INFECTION AND IMMUNITY, Mar. 2006, p. 1800–1808 Vol. 74, No. 3
0019-9567/06/$08.00+0 doi:10.1128/IAI.74.3.1800–1808.2006

Role for the CD28 Molecule in the Control of *Coxiella burnetii* Infection

Amélie Honstettre,[1] Soraya Meghari,[1] Jacques A. Nunès,[2] Hubert Lepidi,[1] Didier Raoult,[1] Daniel Olive,[2] and Jean-Louis Mege[1]*

Unité des Rickettsies, CNRS UMR 6020, Université de la Méditerranée, Faculté de Médecine, 13385 Marseille Cedex 5,[1] and Institut de Cancérologie de Marseille, INSERM UMR 599, Institut Paoli-Calmettes, 13009 Marseille Cedex,[2] France

Received 21 April 2005/Returned for modification 9 June 2005/Accepted 27 November 2005

Q fever is an infectious disease caused by *Coxiella burnetii*, an obligate intracellular bacterium that replicates in macrophages. As cell-mediated immune response to microbial pathogens requires signals mediated by T-cell receptors and costimulatory molecules such as CD28, we wondered if CD28 is involved in protection against *C. burnetii* infection. CD28-deficient ($CD28^{-/-}$) mice were inoculated with *C. burnetii* by intraperitoneal and intravenous routes. With both wild-type and $CD28^{-/-}$ mice, *C. burnetii* organisms were detected exclusively in spleen and liver. The antibody response against *C. burnetii* was impaired in $CD28^{-/-}$ animals, but, surprisingly, the lack of CD28 decreased *C. burnetii* burden in the infected tissues, whatever the manner of inoculation of bacteria. The CD28 deficiency had no effect on either granuloma formation, which reflects cell-mediated immunity against *C. burnetii*, or the production of gamma interferon and tumor necrosis factor, two cytokines known to be involved in granuloma formation. On the other hand, the production of interleukin-10 (IL-10) by peritoneal macrophages was highly impaired in $CD28^{-/-}$ mice. The results suggest that CD28 initiates a signal that favors *C. burnetii* replication through the modulation of the IL-10 pathway.

Q fever is a zoonosis caused by *Coxiella burnetii*, an obligate intracellular microorganism that replicates in myeloid cells. In the primary infection, a minority of patients present acute symptoms such as isolated fever, hepatitis, or pneumonia (36). Q fever may become chronic in patients with valve lesions, arterial aneurysm, or arterial prosthesis, and the most frequent clinical manifestation consists of culture-negative endocarditis (7, 13, 31). The control of Q fever depends on host immune response, as manifested by granuloma formation, and systemic immune response with marked proliferative response to *C. burnetii* antigen and antigen-stimulated gamma interferon (IFN-γ) production (22, 23, 26, 32). However, immune response does not lead to *C. burnetii* eradication, and relapses may occur when cell-mediated immunity is depressed (17, 31). In chronic Q fever, cell-mediated immunity is defective, associated with lack of granulomas, impaired antigen-mediated lymphoproliferation and microbial killing, and cytokine dysfunction (10, 15, 21).

The innate immune response is not sufficient for host protection against *C. burnetii* infection since the lack of Toll-like receptor 4 does not modify the clearance of *C. burnetii* (20). The development of a protective immune response against intracellular microorganisms, including *C. burnetii*, requires an adaptive immune response. Indeed, severe combined immunodeficient mice suffer from persistent infection with *C. burnetii* and finally succumb (1). Besides the signal delivered by a peptide antigen after T-cell receptor engagement, T-cell activation requires a second signal provided by costimulatory molecules (24). Among them, CD28 plays an essential role since functional inactivation or clonal anergy of T cells is found without its engagement (30). CD28 ligands, the CD80 and CD86 molecules, are expressed by antigen-presenting cells; their expression is upregulated by the interaction of antigen-presenting cells with infectious pathogens (4). The role of CD28 in immune responses, including protection against intracellular pathogens, has been assessed using mice treated with CD28-specific antibodies (Abs) or CD28-deficient mice, but it remains elusive. $CD28^{-/-}$ mice infected with influenza virus or vesicular stomatitis virus are unable to mount specific antibody responses or to develop specific $CD8^+$ T cells (6). $CD28^{-/-}$ mice infected with herpesvirus clear the infection despite impaired humoral response (2). $CD28^{-/-}$ mice infected with *Mycobacterium bovis* control bacterial growth and mount cell-mediated immune response (19). Similarly, a lack of CD28 did not interfere with the clearance of *Listeria monocytogenes* in models of primary and secondary infection (34). In contrast, $CD28^{-/-}$ mice are susceptible to *Salmonella enterica* serovar Typhimurium and *Trypanosoma cruzi* (35). $CD28^{-/-}$ mice were unable to resist to *Leishmania major* infection according to some authors (9), but the lack of CD28 did not affect the response against *L. major* in other reports (8).

