Defective Monocyte Dynamics in Q Fever Granuloma Deficiency

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Background. The outcome of Q fever, an infectious disease caused by *Coxiella burnetii*, is associated with granuloma formation. Granulomas are present in patients with resolutive Q fever but are lacking in patients with chronic Q fever.

Methods. Study of granuloma formation requires invasive approaches. Here, we took advantage of a recently described method that enables in vitro generation of human granulomas specific for *C. burnetii*.

Results. Circulating mononuclear cells progressively accumulated around beads coated with *C. burnetii* extracts, and complete granulomas were generated in 8 days. Granuloma cells consisted of macrophages, lymphocytes, and, to a lesser extent, epithelioid cells and multinucleated giant cells. Early events that govern granuloma formation were studied using live-imaging microscopy. Monocytes migrated toward *C. burnetii*-coated beads independently of the presence of T lymphocytes and then recruited T lymphocytes. About 90% of patients with chronic Q fever failed to form granulomas. This deficiency was associated with defective migration of monocytes toward coated beads.

Conclusions. Monocytes were involved in the early stages of granuloma formation and recruited T lymphocytes to complete granuloma formation. This article describes a direct relationship between defective granuloma formation and defective migration of monocytes.

Q fever is a zoonosis caused by *Coxiella burnetii*, an obligate intracellular bacterium that inhabits myeloid cells. The natural history of the disease reveals that the primary infection is symptomatic in a minority of exposed individuals. The acute form of Q fever usually results in cure and is associated with protective immune responses and granuloma formation. Q fever may become chronic in patients with valvular damage, in immunocompromised patients with lymphomas, and in pregnant women. In contrast to acute Q fever, chronic Q fever is characterized by defective cell-mediated immunity and granuloma formation [1, 2], suggesting that the formation of granulomas plays a major role in the resolution of Q fever.

Tissue granulomas are defined as a collection of immune cells, including macrophages and related cells such as multinucleated giant cells (MGCs), epithelioid cells, lymphocytes, and, to a lesser degree, neutrophils, eosinophils, and dendritic cells [3]. Tissue granulomas are found during the course of infectious diseases and several noninfectious diseases, including immunological disorders, cancer, and vasculitis. Several key steps characterize the generation of infectious granulomas. The uptake of pathogens by resident macrophages results in the recruitment of circulating leukocytes and in the migration of dendritic cells to lymph nodes, where they present microbial antigens to naïve T cells. Activated lymphocytes are finally recruited to the site of infection and participate in granuloma formation [3]. When granuloma formation results in protection against pathogens, granulomas prevent pathogens from spreading to other sites through an efficient immune response that leads to pathogen degradation. In the majority of patients with tuberculosis, granulomas contain infection through the mineralization of the...
lesions [4]. Thereafter, the attenuation of the immune response via the apoptosis of activated immune cells leads to the progressive disintegration of granulomas [5]. However, granulomas may also participate in the dissemination of pathogens. Indeed, in some patients with tuberculosis, caseum accumulates in the granuloma center, leading to necrosis, collapse of this granuloma center, and the release of infectious bacilli into the airways [4].

A specific feature of Q fever granulomas is the presence of a central clear space and a fibrin ring within granulomas or at their periphery; thus, these structures are referred to as doughnut granulomas [1]. The granulomas observed in the liver of patients with acute Q fever consist of macrophages, lymphocytes, and polymorphonuclear leukocytes [6]. Macrophages with epithelioid morphology, MGCs, and fibrin rings are also found [7]. Bone marrow granulomas similar to those found in the liver are also observed [8]. In contrast to the protective immune response and the presence of granulomas in patients with acute Q fever, the defective immune response observed in patients with chronic Q fever is associated with the absence of granulomas [2]. The major histological pattern found in the liver of patients with chronic Q fever consists of lymphocyte infiltration with foci of spotty necrosis [9].

