis rats received an intravitreal injection of 7000 viable organisms of *Staphylococcus epidermidis* slime-producing ATCC strain 35983, while 72 control rats received an intravitreal injection of sterile normal saline. Eyes were graded daily for signs of clinical inflammation and were removed 6, 12, 24, 48, 72 h, and 7 days after injection. Vitreous was obtained and titers of TNF-α, IL-1β, and IFN-γ were measured with established enzyme-linked immunosorbent assays. *Results:* In the experimental group, the clinical inflammatory score reached maximum (4+) within 24 h, while inflammation was almost abolished by day 7 (score 0–0.5+). Statistically increased levels of TNF-α and IL-1β were detected in the experimental vitreous with maximum levels observed at 12 h. IFN-γ was also detected in the experimental vitreous and reached maximum levels at 48 h. None of the cytokines examined was detected in sera at any time point in experimental or control rats. *Conclusions:* The results of this study suggest that *Staphylococcus epidermidis* experimental endophthalmitis induces the expression of cytokines TNF-α, IL-1β, and IFN-γ in the vitreous. The time course of those cytokine expression levels is closely associated to the clinical presentation of this endophthalmitis model.

Keywords Experimental endophthalmitis · *Staphylococcus epidermidis* · Cytokines · Tumor necrosis factor-α · Interleuken-1β · Interferon-γ · Lewis rats

Introduction

Infectious endophthalmitis is one of the most severe conditions in ophthalmology, often leading to significant loss of visual acuity and sometimes of the eye itself, although modern pharmaceutical and surgical modalities have been introduced. It is usually a postoperative complication and its incidence after cataract surgery has been estimated from 0.07% to 0.13% [15]. The coagulase-negative staphylococci, which are the most common bacterial group in the flora of the ocular surface [2], have become the leading cause of postoperative endophthalmitis

[27] and comprise 70% of the bacterial isolates reported in the Endophthalmitis Vitrectomy Study [10]. *Staphylococcus epidermidis* constitutes the main representative of this group [22].

Although *S. epidermidis* is considered less virulent than other bacteria, *S. epidermidis* endophthalmitis can often result in a poor visual outcome, owing to either an acute process or a delayed diagnosis. A remarkable variation in clinical presentation, with patterns ranging from an acute, moderately severe form to a chronic, relatively mild inflammation, has been described [22]. Presentation symptoms and signs tend to be less acute and less severe than in the cases of other bacterial isolates, and they are often partially suppressed by intensive postoperative corticosteroid medication. About 10% of cases are painless [21]. Consequently, the infection can run insidious, slowly progressive, and diagnosis is often delayed because of a prolonged asymptomatic interval [1, 21]. Thus, despite a generally good visual outcome after treatment, nearly one-third of the cases have a final visual acuity of less than 20/400, and almost 11% have lost light perception [22].

Poor visual outcome enhances the need for additional, more efficient therapeutic options, which could ensue from better study of the host immune response to *S. epidermidis* endophthalmitis. However, until today, the ocular immune response to *S. epidermidis* endophthalmitis has not been fully understood. Judging from results concerning other inflammatory processes [4, 8], it would be reasonable to theorize a role of inflammatory cytokines in that condition. Cytokines are polypeptides that act as intercellular messengers [19, 24], playing an important role in mediating the processes of inflammation and repair. Macrophages, lymphocytes, natural killers, and other immune cells, even endothelial cells, participate in the secretion of numerous signal molecules [9, 26], which, in turn, recruit more inflammatory cells and regulate host immune responses to an injury or antigen invasion [20].

Cytokines include the tumor necrosis factors (TNFs), the interleukins (ILs), the interferons (IFNs), and a number of growth factors [24]. They have been grouped into four categories or phases of inflammatory reaction [16]:

- *recognition* (mainly IL-1 and TNF-α); rapid expression - establishment of cytokine networks
- *recruitment* (called "chemokines" - human IL-8, rat CINC, etc.); elicitation of leukocytes
- *removal* (mainly IFN-γ, IL-2, and IL-6); activation of macrophages (IFN-γ) or lymphocytes (IL-2 and IL-6), and
- *repair* (several growth factors).

