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MyD88-Dependent Activation of B220−CD11b+LY-6C+ Dendritic Cells during Brucella melitensis Infection

Richard Copin,* Patrick De Baetselier,† Yves Carlier,‡ Jean-Jacques Letesson,2,3* and Eric Muraille2,3†

IFN-γ is a key cytokine controlling Brucella infection. One of its major function is the stimulation of Brucella-killing effector mechanisms, such as inducible NO synthase (iNOS)/NOS2 activity, in phagocytic cells. In this study, an attempt to identify the main cellular components of the immune response induced by Brucella melitensis in vivo is made. IFN-γ and iNOS protein were analyzed intracellularly using flow cytometry in chronically infected mice. Although TCRβ+CD4+ cells were the predominant source of IFN-γ in the spleen, we also identified CD11b+LY-6C+LY-6G+MHC-II+ cells as the main iNOS-producing cells in the spleen and the peritoneal cavity. These cells appear similar to inflammatory dendritic cells recently described in the mouse model of Listeria monocytogenes infection and human psoriasis; the TNF/iNOS-producing dendritic cells. Using genetically deficient mice, we demonstrated that the induction of iNOS and IFN-γ-producing cells due to Brucella infection required TLR4 and TLR9 stimulation coupled to Myd88-dependent signaling pathways. The unique role of MyD88 was confirmed by the lack of impact of Toll-IL-1R domain-containing adapter inducing IFN-β deficiency. The reduction of IFN-γ+ and iNOS− cell frequency observed in MyD88−, TLR4−, and TLR9-deficient mice correlated with a proportional lack of Brucella growth control. Taken together, our results provide new insight into how immune responses fight Brucella infection. The Journal of Immunology, 2007, 178: 5182–5191.

Brucella organisms are facultative intracellular Gram-negative coccobacilli that infect humans and animals. They are causative agents of a worldwide zoonosis. Acute human brucellosis is characterized by undulating fever, which, if untreated, may result in localization of bacteria in various tissues leading to chronic disease with serious clinical manifestations, such as arthritis, osteoarthritis, spondylitis, endocarditis, and several neurological disorders (1–4). Brucella melitensis is the most frequent cause of human brucellosis (5).

Brucella resides and replicates in a vacuolar compartment within macrophages of the infected host (6–8). As for other intracellular microbial pathogens, IFN-γ, a Th1 cytokine produced by activated NK, CD4+ T cells, and CD8+ T cells, contributes to control the infection (9–11). One of its major functions is the stimulation of Brucella-killing effector mechanisms, such as the production of a reactive nitrogen intermediate (RNI) and a reactive oxygen intermediate (ROI) in phagocytic cells (12, 13). A previous study (14) has reported that gp91phox−/− mice and iNOS/NIK2−/− mice are both susceptible to Brucella abortus infection but that inducible NO synthase (iNOS) deficiency more drastically affects the resistance, suggesting a predominant role of RNI in Brucella killing. BALB/c mice are less able to control Brucella infections than C57BL/6 mice, having an ~10-fold increase in bacteria in their spleens during the plateau phase of the infection which occurs between 1 and 6 wk postinfection (15, 16). It has been suggested that BALB/c susceptibility is due to a cessation of IFN-γ production that begins after the first week of infection. This has been mainly demonstrated by measuring in vitro IFN-γ secretion by spleen cells isolated from infected mice and cultivated in the presence of Brucella Ag (15, 11). Despite progresses in mouse models of brucellosis, much remains unknown regarding cellular components of the innate and adaptive immune response induced by B. melitensis infection. In particular, the nature and cell surface phenotype of innate immune effectors implicated in Brucella control are largely undetermined.