The study of granulomas in humans requires invasive approaches such as biopsies and necropsies. Here, we took advantage of a recently described method to generate human granulomas in vitro by incubating peripheral blood mononuclear cells (PBMCs) with beads coated with C. burnetii extracts [10, 11]. Granuloma formation was efficient in healthy individuals. We also showed that monocytes first migrated toward C. burnetii–coated beads and then recruited T lymphocytes. In contrast, most patients with chronic Q fever exhibited defective granuloma formation associated with impaired migration of monocytes toward C. burnetii–coated beads. This study, therefore, revealed the critical role of the initial migration of monocytes in granuloma formation and demonstrated that the deficient granuloma formation observed in chronic Q fever was related to the defective migration of monocytes.

**MATERIALS AND METHODS**

**Patients With Q Fever**

Written informed consent was obtained from each subject, and the study was approved by the Ethics Committee of the Université de la Méditerranée. Fifty-three patients with Q fever were included in the study. The diagnosis of acute and chronic Q fever was based on standardized questionnaires that included epidemiological, clinical, and serological features, as previously described [12]. Patients with acute Q fever included 6 men (ranging in age from 48 to 82 years) and 2 women (49 and 54 years old). Patients with chronic Q fever included 31 men and 14 women, with a mean age of 55.5 years (range, 28–83 years). Among these patients, 34 (25 men, 9 women) had established Q fever endocarditis, the major manifestation of Q fever [1].

Patient blood samples were collected in 5 mL ethylenediaminetetraacetic acid (EDTA) tubes, and blood from healthy volunteers was provided by the Etablissement Français du Sang.

**Coupling of Beads With Bacterial Extracts**

The RSA493 Nine Mile strain of C. burnetii, was genotyped as recently described [13]. Phase II (avirulent) organisms were cultured in mouse L929 fibroblasts by repeated passages, and phase I (virulent) organisms were recovered from mouse spleens at 10 days postinfection [14]. Infected cells were sonicated and centrifuged at 300g for 10 minutes to discard cell debris; bacteria were collected after centrifugation at 10000g for 10 minutes. The content in phase I and phase II bacteria of bacterial preparations was determined by indirect immunofluorescence [14]. Only preparations that contained more than 90% phase I bacteria were used. The Mycobacterium bovis bacille Calmette-Guérin (BCG) strain was provided by the Institute Pasteur (CIP105050) and cultured on Middlebrook 7H10 agar medium. The coupling of sepharose beads with bacterial extracts was performed as recently described [11]. Briefly, bacteria were sonicated, and bacterial extracts (0.5 mg proteins corresponding to about 2.5 × 10^8 bacteria) or glycine (0.5 mg), which was used as a control, were added to 10 mg (4 × 10^4 beads) of cyanogen-bromide–activated sepharose 4B beads (GE Healthcare) for 18 hours. The efficiency of coupling bacterial extracts to beads was determined by measuring the protein content of supernatants. Beads were numbered by microscopic examination and stored in phosphate-buffered saline for 1 month at 4°C.

**Formation of Granulomas**

PBMCs were recovered from the Ficoll-Hypaque interface (MSL, Eurobio). Monocytes and T lymphocytes were isolated from PBMCs by positive selection (Miltenyi Biotec) using magnetic beads coated with anti-CD14 and anti-CD3 antibodies, respectively. The purity of monocyte and T-cell fractions was higher than 98%, as determined by flow cytometry. Granuloma formation was studied as recently described [11]. Cells (2.5 × 10^6 PBMCs, 5 × 10^5 monocytes, or 2 × 10^6 T lymphocytes in a 3 mL volume) were suspended in Roswell Park Memorial Institute 1640 medium containing 10% heat-inactivated type AB human serum and antibiotics (Invitrogen) and incubated in 6-well plates with 800 coated beads for different periods of time at 37°C. Granulomas were defined as collections of PBMCs that entirely covered beads. The percentage of beads that formed granulomas was determined by whole optical examination of at least 3 wells per experiment.