Assuming that both the bacteria and host immune response are responsible for the destructive processes of endophthalmitis, knowledge of cytokine expression characteristics in *S. epidermidis* endophthalmitis is of great importance and may enable an efficient combined treatment in the future. Thus, in the present study, we aimed to investigate the expression of two early-response inflammatory cytokines (TNF-α and IL-1β) and one late-activation cytokine (IFN-γ) in the vitreous after experimentally induced endophthalmitis using an established slime-producing *S. epidermidis* strain.

Materials and methods

Animals—bacterial strain—intravitreal injections

The study included 144 inbred male Lewis rats 9–11 weeks in age, weighing 250–300 g, maintained in a 12-h light/12-h dark cycle under pathogen-free conditions, and fed ad libitum. Two groups were formed: an experimental and a control one, each containing 72 rats, randomly selected. *Staphylococcus epidermidis* slime-positive ATCC strain 35983 was used to infect the experimental group as follows. Each one of the 72 experimental rats received a 50-μl intravitreal pars plana injection of approximately 7000 viable organisms in the right eye according to a previously described model [23], while a similar sterile normal saline injection was given to each one of the 72 control rats in the right eye (placebo group). Each eye had been dilated with sterile 1% atropine eye drops so as to watch needle placement and to prevent nicking of the lens. All experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Clinical inflammatory scores—cytokine detection—statistical analysis

Eyes were graded daily for signs of clinical inflammation with a Haag-Streit slit-lamp biomicroscope, and scores were given according to a scale described by Chan et al. [3] varying from 0 to 4+. Sera were obtained before injection and just before sacrifice. The animals were killed 6, 12, 24, 48, 72 h, and 7 days after injection in six groups. At each one of the six time points described, 12 experimental and 12 control rats were killed. Eyes were removed immediately after death using an operating microscope. The removed globes were bisected posterior to the iris, the lens was carefully lifted out and the vitreous was removed and placed in conical tubes on ice. Samples were centrifuged at 10,000*g* for 5 min at room temperature. The supernatant was stored frozen at −70°C until used.

Titers of rat TNF-α and IFN-γ were measured using standard solid phase sandwich enzyme-linked immunosorbent assay (ELISA) kits from Diaclone Research (Besançon, France), and those of rat IL-1β were measured using an ELISA kit from R&D Systems, Inc. (Minneapolis, USA). Recombinant rat TNF-α, IFN-γ, and IL-1β proteins

provided by the manufacturing companies of the ELISA kits were used as standards. As no eye gave enough vitreous for individual ELISA measurement, each vitreous sample used for ELISA consisted of the vitreous of three random rats belonging to the same group. Serum and vitreous cytokine titers were compared between experimental and control rats. Results were statistically analyzed using the Student's *t*-test. Differences at the level of $P<0.05$ were considered statistically significant. In addition, the Bonferroni adjustment was applied to avoid differences resulting from chance alone.

Results

Clinical inflammation

Clinical inflammatory scores in the experimental and in the control group are presented in Fig. 1a,b.

At 6 h after the injection, most rats showed mild dilatation of the iris vessels and flare in the anterior chamber (score 0.5+). Control rats did not show any signs of inflammation.

At 12 h, clinical inflammation in the experimental group increased. Most animals had cells in their anterior chamber and fibrin at the pupillary margin (score 1+). In the control group, seven of 12 rats showed mild dilatation of the iris vessels (score 0.5+), but the difference between the experimental and the control group was statistically significant ($P<0.001$).

At 24 h, clinical inflammation in the experimental group reached maximum. Five rats had fibrin in the anterior chamber and iris synechiae (score 2+), and one rat presented a hypopyon as well as a loss of red reflex (score 4+). In the control group, most rats showed no signs of inflammation. The difference between the experimental and the control group was still statistically significant ($P<0.001$).

At 48 and at 72 h, clinical inflammation in the experimental group decreased. Most rats had a score of 0.5+. No control rat showed any sign of inflammation.

Finally, by day 7 post-injection, clinical inflammation was almost abolished in the experimental group. The majority of rats had no sign of inflammation. Control rats also did not show any signs of inflammation.