The initial host defense to infection is stimulated by pathogen-associated molecular patterns (PAMPs) which are common to different groups of pathogens. PAMPs are known to be recognized by a battery of germ-line-encoded host receptors which rapidly detect and signal microbial infections. Over the past few years, the TLR family has emerged as a major group of signaling receptors for PAMPs (17). Mammalian TLRs comprise a large family consisting of at least 11 members. TLRs detect multiple PAMPs, including

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LPS—the major glycolipidic component of Gram-negative bacteria (detected by TLR4), bacterial lipoproteins, and lipoteichoic acids (detected by TLR2), and the unmethylated CpG DNA of bacteria (detected by TLR9). TLRs recruit adaptor molecules that subsequently activate downstream signaling pathways and NF-kB. Four adaptor molecules have been identified: MyD88 (18), Toll-IL-1R domain-containing adaptor protein (TIRAP), Toll-IL-1R domain-containing adaptor inducing IFN-β (TRIF) (19), and Toll-IL-1R domain-containing adaptor molecule-2 (TICAM-2). Although the role of the various adaptors in the activation of specific signaling pathways still remains controversial, recent studies (17, 20) suggest that Toll-IL-1R domain-containing adaptor protein/MyD88 and TRIF-related adaptor molecule/TRIF regulate independent pathways. TLR2 (21–23), TLR4 (21–23), TLR9 (24), and MyD88 (21, 22) have been found to be involved in the activation of immune response by B. abortus. Although the role of the MyD88 adaptor has been well-established (21, 22), several studies (21–23) have reported contradictory results about the roles of TLR2 and TLR4. Furthermore, the impact of TLR9 and TRIF adaptor deficiency in the course of infection remains undetermined. In this work, our aim is to characterize the main cellular components of the innate and adaptive immune response induced by mice infected with B. melitensis and to determine which TLRs and adaptor molecules are involved in the activation of these cells.

Materials and Methods

Mice and reagents

Six- to 8-wk-old female BALB/c and C57BL/6 mice were purchased from Harlan. TLR2−/− (25), TLR4−/− (26), and TLR2,4−/− mice with the BALB/c background and TLR9−/− (27), MyD88−/− (18), TRIF−/− (Ref. 19; B. Beutler, The Scripps Research Institute, La Jolla, CA) mice on the C57BL/6 background were bred in the animal facility of the Free University of Brussels (ULB). The maintenance and care of mice complied with the guidelines of the ULB Ethic Committee for the use of laboratory animals.

B. melitensis strain 16M (Biotype 1; American Type Culture Collection ATCC 23456) was isolated from an infected goat and grown in biosafety level III laboratory facility. Overnight culture grown with shaking at 37°C in 2YT medium (Luria-Bertani broth with double quantity of yeast extract) to stationary phase was washed twice in PBS (3500 × g, 10 min) before use in the inoculation of mice as previously described (28).

Mouse infection

Mice were injected i.p. with 4 × 10⁶ CFU of B. melitensis in 500 μl of PBS. Control animals were injected with the same volume of PBS alone. The infectious doses were validated by plating serial dilutions of the inoculums.

Harvest of spleen and peritoneal exudates

At selected time intervals, mice were sacrificed by cervical dislocation. Immediately after being killed, mice were peritoneally washed with 10 ml of ice-cold HBSS and the spleen harvested. Before cytofluorometric analysis, spleens were cut in very small pieces and incubated (37°C, 30 min) into HBSS (Invitrogen Life Technologies) medium containing 4000 U/ml collagenase IV (Roche) and 0.1 mg/ml DNase I fraction IX (Sigma-Aldrich). This procedure was performed in all experiments of this work.

Bacteria titers in spleens and peritoneal exudates of infected mice

Spleen cells or peritoneal exudates were recovered in PBS/0.1% Triton X-100 (Sigma-Aldrich). We performed successive serial dilutions in PBS to get the most accurate bacterial count and we plated them onto 2YT medium plates. CFU were counted after 3 days of culture at 37°C.

Statistical analysis

ANOVA I was used for data analysis after testing the homogeneity of variance (Bartlett test). Average comparisons were performed by pairwise Scheffe’s test. Error bars represent the 95% interval of confidence of the mean (computed from residual mean square and Student’s t, 0.95).

