Neutrophils dampen adaptive immunity in brucellosis

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ABSTRACT

Brucella organisms are intracellular stealth pathogens of animals and humans. These bacteria overcome the assault of innate immunity at early stages of the infection. Removal of neutrophils (PMNs) at the onset of adaptive immunity against Brucella abortus, favored the bacterial elimination in mice. This was associated with higher levels of IFN-γ, and higher proportion of cells expressing IL-6 and iNOS, compatible with M1 macrophages, in the PMN-depleted B. abortus infected mice (PMNd-Br-mice). At later times of the acute infection phase, the amounts of IFN-γ lowered while the IL-6, IL-10 and IL-12 became the predominant cytokines in the PMNd-Br-mice. IL-4, IL-1β and TNF-α remained at background levels at all times of the infection. Depletion of PMNs at the acute stages of infection promoted the premature resolution of spleen inflammation. The efficient removal of bacteria in the PMNd-Br-mice was not due to the increase of antibodies, since the immunoglobulin isotype responses to Brucella antigens were dampen. Anti-Brucella antibodies abrogated the production of IL-6, IL-10 and IL-12 but did not affect the levels of IFN-γ at later stages of infection in the PMNd-Br-mice. These results demonstrate that PMNs have an active role in modulating the course of B. abortus infection, after the adaptive immune response has already developed.
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INTRODUCTION

Polymorphonuclear neutrophils (PMNs) are essential elements of innate immunity and the first line of defense against microbial invaders. These cells phagocytize and destroy bacteria, release cytokines and activate elements of the innate immune response (1). However, PMNs also modulate components of adaptive immunity, a phenomenon that has gained considerable attention in the last years (2, 3).

Neutropenic murine models have been used to dissect the role of PMNs during innate and adaptive immune responses against microbial infections. The selective depletion of PMNs by means of antibodies is the most common and widespread model (4–8). A second model includes a mutant mouse strain named Genista, which is devoid of mature PMNs (4, 9–11). Both models have their advantages and drawbacks; though, they generally display good correlation and render similar results (4, 11). Neutopenia in the anti-PMN depletion model is transient and cannot be maintained beyond one week. Still, the advantage of this model is that the neutropenic condition can be induced at any stage of the infection (12–14).

We have used both, the Genista and the anti-PMN models to explore the role of PMNs and innate immune response during the onset of Brucella abortus infection (4, 15). Brucella organisms are intracellular stealth pathogens of animals and humans that avoid the activation of innate immunity, remaining in several tissues for protracted periods of time (15–17). B. abortus readily invade PMNs, resisting the killing action of these leucocytes (15, 18–22). This correlates with the endurance and modification of the bacterial cell
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evelope components, which barely promote the generation of reactive oxygen species and proinflammatory cytokines in the infected PMNs (15, 19). In addition, through its lipopolysaccharide (Br-LPS), B. abortus mediates in a non-phlogistic manner the premature cell death of PMNs and induces the expression of “eat me” signals on these cells (19, 21). The absence of PMNs at the onset of B. abortus infection, stimulates the recruitment of monocyte/dendritic cells, favors the activation of B and T lymphocytes, and promotes the production of Th1 cytokines (4).

The course of human brucellosis parallels that observed in mice (16, 23). In the mouse model, brucellosis is divided into four phases according to the bacterial colonization of the target organs, the pathological signs, and the profile of the immune response (17, 23). The first phase corresponds to the onset of infection (also known as the incubation stage) which typically extends 2 to 3 days. During this phase, the production of proinflammatory cytokines and the activation of innate immunity are negligible (4). The acute phase follows, extending 2-3 weeks. Active bacterial replication and high levels of Th1 cytokines characterize this phase (23, 24). Then, the chronic steady phase, lasting from 8 to 11 weeks, corresponding to the plateau of the infection. Finally, the chronic declining phase depicted by the gradual elimination of bacteria. This phase may last months or even years (23, 24). During the acute and chronic phases high amounts of anti-Br-LPS antibodies are produced (25). At these stages, the bone marrow (BM) is colonized by Brucella organisms, keeping for protracted periods of time high bacterial loads within BM-PMNs and to a minor extent in monocytes and stem cells (17). This is significant, since PMNs in other target organs such as the spleen, do not harbor Brucella (26).
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Here, we describe how PMNs modulate adaptive immunity at the initial stages of acute murine brucellosis. The results presented here reinforce our previous hypothesis (4) and give new insights into the role that PMNs have in shaping the immune response during brucellosis.