**Characterization of Granuloma Cells**

Individual granulomas were collected with a micromanipulator InjectMan NI2 (Eppendorf). They were then incubated...
in 2 mM EDTA (Invitrogen) for 15 minutes at 4°C to dissociate cells. Granuloma cells were analyzed by May-Grünwald-Giemsa staining to identify epithelioid cells and MGCs and by flow cytometry to identify T cells (mouse antihuman CD8-FITC/CD4-PE/CD3-PC5 mAbs), monocytes (CD14-PE mAbs), macrophages (CD68-PE and CD163-FITC mAbs), and dendritic cells (CD11c-PE mAbs) with isotype-matched fluorophore-conjugated immunoglobulin G (IgG) as controls. The percentage of positive cells was determined using a Canto II flow cytometer (Becton Dickinson).

**Characterization of Granuloma Cells**

We investigated the cellular composition of *C. burnetii* granulomas with May-Grünwald-Giemsa staining and immunophenotyping. On day 0, PBMCs were only composed of lymphocytes and monocytes (Figure 2A). On day 10, each granuloma was composed of 705 ± 64 cells, about 40% of which were lymphocytes, 40% of which were macrophages (Figure 2B and 2E), 10% of which were epithelioid cells (Figure 2C and 2E), and about 3% of which were MGCs containing 2–3 nuclei (Figure 2D and 2E). The cellular composition of *C. burnetii* granulomas was similar to that of BCG-induced granulomas (compare Figure 2E with Figure 2F); however, the proportion of MGCs was significantly (*P < .05) lower in *C. burnetii* granulomas than in BCG granulomas.

**Immunophenotyping (Figure 2G and 2H)** revealed that T lymphocytes (CD3<sup>+</sup> cells) represented 33.4% of the total number of granula cells, and about 90% of these T cells were CD4<sup>+</sup> T cells. CD14<sup>high</sup> monocytes were absent from *C. burnetii*-induced granulomas (less than 1%). They were replaced by CD68<sup>high</sup>/CD14<sup>low</sup> macrophages, which represented directionality index (Di) was calculated using the Chemotaxis Tool plugin as the ratio of the distance between the starting point and the ending point of the cell trajectory and the total distance covered by this cell. At least 10 monocytes and 10 lymphocytes were analyzed per experiment, and each experiment was performed 3 times.

**Statistical Analysis**

Results are presented as the mean ± SD and were compared using the Mann–Whitney U test. Differences were considered significant when *P* < .05.

**RESULTS**

**In Vitro Formation of Granulomas**

PBMCs were incubated with beads coated with phase I *C. burnetii* for different periods of time. PBMCs were not associated with beads on day 0 (Figure 1A), but almost all beads were entirely covered by PBMCs on day 8 (Figure 1B). Thereafter, the number of granulomas dramatically decreased, and granulomas were completely dissociated after 24 days (Figure 1C). In contrast, glycine-treated beads were unable to generate granulomas (Figure 1D). The formation of granulomas was related to *C. burnetii* virulence because phase II bacteria did not induce the formation of granulomas (Figure 1E). As a consequence, *C. burnetii* refers only to phase I *C. burnetii* in the subsequent text. Notably, the time courses of the formation of granulomas induced by *C. burnetii* (see Figure 1C) and BCG (Figure 1F) were similar. These results showed that naive PBMCs are able to form granulomas in response to *C. burnetii* organisms.
the main population of granuloma cells (50.9 ± 2.5%). A fraction of these CD68<sup>high</sup> macrophages expressed CD163. Dendritic cells, defined as CD11c<sup>-</sup>CD68<sup>-</sup> cells, were absent from granulomas, representing less than 1% of total cells. C. burnetii– and BCG-induced granulomas shared similar proportions of T lymphocytes, but the proportions of CD68<sup>high</sup>CD14<sup>low</sup> macrophages and CD68<sup>high</sup>CD163<sup>+</sup> macrophages were significantly (P < .05) increased in C. burnetii–induced granulomas compared with BCG-induced granulomas. These results show that the cellular composition of C. burnetii granulomas was specific and was characterized by the predominance of myeloid cells.