As CD28 is likely critical for T-cell activation in bacterial infections, we investigated its role in a murine model of *C. burnetii* infection. We show that $CD28^{-/-}$ mice exhibited lower *C. burnetii* burden in tissues than wild-type (wt) mice and impaired specific IgG switch but that the lack of CD28 had no effect on granuloma formation. Whereas the production of inflammatory cytokines was not affected by the lack of CD28, the production of interleukin-10 (IL-10) was decreased. These results suggest that CD28 initiates a signal that favors *C. burnetii* replication, likely through the modulation of the IL-10 pathway.

* Corresponding author. Mailing address: Unité des Rickettsies, Université de la Méditerranée, Faculté de Médecine, 27 Blvd. J. Moulin, 13385 Marseille Cedex 5, France. Phone: (33) 4 91 32 43 75. Fax: (33) 4 91 38 77 72. E-mail: Jean-Louis.Mege@medecine.univ-mrs.fr.

MATERIALS AND METHODS

***C. burnetii* preparation.** *C. burnetii* organisms (Nine Mile strain) were cultured as previously described (12). BALB/c mice were injected intraperitoneally (i.p.) with 10^8 organisms. Ten days later, mice were sacrificed, and their spleens were

homogenized. Spleen homogenates were added to L929 cells, and cultures were maintained in antibiotic-free Eagle minimal essential medium supplemented with 4% fetal bovine serum and 2 mM L-glutamine (Invitrogen, Eragny, France) for two passages. Infected cells were sonicated, and the sonicates were centrifuged at 8,000 × *g* for 10 min. Bacteria were layered on 25 to 45% linear Renografin gradient, and the gradients were centrifuged. Purified bacteria were then collected, washed, and suspended in serum-free Hank's balanced salt solution (Invitrogen) before being stored at −80°C. The concentration of organisms was determined by Gimenez staining, and the bacterial viability was assessed using a LIVE/DEAD *Bac*Light bacterial viability kit (Molecular Probes, Eugene, Oreg.), as recently described (16). Only *C. burnetii* preparations containing more than 90% of viable organisms were used. In some experiments, *C. burnetii* organisms were inactivated by a 100°C treatment for 2 h.

Bacterial infection of mice. The experimental protocol was approved by the Institutional Animal Care and Use Committee of the Université de la Méditerranée. $CD28^{-/-}$ mice were purchased from the Jackson Laboratories (Bar Harbor, Maine). $CD28^{-/-}$ mice were backcrossed to C57BL/6 mice for 12 generations, as described elsewhere (38). Female wt C57BL/6 and $CD28^{-/-}$ mice were infected i.p. or intravenously (i.v.) with 5×10^5 *C. burnetii* organisms. All of the infections were done at the same time, and the clinical status of mice was recorded daily. Mice were serially killed before infection (day 0) and after different infection times. As a control, C57BL/6 and $CD28^{-/-}$ mice were infected i.p. with 5×10^4 to 5×10^6 *C. burnetii* organisms, and the infection was measured at day 7 by real-time PCR. In another set of experiments, mice were infected, and reinfection with 5×10^5 *C. burnetii* organisms was done 30 days after the initial infection. Mice were killed until 21 days after reinfection. Blood was collected by retroorbital puncture at the time of sacrifice. Spleen and liver were aseptically excised. Tissue samples were fixed or not with 10% formalin and then embedded in paraffin. Spleens were dilacerated, and splenocytes were recovered after lysis of erythrocytes. Peritoneal macrophages were obtained after washing of the peritoneal cavity of mice with 10 ml of warm phosphate-buffered saline (PBS).