MATERIALS AND METHODS

Ethics. Experimentation in mice was conducted following the guidelines of the “Comité Institucional para el Cuido y Uso de los Animales” of the Universidad de Costa Rica (CICUA-019-16), and in agreement with the corresponding law, “Ley de Bienestar de los Animales, de Costa Rica” (law 9458 on animal welfare). Mice were housed in the animal facility of the Veterinary Medicine School of the National University, Costa Rica. Mice were kept in cages with food and water ad libitum under biosafety conditions.

Generation of neutropenic mice. Inbred C57BL/6 (18 to 21 g) were used in the experiments. Neutropenic mice were generated as previously described (4, 15). Briefly, mice were depleted of PMNs by means of intraperitoneal (i.p.) injection of 100 μg of rat anti-mouse Ly-6G/Ly-6C (Gr-1) (clone RB6-8C5) (Bio X Cell) or 500 μg of anti-mouse Ly6G (clone 1A8) (BD Biosciences) in 0.1 mL PBS. PMN depletion was confirmed by the absence of CD11b+ Ly6G+ cells by flow cytometry and microscopic examination of blood, spleen, lymph nodes and BM (4, Table S1). A single i.p. injection of anti-PMN antibody resulted in the depletion of PMNs from blood, spleen and lymph nodes for at least three
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days (Table S1). PMN depletion in the BM, was achieved only to 25-30% (Table S1). Differences in depletion were observed between RB6-8C5 and 1A8 antibodies. In order to maintain the neutropenic stage, mice were injected with the indicated antibody, every three days according to the different protocols: i) depletion at the onset of innate immunity ii) depletion at the onset of adaptive immunity and, iii) depletion at the acute phase of infection (Fig. S1). In all experiments, non-immune rat IgG was used as a “mock-control” and administered at the same concentrations and route as the anti-PMN antibodies. After eight days of the first anti-PMN injection, mice developed antibodies against the anti-PMN antibody (4). A detailed time course protocol and kinetics for the RB6-8C5-PMN depletion after B. abortus infection has been reported (4).

Brucella abortus infection. Mock-treated and PMN-depleted mice were i.p. infected with 0.1 mL of PBS containing 10⁶ CFUs of virulent B. abortus 2308W (27) as described elsewhere (4). Bacterial colonization was determined in spleens and BM of mice collected at the indicated times following previous protocols (4, 17, 28). Serial dilutions of infected macerated tissues were plated on trypticase soy agar, incubated at 37°C for 72 hours in the presence 5% CO2 and bacterial colony forming units (CFUs) determined (28). Spleens from mice were processed for histopathological studies as described before (29). Blinded evaluation of histopathology slides was performed. The inflammatory stage was evaluated using a semi-quantitative scoring system (30).
Antibodies and cytokines determination. Murine hyper-immune serum production against Brucella antigens and IgG purification were carried out following previous protocols (21). PMN-depleted and the mock-control mice were bled at different times, sera separated from cells, and antibody titration carried out in 96-well round-bottom plastic plates, as described before (21). After titration, immune sera were stored at -20°C in aliquots. Western blotting revealed that most antibody recognition was directed against Br-LPS. For isotype antibody determination against Br-LPS, ELISAs were performed on 96-well plates as previously described (31). Briefly, 96-well plates (Nunc) were coated with 0.1 mL of 10 µg/mL of Br-LPS. Mouse serum was diluted 1:200 in blocking buffer (PBS with 0.4% BSA, 0.05% Tween 20) and then incubated on plates for 1h at 37°C, followed by extensive washings (PBS with 0.05% Tween 20). Secondary horseradish peroxidase antibody conjugates against mouse IgG, IgM, IgG1, IgG2a, IgG2b, and IgA (all from Sigma-Aldrich) at the adjusted dilution in the blocking buffer were used for immunoglobulin isotyping. After washing the plates, the reaction was developed with HRP substrate (Sigma-Aldrich) and the optical density measured at 450 nm. Serial dilutions of the murine hyper-immune serum (positive control serum) and the respective conjugates were performed in order to establish the optimal cut off values for each conjugate, in comparison to sera from uninfected mice. The negative serum optical density for each conjugate was adjusted at 0.110 ± 0.025 nm, while the positive control serum optical density was adjusted at 1.200 ± 0.150 nm. The cut off value was estimated at 0.200 nm.
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The levels of IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12, IFN-γ, TNF-α, cytokines were measured in sera by ELISA (eBioscience), according to the manufacturer’s specifications.