Analysis of Early Movements of PBMCs

To understand how monocytes interact with lymphocytes, we chose to investigate the early stages of granuloma formation using a transmitted light live-imaging method. This method avoids the major drawbacks of using fluorescence in long live-imaging experiments such as heterogeneous labeling, fading, and phototoxicity [11], which may alter the mobility of cells in the vicinity of C. burnetii–coated beads. Monocytes and lymphocytes were easily identified using morphological criteria (Figure 3A), and their trajectories were tracked in white and black, respectively (Figure 3B and 3C), with the starting point of each trajectory located at the origin of the graph (0, 0). In the absence of C. burnetii–coated beads, monocytes covered a distance of less than 10 μm (Figure 3D; see also Supplementary Movie 1); however, the distance traveled by monocytes markedly increased in the presence of C. burnetii–coated beads (Figure 3D; see also Supplementary Movie 2). The distances traveled by monocytes in the absence or presence of C. burnetii–coated beads were significantly different (P < .05; Figure 3D). The trajectories of lymphocytes were completely different from those of monocytes. In the absence of beads, lymphocytes moved in a linear and unidirectional fashion (Figure 3E; Supplementary Movie 1). However, in the presence of C. burnetii–coated beads, the majority of lymphocytes turned around the bead and briefly interacted with beads (Figure 3E; Supplementary Movie 2). The value of lymphocyte Di was significantly (P < .05) lower in the presence of C. burnetii–coated beads than in the absence of the beads (Figure 3E).

Figure 2 continued. The results are presented as the mean ± SD and are representative of 3 experiments. G, Cell phenotyping was performed by flow cytometry. In gray areas, isotype controls; in black, PBMCs on day 0; in red, granuloma cells. CD68<sup>high</sup> macrophages were analyzed according to the expression of CD163. Representative experiments are shown. H, The results of flow cytometry experiments, expressed as the percentage of positive cells, are representative of 4 experiments. Results were compared using the Mann–Whitney U test. *P < .05. Abbreviations: BCG, Mycobacterium bovis bacille Calmette-Guérin; EDTA, ethylenediaminetetraacetic acid, MGCs, multinucleated giant cells; PBMCs, peripheral blood mononuclear cells.
To determine whether the changes in monocyte and lymphocyte trajectories were due to the beads or to the bacterial extracts, we studied these cells’ trajectories in the presence of glycine-coated beads. Lymphocyte and monocyte trajectories in the presence of glycine-coated beads were similar to those found in the absence of *C. burnetii*-coated beads (Figure 3D and 3E, respectively), demonstrating that the observed changes in monocyte and lymphocyte trajectories were dependent on *C. burnetii*.

**Monocytes Initiate Granuloma Formation**

Because monocytes and T lymphocytes were recruited differently by *C. burnetii*-coated beads, we investigated their respective roles in granuloma formation. Monocytes, but not T lymphocytes, induced granuloma formation (Figure 4A). The percentage of beads that formed granulomas was lower when monocytes were used alone than in PBMCs. In addition, the number of granuloma cells per bead (132 ± 36 vs 705 ± 64) was decreased. This pattern suggests that efficient granuloma formation required both monocytes and lymphocytes. The number of epithelioid cells was significantly lower (*P* < .05) in monocyte-generated granulomas than in granulomas formed in the presence of both monocytes and lymphocytes (10 ± 6 vs 70 ± 12 per granuloma), suggesting that lymphocytes were necessary for epithelioid cell differentiation. In contrast, the number of MGCs and the number of nuclei per MGC were similar in both types of granulomas, suggesting that MGC differentiation did not require lymphocytes.
Next, we investigated the role of isolated monocytes by recording the early events of granuloma formation. To achieve this, we performed live imaging during the first 2 hours of granuloma formation. In the presence of C. burnetii–coated beads, monocytes (tracked in white) moved toward the beads (Figure 4B and 4D), and the distance they covered was similar to that covered by monocytes present in PBMCs (Figure 4F). The trajectories of isolated T lymphocytes (tracked in black, Figure 4C) incubated with C. burnetii–coated beads were linear (Figure 4E), with a Di value significantly different (P < .05) from that of T lymphocytes present in PBMCs (Figure 4G). These results indicated that granulomas were initiated by the interaction of monocytes with C. burnetii–coated beads and that complete granuloma formation required the early recruitment of T lymphocytes.