Cytofluorometric analysis

Peritoneal cells and spleen cells were first incubated in saturating doses of 2.4G2 (a rat anti-mouse FcR mAb; ATCC) for 10 min to prevent Ab binding to FcR. A total of 3–5 × 10⁶ cells were stained with various fluorometric mAbs combinations in 200 μl of PBS, 0.5% BSA, 0.02% NaN3 (FACS buffer), and further collected on a FACSCalibur cytofluorometer (BD Biosciences). We purchased the following mAbs from BD Pharmingen: fluorescein (FITC)-coupled M1/70 (anti-CD11b), H57-597 (anti-CD11c), and further collected on a FACSCalibur cytofluorometer (BD Biosciences). We purchased the following mAbs from BD Pharmingen: fluorescein (FITC)-coupled M1/70 (anti-CD11b), H57-597 (anti-CD11c), 2.4G2 (a rat anti-mouse FcR mAb; ATCC) for 10 min to prevent Ab binding to FcR. A total of 3–5 × 10⁶ cells were stained with various fluorescent mAbs combinations in 200 μl of PBS, 0.5% BSA, 0.02% NaN3 (FACS buffer), and further collected on a FACSCalibur cytofluorometer (BD Biosciences). We purchased the following mAbs from BD Pharmingen: fluorescein (FITC)-coupled M1/70 (anti-CD11b), H57-597 (anti-CD11c), PE-coupled M5/114.15.2 (anti-IA/IE), HL3 (anti-CD11c), AL-21 (anti-LY-6C), 1A8 (anti-LY-6G), RM4-5 (anti-CD4), 53-6.7 (anti-CD8a). Cells were gated according to size and scatter to eliminate dead cells and debris from analysis.

Intracellular cytokine staining

Peritoneal and spleen cells were incubated for 4 h in RPMI 1640, 5% FCS with 10 μg/ml Golgi Plug (BD Pharmingen) at 37°C, 5% CO₂. The cells were washed with FACS buffer and stained for cell surface markers before fixation in PBS/1% PFA for 15–20 min on ice. These cells were then permeabilized for 30 min using a saponin-based buffer (1× Perm/Wash; BD Pharmingen in FACS buffer) containing one of the following intracellular staining mAbs: allophycocyanin-coupled XMG1.2 (anti-IFN-γ), allophycocyanin-coupled MP6-XT22 (anti-TNF-α), purified M-I9 (anti-NOS), Santa Cruz Biotechnology stained with Alexa Fluor 647 goat anti-rabbit (Molecular Probes). After final fixation in PBS/1% PFA, the cells were analyzed on a FACSCalibur cytofluorometer. No signal was detectable with isotype controls.
Results

Analysis of C57BL/6 and BALB/c mice infected with B. melitensis 16M

C57BL/6 and BALB/c mice were infected with $4 \times 10^4$ CFU of B. melitensis (strain 16M) by i.p. inoculation. Bacterial titers inside peritoneal cavity and spleen were used to evaluate infection (Fig. 1). As illustrated (Fig. 1 and data not shown), infection peaks in peritoneal cavity and in spleen occurred at 2 and 5 days, respectively. This was followed by a rapid decrease in the peritoneal cavity against a substantial persistent level in the spleen. BALB/c mice susceptibility is therefore confirmed in our experimental model (Fig. 1) with marked susceptibility mainly observed during the plateau phase of infection in spleen. Meanwhile, both C57BL/6 and BALB/c mice could control early infection development with the same efficacy, as evidenced by the similar kinetic of bacterial titer inside the peritoneal cavity and the spleen during 5 days after infection (Fig. 1).