Flow cytometry. Flow cytometry was carried out as previously described (4). PE anti-CD11b (M1/70), Alexa Fluor 488 anti-Ly6C (AL-21), PE Cyanine 5.5 anti-Ly6G (1A8), antibodies were purchased from BD Biosciences, and 1A8 and RB6-8C5 neutralizing antibodies were from Bio X Cell. Blood, spleen and bone marrow cells were prepared as described before (4, 17). Popliteal, inguinal and mesenteric lymph nodes were prepared as described elsewhere (32) and processed for flow cytometry as described before (4). Intracellular staining was performed with APC anti-iNOS (clone CXNFT) and PE anti-IL6 (clone MP5-20F3) with the respective isotype controls all from Invitrogen. Before staining with different antibody mixes, cells were pre-incubated on ice for at least 10 min with the anti-mouse CD16/CD32 (clone 2.4G2) monoclonal antibody to block Fc receptors (BD Biosciences). Multiparameter FACS analysis was performed using a Guava easyCyte flow cytometer (Millipore). FACS data were analyzed using (Flow Jo software, version 10.4).

For each experiment, control mice were included to define the proper gates. Blood was stained directly with the antibodies and lysed with BD FACS lysing buffer (BD-Biosciences). If mice had been previously treated with PMN depleting antibodies, blood samples were washed thoroughly (four times) with PBS to remove anti-Ly6G from serum, before the staining process. All samples were washed and resuspended in PBS prior to acquisition.
Statistics. The data was processed in Microsoft Office Excel. To determine statistical significance, comparison of two samples was performed by a Mann-Whitney test and multiple comparisons were established by Kruskal-Wallis test using the GraphPad software package (version 7.0 GraphPad Prism software, La Jolla, CA, USA). For antibody isotype comparison values were normalized by adjusting the measurements of the different scales to a notionally common scale. For all tests, values of p<0.05 (*) and p<0.01 (**) were considered statistically significant.

RESULTS

The absence of PMNs enhanced the removal of *B. abortus* in mice. We have shown that the absence of PMNs at the onset of *B. abortus* infection, enhances the bacterial removal after several days (4). Following this, we explored whether the absence of PMNs have any influence at the onset of adaptive immunity, once Th1 cytokines and specific antibodies have developed (21). For this, the protocols described in Fig S1 A and B were followed. After the sixth day of infection (one day after PMN-depletion), we observed an initial increase of bacterial loads in the spleens of PMNd-Br-mice (Fig 1A). This outcome agreed with our previous results (4). After 14 days of *B. abortus* infection (nine days post-PMN depletion) the number of CFUs in the spleen of PMNd-Br-mice reached similar values as the non-PMN depleted controls (Fig 1A); though, PMNd-Br-mice showed more efficient
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bacterial removal rates (Fig 1B). This phenomenon was more conspicuous after 30 days of infection (15 days of PMN depletion) (Fig 2).

RB6-8C5 antibody partially depletes a subpopulation of monocytes (33, Table S1). Therefore, we repeated the experiment using the anti-PMN antibody from the 1A8 clone, claimed to be highly specific for murine PMNs (33). Similar results using this antibody were observed (Fig S2). However, the elimination of bacteria was more evident in the BM, regardless of the antibody used to deplete PMNs (Fig 3). This was striking, since during the chronic stages the presence of Brucella organisms in the BM is marked (17) and in contrast to other tissues the BM keeps a proportion of PMNs after depletion of these cells (Table S1).

It is worth mentioning that the 1A8 antibody has several drawbacks in comparison to the RB6-8C5 antibody. To achieve a significant PMN depletion, very high doses of 1A8 antibody (500 µg/mouse) were required. In spite of this, depletion seldom reached more than 95% of blood PMNs (Table S1) and neutropenia was not as steadily maintained as with the RB6-8C5 antibody. Similar results have been recorded by other authors (33). Regardless of this, the overall elimination of bacteria was more efficient in the PMNd-Br-mice than in the infected controls.