**Defective Granuloma Formation in Patients With Chronic Q Fever**

The ability to form granulomas has a critical effect on the outcome of Q fever, but granuloma formation cannot be investigated without the use of invasive approaches. Using an in vitro method, we were able to quantitatively assess granuloma formation in patients over an 18-day period. In 12 patients (8 with acute Q fever and 4 with chronic Q fever), the kinetics of granuloma formation were similar to those of control PBMCs. In contrast, 30 patients with chronic Q fever were unable to form granulomas during a period that ranged from 1 to 18 days (Figure 5A), and the PBMCs of 11 patients with chronic Q fever displayed partial granulomatous responses (Figure 5B). The mechanisms leading to defective granuloma formation were investigated in 3 patients with chronic Q fever. The trajectories of monocytes and lymphocytes were tracked in black and white, respectively (Figure 5C). Monocytes from these chronic Q fever patients covered short distances (Figure 5D). The distances covered by these monocytes were significantly shorter (P < .05) than those covered by monocytes from healthy subjects (Figure 5E) and were similar to those covered by monocytes from healthy donors in the absence of C. burnetii–coated beads (see Figure 3D). About 80% of lymphocytes moved with linear trajectories (Figure 5F), and the differences in Di values between patients with chronic Q fever and healthy subjects were significant (P < .05; Figure 5G). Taken together, these results indicate that monocytes from patients with chronic Q fever were unable to move efficiently toward C. burnetii–coated beads.

**DISCUSSION**

In this article, we showed that granulomatous-like structures formed in vitro appeared in some days, mimicking the granuloma formation observed in patients with acute Q fever [1, 6, 7]. The in vitro–generated granulomas consisted largely of...
macrophages, CD4⁺ T lymphocytes, and, to a lesser degree, epithelioid cells and MGCs that likely resulted from macrophage fusion [15]. The cellular composition of C. burnetii–induced granulomas was at least partially specific, with higher proportions of macrophages expressing CD163 and lower proportions of MGCs than were observed in BCG-induced granulomas. CD163, which is exclusively expressed on myeloid cells, is considered a marker of M2 alternatively activated macrophages, and its expression is upregulated by immunoregulatory cytokines such as interleukin-10 [16, 17]. We previously reported that the interaction of human macrophages with C. burnetii polarizes them toward an atypical M2 program [18]. The in vitro–generated granulomas disintegrated after 3 weeks. A similar time course of granuloma disintegration has been found in mice infected with C. burnetii [19]. The mechanisms governing the disintegration of C. burnetii–induced granulomas may include nutrient exhaustion, imbalance in the release of immunomodulatory cytokines, [18] and/or cell apoptosis [5].

Interestingly, virulent C. burnetii induced granuloma formation, but avirulent variants did not. The main difference between phase I and phase II bacteria is the composition of their lipopolysaccharides (LPSs) [20]. This difference leads to dramatic consequences for myeloid cells, including LPS recognition by Toll-like receptors (TLRs) [21, 22], bacterial uptake [23], and cytokine production [24]. It has been shown that the LPS from phase I C. burnetii is involved in granuloma formation in mice through its interaction with TLR4 [21, 25]. Other bacterial components may play a role in granuloma formation. Indeed, the internalization of phase I C. burnetii by monocytes requires αvβ3 integrin, whereas that of phase II bacteria requires αvβ3 and α2β2 integrins [23], suggesting that different bacterial ligands are recognized by monocytes. New genetic tools suggest that predicted virulence factors include the type IV secretion system [26]. It is likely that the interaction of circulating monocytes (see below) with phase I and phase II bacterial extracts induces different signals, some of which lead to granuloma formation.