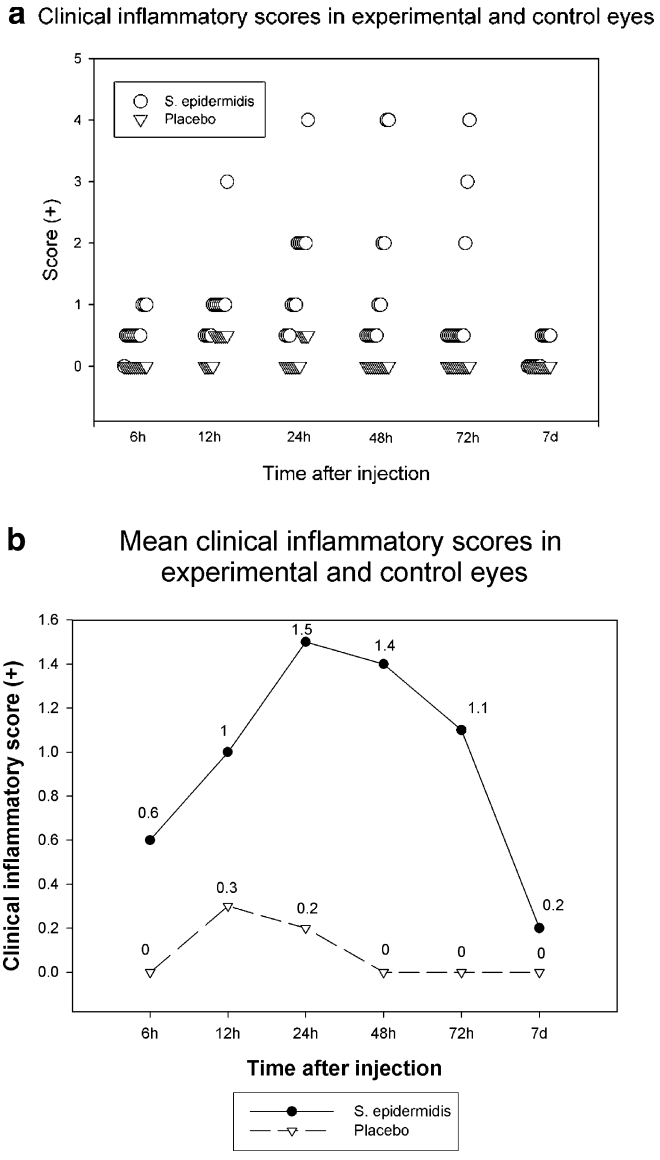

Fig. 1 Comparison between clinical inflammatory scores of the eyes injected with *S. epidermidis* (n=72) versus placebo (n=72). Each time point group consisted of 12 experimental and 12 control eyes. **a** Plotting of all animals. **b** Mean scores. Mean clinical inflammatory score in the experimental group was maximum at 24 h. Score system used (Chan et al. 1994): *0* no inflammation. *0.5+* mild dilatation of iris vessels and/or flare in the anterior chamber (AC). *1+* cells in the AC and/or fibrin at the pupillary margin. *2+* fibrin in the AC and/or synechiae. *3+* fibrin clots in the AC and/or corneal edema. *4+* hypopyon, hyphema, and/or loss of red reflex

Cytokines

TNF-α was detected at all time points (6, 12, 24, 48, 72 h, and 7 days post-injection) in all vitreous samples of the experimental and the control group. Levels in the control group were very low and did not vary significantly between time points ($P>0.05$). At 6 h after the injection, TNF-α levels in the experimental group showed a 74% increase as compared with those in the control group ($P<0.005$) (Fig. 2). At 12 h, maximum levels of TNF-α were observed in the vitreous samples of the experimental group, 251% higher than those in the control group ($P<0.001$) (Fig. 2). At 24, 48, and 72 h, TNF-α decreased but was significantly