B. abortus infection enhanced cytokine production in neutropenic mice. At the onset of B. abortus infection, the levels of proinflammatory cytokines are negligible. This event agrees with the furtive strategy of Brucella (15). However, once the infection has been established (after five days), there is an increase in IFN-γ production; the most relevant
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cytokine for mounting an efficient immune response against *Brucella* sp. infections (34, 35). As expected, after six days of infection the levels of IFN-γ were already high in the mock-control mice (Fig 4). Still, the amounts of IFN-γ doubled in PMNd-*Br-*mice (one day of PMN depletion), with negligible or low production of other cytokines (Fig 4). After 14 days of infection (nine days of PMN depletion), the levels of IFN-γ decreased, but the regulatory IL-10 and other cytokines such as IL-12 and IL-6 significantly increased (Fig 4). Similar results in the levels of IFN-γ were observed using the 1A8 antibody for PMN depletion (Fig S3).

Unexpectedly, the day after PMN depletion (six days of *B. abortus* infection) the PMNd-*Br-*mice showed clinical symptoms such as lethargy, piloerection, anorexia, and general malaise. Weight loss of the PMNd-*Br-*mice was evident after 14 days of infection (nine days of PMN depletion) (Fig 5). However, 30 days after infection (25 days of PMN depletion) the weight of the PMNd-*Br-*mice increased in comparison to the mock-controls, suggesting health improvement due to better bacterial removal (Fig 5). Similar results were obtained with the 1A8 antibody.

The absence of PMNs promoted the premature resolution of spleen inflammation in infected mice. The removal of PMNs at the onset of *B. abortus* infection induces premature granulomatous, inflammation and follicular hyperplasia of the spleen, characterized by augmented infiltration of epithelioid histiocytes (4). In contrast, the removal of PMNs after the immune response has been established, induced a different pathological effect in the infected mice (Fig 6). As expected, after six days of infection (one day of PMN depletion),...
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PMNd-Br-mice showed no significant differences in spleen inflammation (Fig 6 A and B). However, the absence of PMNs at the acute stages of adaptive immunity favored the fast resolution of spleen inflammation (Fig 6). Indeed, after 14 days of infection (nine days of PMN depletion), PMNd-Br-mice showed lower number of granulomas, reduced vasodilation, lower follicular hyperplasia, and less hyperemia (Fig 6 C and D) than the spleens of the mock-controls (Fig 6 E). As shown before (4), the sole depletion of PMNs did not induce pathological alterations in the target organs of non-infected mice.

Neutropenic mice showed lower antibody responses against B. abortus antigens. It has been demonstrated that IFN-γ influences the immunoglobulin isotypes against Brucella antigens (Finkelman et al., 1988). Following this, we investigated if the reduced bacterial loads in the neutropenic mice could be due to higher antibody titers or to an increase of specific antibody isotypes against Br-LPS, the most relevant antigen in brucellosis (16). In comparison to the mock-controls, the PMNd-Br-mice displayed lower antibody agglutination titers after 21 and 30 days of infection (16 and 25 days of PMN depletion, respectively) (Fig. 7A). These titers correlated with the general lower amounts of the immunoglobulin isotypes against Br-LPS, at both times; being more evident after 30 days of infection (25 days of PMN depletion) (Fig. 7 B). A similar trend was recorded after 30 days of infection (25 days of PMN depletion) when the 1A8 antibody was used for PMN depletion. However, it was less conspicuous than that observed with the RB6-8C5 antibody and a significant increase of IgG3 production was observed (Fig S4).
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The absence of PMNs promoted M1 macrophage polarization. Brucella organisms manipulate the PPARγ pathway to avoid M1 macrophage polarization and benefit from a nutrient rich environment of alternatively activated M2 macrophages (30). Since M1>M2 polarization is promoted by IFN-γ, we explored the proportion of M1 cells in the PMNd-Br-mice. As shown in Fig 8 (A and B), the relative amounts of lymph node Ly6C+/Ly6CHi cells were enhanced in the PMNd-Br-mice, after 9 days of infection. When Ly6C+/Ly6CHi cells were analyzed for intracellular IL-6 and iNOS (markers for M1 macrophages), the proportion of macrophages displaying these markers was higher in the PMNd-Br-mice (Fig 8 C and D).