Histologic analysis and immunohistologic detection of *C. burnetii*. All of the slides were coded and read in a blinded manner. The 5-μm sections of paraffin-embedded tissues were stained with hematoxylin-eosin-saffron to assess the presence of granulomas, defined as collections of 10 or more macrophages and lymphocytes within the organs. Their number was determined after whole optical examination of at least three tissue sections of each organ. They were quantified using the image analyzer SAMBA 2005 (SAMBA Technologies, Alcatel TITN, Grenoble, France) (29). The results are expressed as the number of granulomas found per surface unit (mm^2). Formalin-fixed, paraffin-embedded tissue sections were deparaffinized in xylene, rehydrated in graded alcohol for 2 min each, and rinsed in PBS for 5 min. Each tissue section was incubated with rabbit Abs to *C. burnetii* (diluted at 1:200) for 60 min, as previously described (27). Bacteria were revealed using an Immunostain-*Plus* kit (Zymed, CliniSciences, Montrouge, France) according to the manufacturer's instructions. Briefly, slides were incubated with biotin-conjugated Abs directed against rabbit immunoglobulins, followed by peroxidase-labeled streptavidin with amino-ethylcarbazole as the substrate. Slides were counterstained with Mayer hematoxylin for 10 min. Negative controls were performed using normal rabbit serum. The bacteria were numbered by optical examination, and the tissue area was determined by image analysis. The results are expressed as the number of bacteria found per mm^2 of tissue.

Tissue detection of *C. burnetii* by real-time PCR. DNA was extracted from spleens and livers from mice infected with different doses of *C. burnetii* at day 7 postinfection by using a QIAamp DNA MiniKit (QIAGEN, Hilden, Germany). The tissues were weighed (10 mg and 25 mg for the spleen and the liver, respectively), dilacerated, and incubated with 180 μl of detergent lysis buffer and 20 μl of proteinase K for 3 h at 56°C. The extraction protocol was followed as recommended by the manufacturer. Quantitative PCR (qPCR) was performed using a LightCycler FastStart DNA SYBR green system (Roche, Mannheim, Germany) and carried out with the primers FAF216 (5′-GCACTATTTTTA GCCGGAACCTT-3′) and RAF290 (5′-TTGAGGAGAAAAACTGGATGA GA-3′), which amplify a 74-bp fragment of the *C. burnetii com1* gene (GenBank accession no. AF318146) and are highly conserved among *C. burnetii* strains (42). The specificity of PCR products was confirmed by sequencing. In each qPCR run, a standard curve was generated using serial dilution ranging from 10^8 to 10^4 copies of the intergenic spacer region as described previously (14) and calculated by LightCycler software (LC-Run version 5.32; Roche).

Antibody determination. Blood was allowed to clot at room temperature and centrifuged at 700 × *g* for 10 min. The resulting serum was stored at −20°C until it was analyzed for the presence of anti-*C. burnetii* Abs. Slides with smears of formaldehyde-inactivated organisms were incubated with serial dilutions of serum for 30 min. After being washed in PBS, the bacteria were labeled with fluorescein-conjugated goat Abs directed against mouse immunoglobulin G (IgG) (Beckman Coulter, Villepinte, France) and rat Abs against mouse IgG1, IgG2a, IgG2b, IgG3, and IgM (BD Pharmingen, Le Pont de Claix, France) at a 1:100 dilution for 30 min. The slides were then washed in PBS and examined by fluorescence microscopy. The starting dilution for the serum sample was 1:25, and titers of samples were determined to end point as previously described (39).