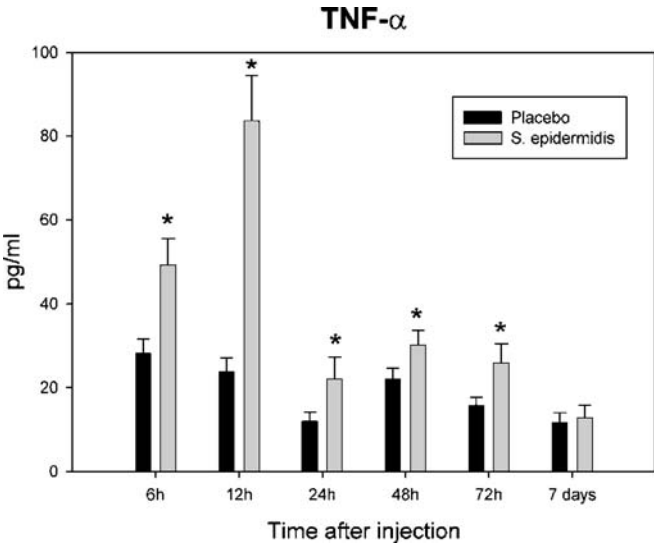

Fig. 2 Mean (±SD) TNF-α levels in eyes injected with *S. epidermidis* (n=72) and in eyes of the placebo group (n=72). Each time point group consisted of 12 experimental and 12 control eyes. All differences, except that observed at 7 days, were statistically significant (*P<0.05 versus placebo, Student's t-test with Bonferroni adjustment). A peak in mean TNF-α in the experimental group was observed at 12 h

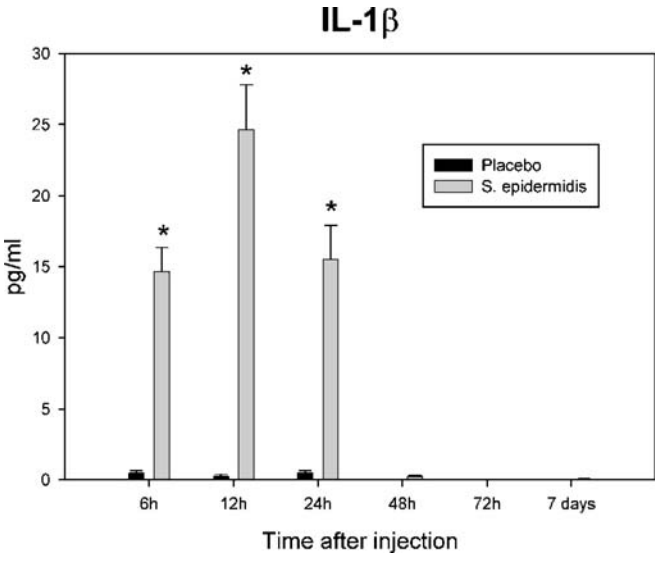

Fig. 3 Mean (±SD) IL-1β levels in eyes injected with *S. epidermidis* (n=72) and in eyes of the placebo group (n=72). Each time point group consisted of 12 experimental and 12 control eyes. Differences at 6 h, 12 h, and 24 h were statistically significant (*P<0.001 versus placebo, Student's t-test with Bonferroni adjustment). A peak in mean IL-1β in the experimental group was observed at 12 h

higher in the experimental than in the control group (P<0.05). By day 7, there was only a 7% difference in the vitreous TNF-α levels between the experimental and the control group, which was not statistically significant (P>0.05). No TNF-α was measurable in the sera of experimental and control rats at any time point.

IL-1β was detected at all time points (6, 12, 24, 48, 72 h, and 7 days post-injection) in all vitreous samples of the experimental group. It was also present at very low levels in most vitreous samples of the control group at 6, 12, and 24 h. IL-1β levels in the experimental group showed a similar curve as that of TNF-α (Fig. 3). They were 2984% higher than control IL-1β levels at 6 h (P<0.001), reached maximum levels at 12 h showing a 9760% increase as compared with those of the control group (P<0.001), decreased at 24 h, and declined to non-detectable levels at 48, 72 h, and 7 days, with no significant difference from those of the control group at the last three time points (P>0.05) (Fig. 3). No IL-1β was detected in the sera of experimental and control rats at any time point.

