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Pathogenesis and toxins

Immune discrepancies during *in vitro* granuloma formation in response to *Cutibacterium* (formerly *Propionibacterium*) *acnes* infection

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ABSTRACT

Cutibacterium (formerly *Propionibacterium*) *acnes* is involved in chronic/low-grade pathologies such as sarcoidosis or prosthetic joint infection (PJI). In these diseases, granulomatous structures are frequently observed. In this study, we induced a physiological granulomatous reaction in response to different well-characterized clinical *C. acnes* isolates in order to investigate the cellular process during granuloma formation. Three *C. acnes* isolates selected according to their origin (PJI, sarcoidosis and acne) were typed by MLST. All *C. acnes* isolates generated granulomatous structures in our experimental conditions. The bacterial burden was better controlled by granulomas induced by the sarcoidosis *C. acnes* isolate. The PJI *C. acnes* isolate, belonging to CC36, promoted the recruitment of $CD8^+$ lymphocytes inside the granuloma. In contrast, the acne and sarcoidosis *C. acnes* isolates, belonging to phylotypes IA_1/CC18 and IA_2/CC28, respectively, generated a higher number of granulomas and promoted the recruitment of $CD4^+$ lymphocytes inside the granuloma. Our results provide new evidence supporting the role of *C. acnes* in the development of sarcoidosis and new explanations concerning the mechanisms underlying PJI due to *C. acnes*.

1. Introduction

Cutibacterium acnes is an important part of the normal flora of human skin, living in and around sweat glands and sebaceous follicles. The pathogenicity of *C. acnes* has long been restricted to skin conditions [1]. Although described as a commensal bacterium with a low pathogenicity, its involvement has been reported in many clinical entities [2]. In the context of chronic/low-grade pathologies, the two main features of *C. acnes* infection are sarcoidosis and prosthetic joint infection (PJI). The link between these two pathologies is the presence of granulomatous structures in response to the infection.

Sarcoidosis is a systemic disease with an unknown etiology, which is characterized by non-caseating granulomas found primarily in the lung [3]. While the accurate cause of sarcoidosis remains unknown, it seems that the pathogenesis results from the combination of genetic susceptibility and exposure to specific antigens, either environmental or infectious [3]. Nevertheless, *C. acnes* has been suspected of being involved in the development of sarcoidosis and has frequently been cultured out of lymph nodes from sarcoidosis patients [4,5] and localized within sarcoidosis granulomas [6]. Additionally, *C. acnes* has been shown to drive differential cytokine responses in the peripheral blood mononuclear cells of sarcoidosis patients [7]. In 2–14% of cases, *C. acnes* has also been identified as the cause of various implant-associated infections, including PJI, particularly in shoulder prostheses, spine implant surgery, and hip and knee prostheses [8,9]. We previously reported that *C. acnes* isolates belonging to clonal complex (CC) 36 were more frequently observed in PJI [10]. These PJI are also characterized by granulomatous structures in the tissues next to the material [11–13].

Some granuloma-producing animal models of sarcoidosis have been proposed using different bacteria and bacterial products

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[14–16]. On the other hand, host-bacterial interaction in PJI is usually assessed using a foreign-body infection model with guinea pigs or rabbits [17,18]. To date, no study has focused on the granuloma formation next to the material implanted. Moreover, animal models remain technically and ethically unsuitable to evaluate a large collection of isolates.

Thus, the cellular organization and host-bacteria interactions within complex granulomas have not been well-described to date. To our knowledge, there is no relevant model that illustrates *in vitro* granulomas leading to clinical extrapolation. To develop such a model, we used freshly collected human peripheral blood mononuclear cells (PBMCs) from healthy individuals. This enabled us to evaluate the relevance of an *in vitro* persistence model of *C. acnes* in human blood cell phagocytes in a cellular and physiological environment that mimics the *in vivo* situation. In this study, we induced a physiological granulomatous reaction in response to different *C. acnes* isolates in order to investigate the cellular process during granuloma formation.

2. Material and methods

2.1. Bacterial strains

The *C. acnes* ATCC6919 isolate (CC18, phylotype IA1) came from the American Type Culture Collection (ATCC). Its clinical origin is acne lesion and its genome has been sequenced (Genebank accession number 1174878). The *C. acnes* BL clinical isolate (CC36, phylotype IB) was isolated at Nantes University Hospital from a PJI. Its genome was recently sequenced [19]. Lastly, the *C. acnes* S8 strain (CC28, phylotype IA_2) was kindly provided by Professor Y. Eishi of the Department of Human Pathology, Tokyo Medical and Dental University, Tokyo, Japan. This strain was isolated from the lymph node of a sarcoidosis patient [20]. These isolates have previously been typed by MLST [20,21].