Anti-Brucella antibodies abrogated IFN-γ production at the onset but not at later times of infection. Since the high amounts of IFN-γ inversely correlated with the antibody titers in the PMNd-Br-mice, then we injected non-sterilizing amounts of anti-Brucella antibodies at different infection times. Anti-Brucella serum given one day before infection, completely abrogated the IFN-γ response in mice and lowered the bacterial loads (Fig 9), regardless of the presence or absence of PMNs. However, if the same antibody regime was given after 6 days of infection, the levels of IFN-γ remained unchanged (Fig 9 B).

Moreover, after treatment with the corresponding antibodies at 6, 9 and 12 days after infection (Fig S1), the levels of IFN-γ were not significantly different at 14 days of infection (Fig 9 C); though, the bacterial loads were still lower in relation to the mock-controls (Fig 9 E).
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Anti-Brucella antibodies abrogated IL-6, IL-10 and IL-12 cytokines in neutropenic mice. Since IL-6, IL-10 and IL-12 cytokines considerably raised in the PMNd-Br-mice at later stages of acute infection (day 14 of infection, day 9 post depletion, Fig 4), then we explored the effect of anti-Brucella antibodies at these times in the PMNd-Br-mice. In contrast to IFN-γ, anti-Brucella antibodies dampen the levels of IL-6, IL-10 and IL-12 in the PMNd-Br-mice (Fig 10). Other cytokines such as IL-4, TNF-α and IL-β remained close to background levels at all times and in all experiments (Fig 4 and Fig 10).

DISCUSSION

We have shown that PMN removal before the development of adaptive immunity promotes the elimination of B. abortus from the target organs at the onset of infection (4). This phenomenon is linked to the efficient recruitment of macrophages and dendritic cells, stronger activation of CD4+ and CD8+ T lymphocytes and the concomitant increase of IFN-γ (4). Here, we have complemented these findings and demonstrated that the absence of PMNs after the adaptive immunity has fully developed, also favored the efficient elimination of B. abortus in mice.

These results seem counterintuitive, mainly when they are compared to the positive role that PMNs play in controlling other bacterial infections, such as Salmonella, Yersinia, Legionella and Listeria organisms (7, 8, 11). In the case of Brucella sp. infections the primary microbicidal function of PMNs is not achieved. Rather, Brucella organisms induce the premature cell death of PMNs in a non-phlogistic manner (19) and dampen the
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regulatory influence that PMNs have on adaptive immunity at different stages of the infection.

It is known that M1 macrophages are the first line of defense against intracellular pathogens, including *Brucella* organisms (36, 37). The higher production of IFN-γ correlated with the activation of these cells, the resolution of inflammation and the efficient elimination of the bacteria in the PMNd-Br-mice. Under the effect of IFN-γ, M1 macrophages differentiate, increase their microbicidal activity and amplify Th1 polarization of CD4+ lymphocytes by IL-12 production (37, 38).

The higher secretion of IFN-γ was a common feature in both, the PMNd-Br-mice at the onset of infection (4) and PMNd-Br-mice after adaptive immunity has emerged. In spite of this, some significant differences were observed. For instance, the levels of IFN-γ produced at the onset of infection were lower (4) than those recorded once adaptive immunity has developed. While in the former case the levels of INF-γ were not associated with sickness, in the latter case weight lost and cachexia were observed in the neutropenic infected mice. This was an unexpected clinical feature. Indeed, *Brucella* infected mice seldom show sickness during the early days of infection (23). It seemed, therefore, that the very high levels of IFN-γ (close to 7500 pg/mL) and the subsequent activation of the immune system was not without a price (39).

It has been shown that IL-12 is an essential cytokine to keep Th1 response in brucellosis (40). The higher levels of IL-12 at later times of the acute infection in the PMNd-Br-mice, revealed no shift towards Th2 response. Moreover, cytokine IL-4 always remained close to background levels. The negligible amounts of IL-4 in the sera and spleen cells of infected
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neutrophils during brucellosis is a well-known feature and draws the Th1 predominant immune response (15, 23). It is also known that removal of IL-4 depresses anti-Brucella antibody response, indirectly favoring the Th1 response (41).