IFN-γ (9–11) and iNOS (11, 14) have been implicated in resistance to B. abortus infection. Using IFN-γ−/− and iNOS−/− C57BL/6 mice, we showed that IFN-γ and iNOS are implicated
FIGURE 3. CD11b⁺ LY-6C⁺ cells are the main iNOS-producing cells during *B. melitensis* infection. Wild-type C57BL/6 mice (five per group) were injected i.p. with PBS or $4 \times 10^4$ CFU of *B. melitensis*, as specified. At the indicated time after treatment, mice were sacrificed and peritoneal exudates and spleens harvested. A, Pooled spleen cells were incubated with FITC-coupled anti-CD11b, PE-coupled anti-LY-6G, and anti-iNOS/NOS2 mAb stained with Alexa Fluor 647 goat anti-rabbit. Number indicates the percentage of cells in the selected quadrant. B–E, Pooled peritoneal cells and spleen cells from control or infected mice (as indicated) were stained with FITC-labeled anti-CD11b, anti-iNOS/NOS2 mAb stained with Alexa Fluor 647 goat anti-rabbit and with the following PE-coupled mAbs: anti-CD11c (B), anti-IA/IE (C), anti-LY-6C (D), and anti-LY-6G (E). These results are representative of five independent experiments.
in the resistance to *B. melitensis* infection (Fig. 2A). At 5 days postinfection, we found 10-fold increase CFU in the spleen of infected IFN-γ−/− and iNOS−/− mice when compared with wild-type mice. In contrast, in the peritoneal compartment, while iNOS−/− and wild-type mice displayed similar bacteria levels at all test times, IFN-γ−/− mice displayed 10-fold increase CFU. These data suggest that both IFN-γ and iNOS may have a potential role in the control of *B. melitensis* 16M growth in the spleen whereas only IFN-γ plays a role in the peritoneal cavity.

Flow cytometric analysis of IFN-γ and iNOS cell frequency in peritoneal and splenic compartments in the course of *B. melitensis* infection was used to clarify BALB/c mice susceptibility. It is important to note that cells purified from naive and infected mice were not stimulated in vitro before intracellular staining. Thus, iNOS and IFN-γ detected in these cells derive only from natural in vivo activation during the course of infection. Naive and infected iNOS−/− and IFN-γ−/− C57BL/6 mice were used as internal negative control in these experiments. As expected, significant iNOS and IFN-γ staining were only observed in infected wild-type C57BL/6 mice (Fig. 2, B and C). We observed that the frequency of iNOS+ cells increased slowly during infection of wild-type C57BL/6 mice and peak at 10–15 days in the peritoneal cavity and spleen (Fig. 2, D and E). IFN-γ+ cells were not detected in the peritoneal cavity (data not shown); meanwhile, an impact of this cytokine was observed on the resistance against the bacterial invasion (Fig. 2A). Thus, it is possible that IFN-γ produced in another compartment, such as the spleen, mediated resistance in the peritoneal cavity. In the spleen of C57BL/6 mice, the frequency of IFN-γ+ cells reached its plateau phase between 5 and 15 days postinfection (Fig. 2F). Surprisingly, a similar observation
was found for both iNOS- and IFN-γ-producing cells in infected BALB/c mice (Fig. 2, D–F). However, the frequency of iNOS+ cells and mostly IFN-γ+ cells is lower in BALB/c mice. Thus, we found that BALB/c mice kept the ability to produce IFN-γ during the plateau phase of the infection. However, the frequency of IFN-γ+ cells is weak and can explain the lack of resistance of these mice. Cell surface phenotypes of IFN-γ+ and iNOS+ cells during B. melitensis infection in C57BL/6 and BALB/c mice were later characterized.

CD11bhighLY-6ChighLY-6GlowMHC-IIhigh cells are the main iNOS-producing cells during B. melitensis infection

B. melitensis infection induces a recruitment of CD11bhighLY-6Ghigh cells (granulocyte) and CD11bhighLY-6Glow cells in the spleen of infected C57BL/6 mice (Fig. 3A). Both populations contain iNOS+ cells, but the CD11bhighLY-6Glow population appears as the major iNOS-producing cells at 10–15 days of infection. Further analysis of their cell surface phenotype by flow cytometry showed that these later cells display CD11chighMHC-IIhigh, LY-6Chigh and LY-6Clow (Fig. 3, B–E, respectively). iNOS+ cells in the peritoneal cavity expressed a very similar phenotype with the exception of CD11c which appeared negative (Fig. 3B). Similar results were obtained in infected BALB/c mice (data not shown).