In our study of the early stages of granuloma formation by live imaging, we noted that the presence of C. burnetii–coated beads specifically affected the trajectories of PBMCs according to the following scenario. In the first step, monocytes moved toward and adhered to beads in a lymphocyte-independent way. In the subsequent step, T lymphocytes were recruited by the forming granuloma. These results are consistent with previous animal studies. In mice, M. bovis is cleared from blood through direct capture by liver resident macrophages that nucleate granuloma formation through the recruitment of uninfected resident macrophages and circulating monocytes. In a second step, CD4⁺ T cells migrate in close apposition to myeloid cells [27]. In zebrafish embryos, which lack lymphocytes, mycobacterial infection leads to macrophage aggregation, formation of granuloma-like structures, and macrophage differentiation into epithelioid cells and multinucleated cells [28]. We also showed that monocytes induced the formation of

Figure 5. Granuloma formation in patients with Q fever. PBMCs from patients with Q fever were incubated with beads coated with phase I Coxiella burnetii for 18 days. The percentage of granulomas was determined by optical examination. A and B, The granulomatous response of patients was considered largely inhibited (A) or partially inhibited (B). The results are presented as the mean ± SD of 2 experiments performed in triplicate for each patient. C–G, PBMCs from 1 patient unable to generate granulomas were incubated with C. burnetii–coated beads, and cell trajectories were studied by live imaging. Monocytes and lymphocytes are tracked in white and black, respectively (C). The trajectories of monocytes (D) and lymphocytes (F) were determined, and the distances covered by monocytes (E) and lymphocytes (G) were compared with those of monocytes and lymphocytes present in control PBMCs. The experiments were performed with cells from 3 patients, and at least 10 cells per experiment were tracked. One representative experiment is shown. Abbreviations: PBMCs, peripheral blood mononuclear cells; QF, Q fever.

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granulomas and differentiated into MGCs even in the absence of lymphocytes. Because the proportion of epithelioid cells was smaller than that found in granulomas generated in the presence of T lymphocytes, we can hypothesize that T lymphocytes contributed to macrophage differentiation into epithelioid cells.

Finally, we explored granuloma formation in patients with Q fever. Patients with acute Q fever presented a normal granulomatous response, emphasizing clinical data that demonstrate that defective granulomatous response is only associated with chronic Q fever [1]. In contrast, the granulomatous response was defective in the majority of patients with chronic Q fever. This defective response was not related to gender, age, specific IgG titers, or the clinical presentation of chronic Q fever (endocarditis vs no endocarditis). Granuloma formation could not be used to measure the activity of the disease because some apparently cured patients were unable to mount a granulomatous response. It may be useful to develop a longitudinal approach in which individual patients are followed to relate defective granulomatous responses and prognosis. In our study, defective granuloma formation and defective ability of monocytes to move toward C. burnetii–coated beads were associated. These results emphasize the importance of C. burnetii on myeloid cells. C. burnetii specifically infects myeloid cells and hijacks their microbioidal functions [29]. C. burnetii–infected monocytes [30] and monocytes from patients with chronic Q fever [31] have been reported to display inhibited transendothelial migration due to modulation of chemokine networks [32]. We can hypothesize that in chronic Q fever, the inability of monocytes to move toward the site of infection prevents local lymphocyte recruitment, leading to defective granuloma formation and nonprotective immune responses.

In conclusion, we have shown that monocytes play an essential role in the early stages of granuloma formation in human C. burnetii infection. The defective granuloma formation observed in chronic Q fever may be related to the inability of monocytes to induce the nucleation of nascent granulomas.

Supplementary Data

Supplementary materials are available at The Journal of Infectious Diseases online (http://www.oxfordjournals.org/our_journals/jid/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyrighted. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

Potential conflicts of interest. All authors: No reported conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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