The rise of IL-10 and IL-6 in the PMN-depleted infected mice at later times of infection, correlated with the decreasing levels of IFN-γ. The lack of IL-10 has been related to lower B. abortus survival at early stages of infection and linked to the regulation of INF-γ at later stages (30, 41). Likewise, the IL-6 limits the recruitment of innate immune cells, and therefore represents a critical event in the regulation of inflammation (42). Raise of IL-6 has also been observed at the onset of the infection in PMNd-Br-mice, including the Genista (4).

It is worth noting that anti-Brucella antibodies only damped the IFN-γ when given before the infection. This seems related to the fast removal of bacteria that hampers the development of adaptive immunity, a trend observed before with other bacteria (43, 44).

However, after initiation of adaptive immunity, anti-Brucella antibodies did not influence the levels of IFN-γ, regardless the presence or absence of PMNs or the number of bacteria. This agrees with previous data, showing that the levels of IFN-γ generated are independent of the bacterial loads in B. abortus infected mice (4). In contrast, anti-Brucella antibodies dampen IL-6, IL-10 and IL-12 in neutropenic mice at later stages of the acute infection (compare Fig 4 with Fig 10), a fact that correlates with the lower amounts of bacteria in the treated mice.

It was clear that the efficient elimination of bacteria in the neutropenic mice was not linked to the rise of antibodies, or to increased levels of specific immunoglobulin isotypes against
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Brucella antigens. On the contrary, lower antibodies titers were observed after three and four weeks of infection. This may be the result of a stronger cellular immunity over an antibody response, promoted by the high levels of IFN-γ during the acute phase of infection. The fact that IFN-γ exerts a regulatory influence on the production of immunoglobulin isotypes against Brucella antigens, supports this (45). In addition, mice devoid of B cells (thus, deprived of antibodies), eliminates B. abortus more efficiently; an event that is linked to the higher levels of IFN-γ and to a stronger cellular Th1 response (46). Although the lower bacterial loads could have had some influence on the antibody titers, this seems unlikely. It has been shown that once antibodies are produced, they remain at the same high levels, regardless on the number of Brucella organisms present in the target organs (47). Likewise, an increase in bacterial loads was recorded in the PMNd-Br- mice after the first day of PMN removal (Fig 1 and Fig 2).

The ability of Brucella organisms to develop chronic infections is linked to their long persistence in the BM (17, 48). In humans and mice, the colonization of the BM by Brucella organisms causes neutropenia, thrombocytopenia, anemia, pancytopenia and other pathological signs (17, 48, 49). The bacterium resides within BM monocytes, PMNs and to less extent, granulocyte-monocyte progenitors (17). Therefore, the abrogation of the B. abortus infection in the BM of PMNd-Br-mice was intriguing, considering the significant number of PMNs remaining in the BM after repeated injections with anti-PMN (Table S1). Whether anti-PMN antibodies mostly removed mature and functional PMNs from the BM, remains to be studied.
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The precise routes by which PMNs regulate other cells of the immune system remain elusive and those proposed for other pathogens do not match with our observations (50, 51). For instance, in a murine model of Legionella pneumophila, PMN depletion led to more Th2 skewing and more disease (8). This is striking since in both bacterial diseases IFN-γ plays a central role (34, 35, 52) and the pathogenic mechanisms and intracellular life cycle of these two bacteria display some resemblances (53). Other regulatory mechanisms such as direct contact between PMNs with lymphocytes, macrophages/monocytes and dendritic cells have been discussed before (4). The regulation through PMNs cytokines seems unlikely, since the amounts of proinflammatory cytokines released by B. abortus infected PMNs are negligible (19).

One alternative mechanism that explains the phenomenon observed here, relates to the “Trojan horse” hypothesis (19, 24). This mechanism proposes that prematurely dying Brucella-infected PMNs displaying “eat me” signals are readily phagocytized by cells of the mononuclear phagocytic system in a non-phlogistic manner (19). This opens a window for the brucellae intracellular trafficking to the endoplasmic reticulum and eventual replication in these phagocytic cells. In course, this will delay the activation of the adaptive immune system, allowing this stealthy organism to establish a long-lasting infection (11, 15, 24). In the absence of PMNs, this mechanism will be “shattered”, allowing mononuclear phagocytic cells to interact directly with the bacterium in a proinflammatory manner. In course, this will allow the strong activation of the immune system, reflected by an increased release of IFN-γ by CD4+ and CD8+ and polarization of macrophages towards M1, which are central for combating intracellular parasites. This proposal fits with the
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Occam's razor principle of parsimony, previous experimental data and the results presented here.