Cytokine determination. Splenocytes and peritoneal cells from wt and $CD28^{-/-}$ mice were incubated in RPMI 1640 containing 25 mM HEPES, 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). All media were checked for the absence of endotoxins with *Limulus* amebocyte lysate (Cambrex Bioscience, Emerainville, France). Splenocytes (2×10^6 cells in 1 ml) were incubated in flat-bottomed, 24-well culture plates (Nunc, PolyLabo, Strasbourg, France) with heat-inactivated *C. burnetii* (10 organisms per cell) for 24 h at 37°C. Once collected, supernatants were stored at −80°C until IFN-γ measurement. Peritoneal cells (10^6 cells/assay) were incubated in 24-well culture plates for 1 h and washed to eliminate nonadherent cells. Adherent macrophages were stimulated by heat-inactivated *C. burnetii* (10 organisms per cell) for 24 h at 37°C, and cell supernatants were tested for the presence of tumor necrosis factor (TNF) and IL-10. Cytokine production was measured by immunoassay kits. IFN-γ (detection limit, 37 pg/ml) and IL-10 (detection limit, 37 pg/ml) assay kits were provided by Endogen (BioAdvance, Emerainville, France). TNF (detection limit, 23.4 pg/ml) assay kits were provided by R&D Systems (Abingdon, United Kingdom). The intra- and interspecific coefficients of variation were less than 10%.

Statistical analysis. Results, given as the means ± standard deviations (SD), were compared by Student's *t* test. Differences were considered significant when P was <0.05.

RESULTS

Role of CD28 in *C. burnetii* infection. We wondered if the lack of CD28 affects the susceptibility of mice towards *C. burnetii*. Wild-type and $CD28^{-/-}$ mice were injected i.p. with 5×10^5 organisms, and the infection was recorded. Mortality and morbidity were not observed with wt and $CD28^{-/-}$ mice up to 42 days, suggesting that the lack of CD28 did not modify the resistance of mice towards *C. burnetii*. Increasing injected doses to 5×10^6 *C. burnetii* organisms did not change mortality and morbidity of wt and $CD28^{-/-}$ mice. The course of cellular infection was assessed by immunodetection of *C. burnetii* in tissues. *C. burnetii* organisms were detected only in the spleens and the livers from wt and $CD28^{-/-}$ mice; they appeared as coarse or fine intracellular grains inside granulomas and were never found outside granulomas (Fig. 1A). They were detected after 4 days in the spleens from wt and $CD28^{-/-}$ mice. Bacterial load reached a peak at day 7 postinfection, and bacteria were undetectable after 14 days for wt mice. For $CD28^{-/-}$ mice, bacterial load was very low during the course of the experiments and was significantly lower ($P < 0.02$) than that for wt mice at day 7 (Fig. 2A). In the liver, *C. burnetii* organisms were detected after 4 days for wt and $CD28^{-/-}$ mice. Bacterial load steadily decreased from day 4 to day 21, and no organism was detected thereafter. The bacterial load was significantly lower ($P < 0.05$) in $CD28^{-/-}$ mice than in wt mice at days 4, 7, and 14 (Fig. 2B). When mice were infected i.v. with *C. burnetii*, the results were emphasized. With wt mice, *C. burnetii* organisms were detected only at day 7 in the spleen (Fig. 2C) and at days 4 and 7 in the liver (Fig. 2D). In the spleens (Fig. 2C) and the livers (Fig. 2D) of $CD28^{-/-}$ mice, bacteria were detected only at day 7. We also wondered if the lack of CD28 affects resistance to *C. burnetii* during reinfection. Mice were infected with *C. burnetii* organisms for 30 days, and they received a second i.p. injection with 5×10^5 organisms. This resulted in the presence of low amounts of bacteria in the spleens and in the livers of wt mice. For $CD28^{-/-}$ mice, *C.*