IFN-γ was observed at all time points (6, 12, 24, 48, 72 h, and 7 days post-injection) in all vitreous samples of the experimental and the control group, yet at very low and statistically stable levels in the latter (P>0.05). IFN-γ was very high at 6 h post-injection in the experimental vitreous (164% higher than that of the control vitreous), decreased at 12 and 24 h, peaked again at maximum levels at 48 h (344% higher than that of the control vitreous), decreased at 72 h, and was very low at 7 days (Fig. 4). Differences between the experimental and the control group were statistically significant at all time points (P<0.05) except at 7 days (P>0.05). No IFN-γ was found in the sera of experimental and control rats at any time point.

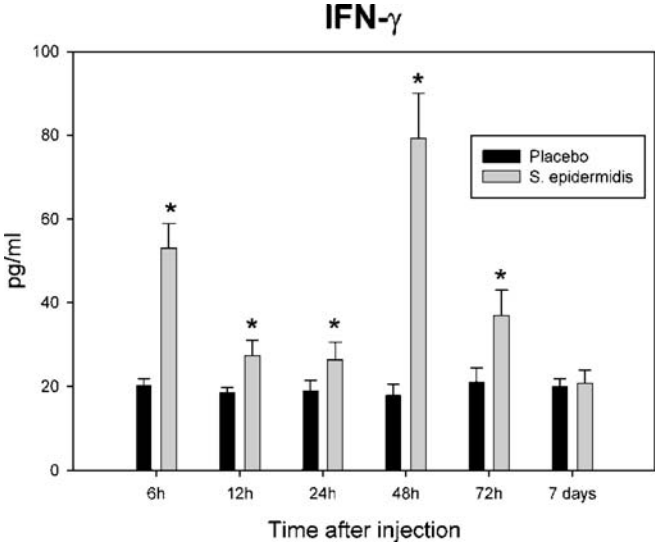

Fig. 4 Mean (±SD) IFN-γ levels in eyes injected with *S. epidermidis* (n=72) and in eyes of the placebo group (n=72). Each time point group consisted of 12 experimental and 12 control eyes. All differences, except that observed at 7 days, were statistically significant (*P<0.05 versus placebo, Student's t-test with Bonferroni adjustment). A first peak in mean IFN-γ in the experimental group was seen at 6 h, whereas a second, more important peak was observed at 48 h

Discussion

To clarify the early inflammatory-immune response to experimental *S. epidermidis* endophthalmitis, we studied the expression of cytokines TNF-α, IL-1β, and IFN-γ in the vitreous of Lewis rats. Our results showed that intravitreal injection of viable *S. epidermidis* slime (+) organisms induced the production of all those three cytokines in the vitreous, as well as clinical signs of endophthalmitis. The latter were present in all experimental eyes at 12, 24, 48, and 72 h post-injection, being maximal at 24 h. In a time-related pattern, TNF-α and IL-1β peaked earlier, at 12 h, while IFN-γ was also detected in the experimental vitreous but reached maximum levels later, at 48 h, after having regressed from a very early peak at 6 h.

It is of interest to note the lack of systemic TNF-α, IL-1β, and IFN-γ in sera, suggesting that their production is local in response to bacteria or bacterial products. As for a slight but insignificant increase in cytokine levels seen in the vitreous of control eyes, it may have been caused by injection trauma.

In the experimental eyes of this study, TNF-α and IL-1β showed the same variation with time after the injection of bacteria. Both of them started to increase early and peaked at 12 h post-injection. This may have been in response to constant inflammatory stimulus present in the vitreous as a result of bacterial growth and release of bacterial products and slime. A large number of host inflammatory cells entering the eye soon after the inoculation may also account for the significant production of those proinflammatory (first phase) cytokines [7, 17].

Clinical signs of endophthalmitis were present in all experimental eyes at most time points and peaked at 24 h post-injection. It is therefore reasonable to propose that the secretion of proinflammatory cytokines (which peaked earlier) mediated the expression of endophthalmitis in the examined animal model. Indeed, numerous cytokines, among which TNF-α, IL-1, IL-6, IL-8, and IFN-γ, have been reported to cause ocular inflammation after intraocular administration [11, 25]. The inflammatory reaction occurs readily, starting a few hours after the cytokine is injected. Following the injection, cytokines set up a chain of events leading to cytokine cascades, each with either an agonist or an antagonist activity. TNF-α and IL-1, both produced by macrophages, are considered to be early initiators of this inflammatory process [6].