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FIGURE LEGENDS

Figure 1. PMN depletion at the onset of adaptive immunity promoted *Brucella* removal. C57BL/6 mice were i. p. infected with 0.1 mL of $10^6$ CFUs of *B. abortus* 2308W. After 5 days of infection, one group of mice was depleted of PMNs by means of i. p. injection of RB6-8C5 anti-PMN. (A) At the indicated times CFU/spleen and spleen weights were determined. Each symbol represents one animal, and the lines represent the median for each group. (B) Rate of change in CFU/spleen ($\Delta$ CFU/spleen) and CFU/spleen weight ($\Delta$ CFU/g of spleen) were calculated over time using the following equations:

$\Delta$CFU/spleen=mean CFUs 14 days/CFUs 6 day ± SD; $\Delta$CFU/g of spleen=mean CFU/ g of spleen 14 days/6 days ± SD. Bars represent standard deviation. Values of $p<0.05$ (*) and $p<0.01$ (**) in relation to the mock-controls are indicated below the bars.

Figure 2. PMN depletion at the acute infection period promoted the *Brucella* removal. C57BL/6 mice were i. p. infected with 0.1 mL of $10^6$ CFUs of *B. abortus* 2308W. After 15 days of infection, one group of mice was depleted of PMNs by means of i. p. injection of RB6-8C5 anti-PMN. (A) Then at the indicated times CFU/spleen and spleen weights were determined. Each symbol represents one animal, and the lines represent the median for each group. (B) Rate of change in CFU/spleen ($\Delta$ CFU/spleen) and CFU/spleen weight ($\Delta$ CFU/g of spleen) were calculated over time using the following equations:

$\Delta$CFU/spleen=mean CFUs 30 days/CFUs 6 day ± SD; $\Delta$CFU/g of spleen= mean CFU/ g of
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spleen 30 days/16 days ± SD. Bars represent standard deviation. Values of p<0.01 (**) in relation to the mock-controls are indicated below the bars.

Figure 3. Late PMN depletion diminished bacterial loads in BM. C57BL/6 Mice were i. p. infected with 0.1 mL of $10^6$ CFUs of *B. abortus* 2308W and at 15 days post-infection one group of mice was depleted of PMNs by means of i. p. injection of 1A8 or RB6-8C5 anti-PMN. At the indicated times CFU/BM was determined in the BM. Each symbol represents one animal. Values of p<0.01 (**) in relation to the mock-controls are indicated above the corresponding group.

Figure 4. PMN depletion after *B. abortus* infection increased the levels of cytokines. C57BL/6 mice were infected by the i. p. route with 0.1 mL of $10^6$ CFUs of *B. abortus* 2308W. After 5 days of infection, one group of mice was depleted of PMNs by means of i. p. injection of RB6-8C5 anti-PMN. The levels of various cytokines were determined by ELISA in the sera of all mice at 6 and 14 days post-infection (one and nine days post-depletion), respectively. Median values are indicated by a horizontal line within the bars. Values of $p<0.05$ (*) in relation to the mock-controls are indicated above the corresponding group.

Figure 5. PMN depletion after *B. abortus* infection induces the reduction in body weight. C57BL/6 mice were i. p. infected with 0.1 mL of $10^6$ CFUs of *B. abortus* 2308W and depleted of PMNs with RB6-8C5 antibodies after five days post-infection. A) Body
Figure 6. PMN depletion during the immune response favored the premature resolution of inflammation. Upper panel: Spleens from *B. abortus* 2308W infected C57BL/6 mice were processed for histological examination, stained with hematoxylin and eosin and pathological parameters observed under the microscope (10×). Lower panel: Semi-quantitative estimation of the spleen inflammation by evaluating the pathological index. The arrows indicate the presence of granulomas. All values in the lower right panel at 14 days of infection (9 days after PMN depletion) were significant at *p*<0.01 in relation to the mock-controls. Bars represent the standard deviation.