2.2. Human peripheral blood mononuclear cells and Cutibacterium acnes co-culture

Peripheral blood samples were obtained from healthy volunteers at the *Etablissement Français du Sang*, Pays de la Loire (Nantes, France). PBMCs were isolated by gradient density sedimentation as previously described [22]. All the analyses presented were performed according to the principles expressed in the Helsinki Declaration. Co-culture protocols were based on previously described *Mycobacterium tuberculosis* granuloma experiments by Altare et al. [23]. In brief, PBMCs were adjusted to a final concentration of 10^6 cells/mL per well. Then, *C. acnes* cells were added to the blood cells and incubated at 37 °C, 5% CO_2. Cellular aggregation was followed daily using light microscopy. Uninfected PBMCs were used as the negative control. At various time points of incubation (day 3 and day 7), granuloma structures were processed for microscopic observation, as well as for flow cytometric analysis.

2.3. Colony-forming unit assay

C. acnes growth was measured using a colony-counting technique (colony-forming unit, CFU). After co-culture at different time points, the cells were washed three times with phosphate-buffered saline. Cells in the wells were lysed with Triton ×100 to release the internalized bacteria. Serial dilutions were determined according to the original bacterial number in the culture. Suspensions were spread on Schaedler plates and *C. acnes* colonies were counted after 5 days of incubation at 37 °C under an anaerobic atmosphere. The CFU/mL data obtained corresponded to the bacterial load within the granulomas.

2.4. Flow cytometry

The granuloma structures from co-culture plates were washed twice in phosphate-buffered saline containing 2% FBS and collected at different time points under light microscopy. The cells were suspended in the same buffer to a final volume of 200 μL and stained with a combined fluorescent-conjugated antibodies mix. The antibodies were specific to CD3-BV421 (clone UCHT1, BD Biosciences), CD4-FITC (clone RPA-T4, BD Biosciences), CD8-APC (clone RPA-T8, BD Biosciences), and NKp46-Pecy7 (clone 9E2/NKp46, BD Biosciences). The cells were incubated for 30 min at 4 °C in the dark, washed twice with 2% FBS in phosphate-buffered saline and analyzed by flow cytometry. After gating on CD3 lymphocytes, the $CD3^+$ population was separated into $CD4^+$ and $CD8^+$ T cells. Natural killer cells were gated out from the $CD3^-$ population. All data were acquired using an LSR II instrument (BD Biosciences) and analyzed with FlowJo software version 9.4.10 (Tree Star Inc.) and DIVA software version 6.2 (BD).

3. Results

3.1. PBMCs infected by the sarcoidosis C. acnes isolate generated a higher number of granulomatous structures

The first step in the development of an *in vitro* human model of *C. acnes* granulomas was to induce recruitment of PBMCs around live *C. acnes*. We first determined the optimal multiplicity of infection (MOI) of *C. acnes* for use in subsequent experiments. The monocyte:bacteria MOI ratios studied were 10,000:1, 1000:1, 100:1 and 10:1. The MOIs 100:1 and 10:1 caused premature destruction of granulomatous structures due to an uncontrolled increase in the bacterial load. By contrast, at an MOI of 10,000:1, no cellular aggregation resembling granulomatous structures was observed under light microscopy. The MOI of 1000:1 was chosen for use in subsequent experiments.

PBMCs from five healthy immunocompetent subjects were challenged with the three *C. acnes* isolates. Cell cultures were maintained for up to 14 days post-infection and the occurrence of immune infiltrates was quantified on day 7 and day 14 by light microscopy observation. Cellular aggregation was visible three days after challenge. Between days 4 and 14, distinguishable multicellular and multilayered structures similar to granuloma-like structures were identified under infection conditions. At that time, all the *C. acnes* isolates tested were able to induce dense aggregates. Fig. 1A shows the representative compact immune infiltrates observed with each of the three clinical isolates of *C. acnes* seven days after challenge. On day 7 post-infection, the average number of these structures was between 50 (*C. acnes* BL) and 74 (*C. acnes* S8) per well (Fig. 1B**)**. On day 14, the size of the structures increased for all isolates with no significant difference compared to day 7. The formation of these structures against *C. acnes* also varied depending on the phylogenetic characteristic of each isolate. Fig. 1B shows that *C. acnes* S8 (CC28) developed significantly more granuloma-like structures than *C. acnes* BL (CC36) on day 7 after infection ($p < 0.05$). *C. acnes* BL developed significantly fewer granuloma-like structures than *C. acnes* ATCC6919 (CC18) and *C. acnes* S8 on day 14 after infection ($p < 0.05$).

3.2. Granulomas induced by the PJI C. acnes isolate exerted a greater infection control

After infection of human immune cells, the *C. acnes* burden was assessed by a colony-counting method at day 7 and day 14. The results of the *C. acnes* burden were expressed by the mean of CFU/mL for five subjects. They showed no significant difference in