In addition, the observed time course of clinical inflammation seems to correspond to previously published reports. For instance, endotoxin-induced uveitis (EIU) in the rat caused by intraperitoneal injection of bacterial lipopolysaccharide (LPS) is characterized by a rapid breakdown of the blood/aqueous barrier (within 3 h) and increased vascular permeability, with clinical signs peaking at 24 h and resolving by 48 h, independently of rat strain [5]. Similar findings have been described in EIU in the rabbit caused by intravitreal injection of LPS, with inflammatory cells continuing to extravasate at 24 h [12]. Moreover, in experimentally induced *S. epidermidis* endophthalmitis in the aphakic rabbit eye, at 24 h after the inoculation of bacteria, all eyes had shown signs of clinical infection [18].

IFN-γ showed a different variation pattern and seems to have contributed both to the expression and to the resolution of the inflammation. In the experimental eyes, IFN-γ variation showed an early peak at 6 h and a late peak with maximal levels at 48 h post-injection. The later activation of lymphocytes (all classes) seems to provide the main source of this third phase cytokine. Moreover, recent reviews consider IFN-γ to be a pleotropic cytokine that plays an essential role in both the innate and adaptive phases of an immune response [28]. Thus, initial production of IFN-γ by macrophages provides an autoregulatory loop that allows innate immunity to begin combating the invading organism at the site of infection prior to the development of the adaptive immune response [28]. IFN-γ is further produced by stimulated T-cells, and skews the response to a Th1 (inflammatory) phenotype [6, 13]. That double origin of IFN-γ would explain the double peak of that cytokine seen in our experiments.

In contrast with the unfavorable natural history of untreated infectious endophthalmitis seen invariably in humans, spontaneous regression of *S. epidermidis* endophthalmitis has been described in the rat, showing its capacity to beat the infection without treatment [23]. Rabbits have shown a similar capacity in eradicating bacteria [14]. Results demonstrated that *S. epidermidis* not only declines in number, but the vitreous cavity is sterile in almost all cases tested after 72 h [18]. This observation was confirmed in our study, since clinical inflammation in the experimental group gradually regressed after the 24-h time point and was almost abolished by day 7 post-injection. In addition, TNF-α and IL-1β vitreous levels in the experimental group gradually decreased after the 12-h time point and no statistically significant differences were detected between the experimental and the control group by day 7 post-injection. Similarly, IFN-γ vitreous levels in the experimental group decreased after the 48-h time point, showing no statistically significant differences with the control group by day 7. Rat eyes seem to have beaten the induced endophthalmitis.

Cytokines seem to be necessary for the initiation and propagation of inflammatory process; nevertheless, their secretion is often associated to cytotoxic effects [11], which may be enhanced due to synergy of numerous cytokines [13, 16]. We might therefore suggest that, as the cytotoxic effects of TNF-α and IL-1β are enhanced in the presence of other cytokines, particularly IFN-γ, the observed decrease in TNF-α and IL-1β levels at the time of IFN-γ increase in our experiments (at 48 h post-injection) would enable repair with less toxicity.

The present study does not offer gene expression data for the cytokines studied, which would have enhanced the importance of ELISA results. Furthermore, it raises the need to investigate additional aspects of immune response

to endophthalmitis. In that context, other Th1 (inflammatory) and Th2 (anti-inflammatory) cytokines will have to be studied to evaluate the balance of the effector immune phenotype in the eye following staphylococcal infection. Last but not least, it would be useful to test whether neutralization of cytokines by antibodies or inhibitory RNA would change the severity of the disease.

In conclusion, the results of this study showed that intravitreal injection of viable *S. epidermidis* slime (+) organisms induces the expression of TNF-α, IL-1β and IFN-γ in the vitreous, as well as clinical signs of endophthalmitis. All three cytokines precede the maximal manifestation of inflammation in our model, while IFN-γ is also expressed at more important levels during the regression of inflammation. As both the bacteria and host immune response are responsible for the destructive processes of endophthalmitis, we believe that knowledge of cytokine expression characteristics in animal models of *S. epidermidis* endophthalmitis is of great importance and may enable an efficient combined antibiotic, anti-inflammatory and anti-cytokine treatment in the future.

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