Figure 7. The specific antibody response was depressed in PMN depleted mice. C57BL/6 mice were i. p. infected with 0.1 mL of 10^6 CFUs of *B. abortus* 2308W and at five days of infection one group of mice was depleted of PMNs by means of RB6-8C5 anti-PMN i. p. injection. (A) Agglutination titer against *Brucella* cells. (B) Isotype antibody responses against *Br*-LPS. Each symbol represents one animal. Blue dashed lines show the average normalized value of the mock-controls and the grey areas represent the standard deviation of the mock-controls. The cut off and range values of ELISAs optical densities
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... are indicated in the Materials and Methods section. Values of $p<0.05$ (*) in relation to the mock-controls are indicated above the corresponding group.

**Figure 8. The absence of PMNs promoted M1 macrophage polarization.** Lymph nodes leukocytes of C57BL/6 mice were analyzed by flow cytometry for CD11b, Ly6C, IL-6 and iNOS markers. (A) Lymph node leukocytes of infected mice sorted by CD11b$^+$/Ly6$^{Hi}$. (B) Lymph node leukocytes from PMN-depleted infected mice sorted by CD11b$^+$/Ly6$^{Hi}$. (C) Ly6$^C$ and Ly6$^{C}H$ cells from lymph nodes were analyzed the presence of intracellular IL-6 by flow cytometry (D) Ly6$^C$ and Ly6$^{C}H$ cells from lymph nodes were analyzed the presence of intracellular iNOS by flow cytometry. The gray areas and the numbers within parenthesis correspond to *B. abortus* infected mice. The red lines demarking areas and the numbers in red, correspond to PMN-depleted infected mice.

**Figure 9. Anti-Brucella antibodies dampen the IFN-$\gamma$ production at the onset but not at later times of infection.** (A) C57BL/6 mice were i. p. injected (+) or not (-), either with immune mouse sera against-*Brucella* or with a mixture of immune mouse sera against *Brucella* and anti-PMN (RB6-8C5) one day before infection and two days after infection, and IFN-$\gamma$ measured in the sera of mice by ELISA after 5 days of infection (Fig 1S C). (B) Mice were i. p. injected (+) or not (-), either with immune mouse sera against-*Brucella* or with a mixture of immune mouse sera against *Brucella* and anti-PMN five days after infection and IFN-$\gamma$ measured after 6 days of infection (Fig 1S D). (B) Mice were i. p. injected (+) or not (-), either with immune mouse sera against-*Brucella* or with a mixture of...
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immune mouse sera against *Brucella* and anti-PMN 6, 9 and 12 days after infection and IFN-γ measured after 14 days of infection (Fig 1S E). (D) Bacterial counts corresponding to experiment “A”. (E) Bacterial counts corresponding to experiment “C”. Median values are indicated by a horizontal line within bars in “A”, “B” and “C”. In “D” and “E” each symbol represents one animal, and the lines represent the median for each group. Values of *p*<0.05 (*) and *p*<0.01 (**) in relation to the mock-controls are indicated above the corresponding group.

Figure 10. Anti-*Brucella* antibodies dampen proinflammatory cytokines in neutropenic mice at acute stages of infection. (A) C57BL/6 mice were i. p. injected (+) or not (-), either with immune mouse sera against *Brucella* or with a mixture of immune mouse sera against *Brucella* and anti-PMN (RB6-8C5) at 6, 9 and 12 days after infection and the various proinflammatory cytokines measured in the sera of mice by ELISA after 14 days of infection. Median values are indicated by a horizontal line within bars. In comparison with cytokine levels in neutropenic infected mice shown in Fig 4 (far right column in each graphic), the levels of IL-12, IL-10 and IL-6 are significantly lower (*p*<0.01) in neutropenic mice treated with anti-*Brucella* antibodies.
Day 14
9 days p. d.

Mice body weight (g)

Mock Anti PMN

Day 30
25 days p. d.

Mock Anti PMN

*
A

Agglutination titer

Mock
Anti-PMN
Mock
Anti-PMN

Day 21
16 day p. d.
Day 30
25 day p. d.

B

Day 21

IgG
IgG1
IgG2a
IgG2b
IgG3
IgA
IgM

Deviation from normal infection

0.0
0.5
1.0
1.5

0 2 4 6

Number of mice

Day 30

IgG
IgG1
IgG2a
IgG2b
IgG3
IgA
IgM

Deviation from normal infection

0.0
0.5
1.0
1.5

0 2 4 6

Number of mice