The majority of iNOS+ cells from both the peritoneal cavity and the spleen expressed CD11bhighLY-6ChighLY-6Glow and MHC-IIhigh cell surface markers similar to inflammatory dendritic cells (DC) recently described during the course of Listeria monocytogenes infection in mouse (28–31) and in human psoriasis (32). Because these cells have been described to produce TNF-α (28–31) and TNF-α has been associated to the control of Brucella infection (33), we investigated the ability of CD11bhighLY-6Chigh cells to produce TNF-α during B. melitensis infection (Fig. 4). Infected TNF-α−/− mice were used as negative control for TNF-α staining in these experiments. In wild-type C57BL/6 mice, a large fraction of CD11bhighLY-6Chigh peritoneal cells appeared TNF-α positive. In contrast, splenic CD11bhighLY-6Chigh cells were almost TNF-α negative.

It is well-known that IFN-γ positively regulate iNOS production in experimental bacterial infectious model (12, 13) and mediate resistance to Brucella infection (Refs. 9–11 and Fig. 2A). We therefore analyzed the impact of IFN-γ deficiency on the activation of CD11bhighLY-6Chigh cell population during B. melitensis infection. As shown in Fig. 4, IFN-γ deficiency strongly decreased iNOS+ cell frequency in peritoneal cavity and spleen. In contrast, TNF-α+ cells frequency was enhanced in peritoneal cavity and in spleen.
In the early course (5 days), IFN-γ was observed to significantly increase the frequency of IFN-γ-producing cells in BALB/c mice (data not shown). It is interesting to note that depleting IFN-γ by the administration of mAbs that neutralize IFN-γ (10) or by the use of IFN-γ-γ− mice (Ref. 11 and data not shown) results in a 10-fold increase in the number of bacteria. Here, for the first time, we characterized by flow cytometry the cell surface phenotype of spleen cells producing IFN-γ in vivo during the course of Brucella infection. At the peak of infection (5 days), IFN-γ is produced mainly by TCRβ NK1.1+ NK cells, TCRβ+CD4+ T cells, and TCRβ+CD8+ T cells. During the plateau phase of infection (10–21 days), CD4+ T cells become the major producing cells (74% of IFN-γ+ cells).

The respective contribution of CD4+ T cells and CD8+ T cells to protective immune response against Brucella has been controversial (reviewed in Ref. 34). Transfer experiments showed an equal role for both subsets. In contrast, β2-microglobulin−/− mice (deficient for CD8+ T cell activation) appeared more susceptible to MHC-II−/− mice (deficient for CD4+ T cell activation) suggesting that CD8+ T cells play a crucial role in protection. However, it is well-known that β2-microglobulin−/− mice are deficient for both MHC-I and CD1d Ag-presentation pathways rendering complex the interpretation of the results in this model. The relatively equal role of NK, CD4+, and CD8+ T cells in IFN-γ secretion at the peak of infection could explain the results of transfer experiments. In addition, the predominant role of CD4+ T cells in IFN-γ secretion during the plateau phase of infection could explain why RAG1 (35) but not β2-microglobulin-deficient mice (11) and NK-depleted mice (36) drastically fail to control Brucella infection.

Experiments in genetically deficient mice have shown that MyD88 adaptor protein is implicated in the resistance to B. abortus infection (21, 22). We demonstrated that MyD88 deficiency strongly reduces the frequency of IFN-γ− cells at all time of B. melitensis infection. The unique role of MyD88-dependent signaling pathways is confirmed by the fact that TRIF-deficient mice do not have a reduced frequency of IFN-γ− cells and display normal resistance to infection. Identification of TLRs coupled to MyD88 and detecting B. melitensis infection appear complex. Contradictory results have been reported concerning the implication of TLR2 and TLR4 in resistance to B. abortus infection. TLR2 has been involved in heat-killed B. abortus-induced TNF-α (22) but not in the resistance to infection (21, 23). TLR4 deficiency has been reported to reduce (23) or have no affect (21) on resistance in...
B. abortus. In our experimental model of B. melitensis infection, we found that TLR4, but not TLR2, significantly decrease resistance to infection. The reasons for these discrepancies are not known but could be due to the different strain of Brucella used. In addition, we reported for the first time that TLR9 deficiency also reduces resistance. This result is in agreement with previous work (24) showing the role of TLR9 in the induction of inflammatory cytokines by heat-killed B. abortus. Collectively, our data suggest that both TLR4 and TLR9 cooperate in the immune system to detect B. melitensis infection. A logical consequence of multiple TLR signaling is that the reduction of IFN-γ cell frequency is moderately or not affected by the deficiency of TLR4 or TLR9 alone.

In various experimental models of infection by intracellular pathogen, a major function of IFN-γ is the stimulation of killing effector mechanisms, such as the production of RNI and ROI in phagocytic cells (12, 13). A previous study (14) reported that both gp91phox−/− mice and iNOS/NOS2−/− mice are susceptible to B. abortus infection but that iNOS deficiency more drastically affects the resistance, suggesting a predominant role of RNI in Brucella killing. Based on these data, we selected iNOS as a marker of protective innate immune response against Brucella. We showed that IFN-γ positively regulates the frequency of iNOS cells in the peritoneal cavity and spleen. Infected IFN-γ−/− mice display a 10-fold reduction of iNOS cells frequency. In contrast, TNF-α deficiency moderately affects this frequency during Brucella infection. This suggests a key role of CD4 T cells, the main IFN-γ cells, in the regulation of Brucella-killing effector mechanisms.

Characterization of cell surface marker expressed by peritoneal and splenic iNOS cells showed that the great majority of these cells expressed the following phenotype: CD11bhighLY-6CnullLY-6GlowMHC-IIhigh. They are negative for T cell (TCRβ, CD8α), B cell (IgD, IgM, B220), mastocyte (c-kit), and NK (NK1.1) cell

**FIGURE 7.** TLR-4, TLR-9, and MyD88, but not TLR-2 and TRIF, regulate frequency of iNOS and IFN-γ cell during B. melitensis infection. Wild-type, MyD88−/−, TRIF−/−, TLR9−/− C57BL/6 mice and wild-type, TLR2−/−, TLR4−/−, TLR2/4−/− BALB/c mice (five per group) were injected i.p. with PBS or 4 × 10^7 CFU of B. melitensis, as specified in the figure. Mice were sacrificed at selected time after treatment and peritoneal exudates and spleens harvested. Cells of each animals were stained with allophycocyanin-labeled anti-IFN-γ or anti-iNOS/NOS2 mAb stained with Alexa Fluor 647 goat anti-rabbit. Data represent the number of positive cells per 10^7 total cells acquired. These results are representative of three independent experiments. Significant differences in relation to wild-type mice are denoted by an asterisk (*) for p < 0.05.
surface markers (data not shown). Splenic iNOS+ cells also express CD11c and thus display a phenotype very similar to inflammatory DC recently described during L. monocytogenes infection in mice (28–31) and psoriasis in human (32). These cells, termed TNF/iNOS-producing DC by the authors, appear as a "classical" DC, various specialized DC subsets have been recently defined. Plasmacytoid DC, expressing B220+CD11b−Ly6C−Ly6C+CD11c+ markers, are specialized in IFN-α production and antiviral function (39). NK DC, expressing CD11b+DX5+ NK1.1+ markers, displayed both cytotoxicity and APC functions (40). The implication of B220+CD11b+Ly6C−NK1.1−iNOS−DC in both Listeria (28–31) and Brucella infection (this study) allows us to propose that these cells can be major effectors in murine Th1 immune response against intracellular bacteria. Moreover, as CD11c+CD11b+Ly6C−DC isolated during Listeria infection display APC function in vitro (30), it is possible that these cells also play an important regulatory role by stimulating and programming T cell responses during these infections. Interestingly, their recent finding in human psoriasis (32) suggests that they can also play an important role in human Th1 immune response. As demonstrated in the Listeria model (31), we also showed that inflammatory DC activation during Brucella infection is strongly dependent on MyD88-signaling pathways. In contrast, TRIF pathways do not appear to be required. As for IFN-γ+ cell, we observed a reproducible but weak impact of TLR-4 and TLR-9 deficiency on iNOS expression in mice. We are indebted to Gre´goire Lauvau for his critical review of the manuscript and to Christian Didembourg and Matthieu Terwagne for their diligent technical assistance. We thank Eric Depiereux for statistical analysis of our results and Bernard Nkengfack for relevant English corrections.

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