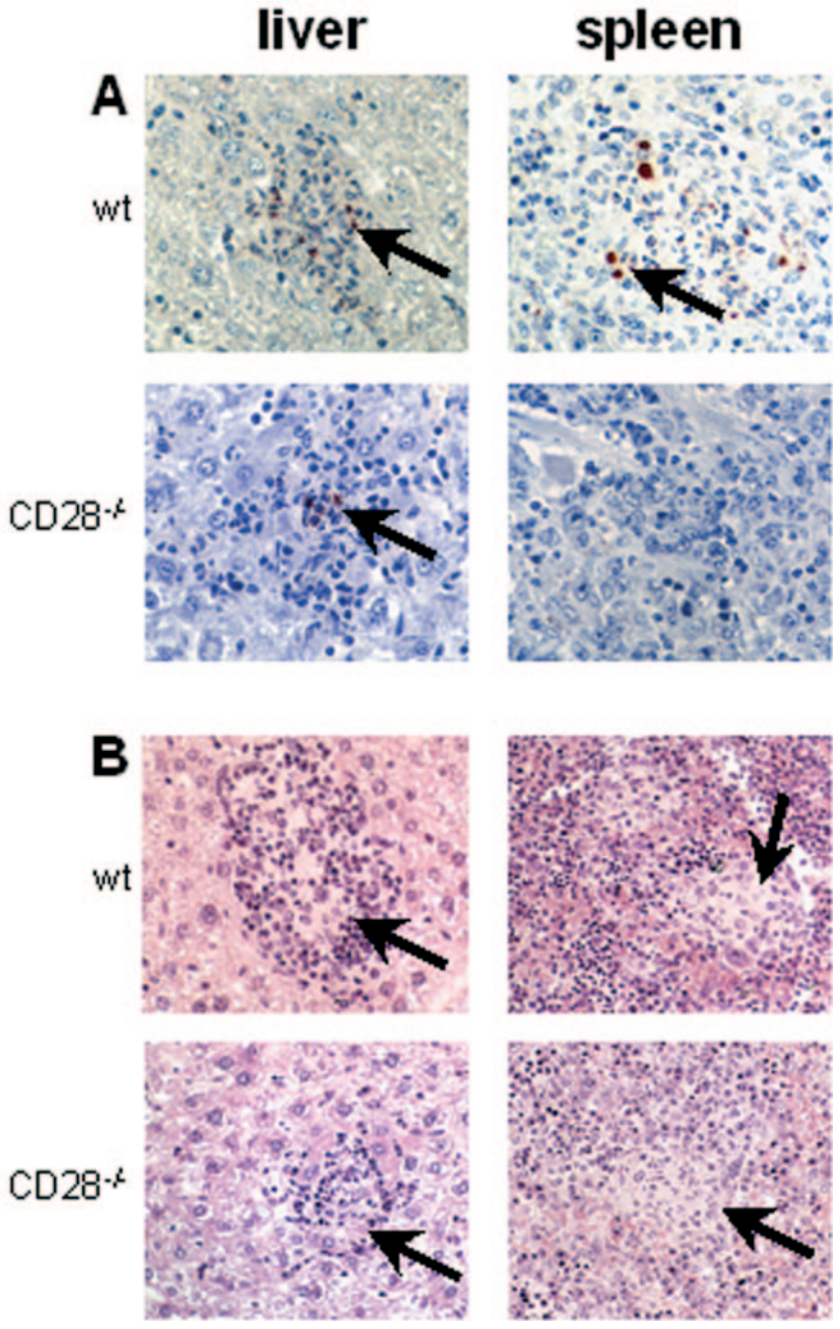


FIG. 1. *C. burnetii* and granulomas in tissues. Wild-type and CD28$^{-/-}$ mice were infected i.p. with 5×10^5 *C. burnetii* organisms and killed at day 7. (A) Bacteria were revealed by immunostaining of the spleen and the liver. (B) Granulomas were revealed by histochemical analysis of the spleen and the liver. Representative micrographs are shown.

burnetii organisms were never found in spleen or liver (data not shown). In order to assess the role of inoculum size on *C. burnetii* infection, wt and CD28$^{-/-}$ mice were injected i.p. with different doses of *C. burnetii* organisms (5×10^4 to 5×10^6 organisms), and the tissue infection was measured by qPCR. With wt mice, the bacterial DNA copy number steadily increased in liver and reached a plateau in spleen in response to 5×10^5 and 5×10^6 organisms. With CD28$^{-/-}$ mice, the bacterial DNA copy number steadily increased when *C. burnetii* doses varied from 5×10^4 to 5×10^6 organisms. It was significantly lower ($P < 0.002$) than that for wt mice for all tested doses of bacteria (Fig. 2E and F). Taken together, these results show that the lack of CD28 decreases *C. burnetii* burden in the spleens and the livers of infected mice.

Role of CD28 in Ab response to *C. burnetii* infection. *C. burnetii*-specific Abs were measured in sera from wt and CD28$^{-/-}$ mice infected with 5×10^5 *C. burnetii* organisms. During primary infection, specific IgM were detected after 7 days, reached maximum titers after 21 days, and were undetectable thereafter (Fig. 3A). *C. burnetii*-specific IgG were de-

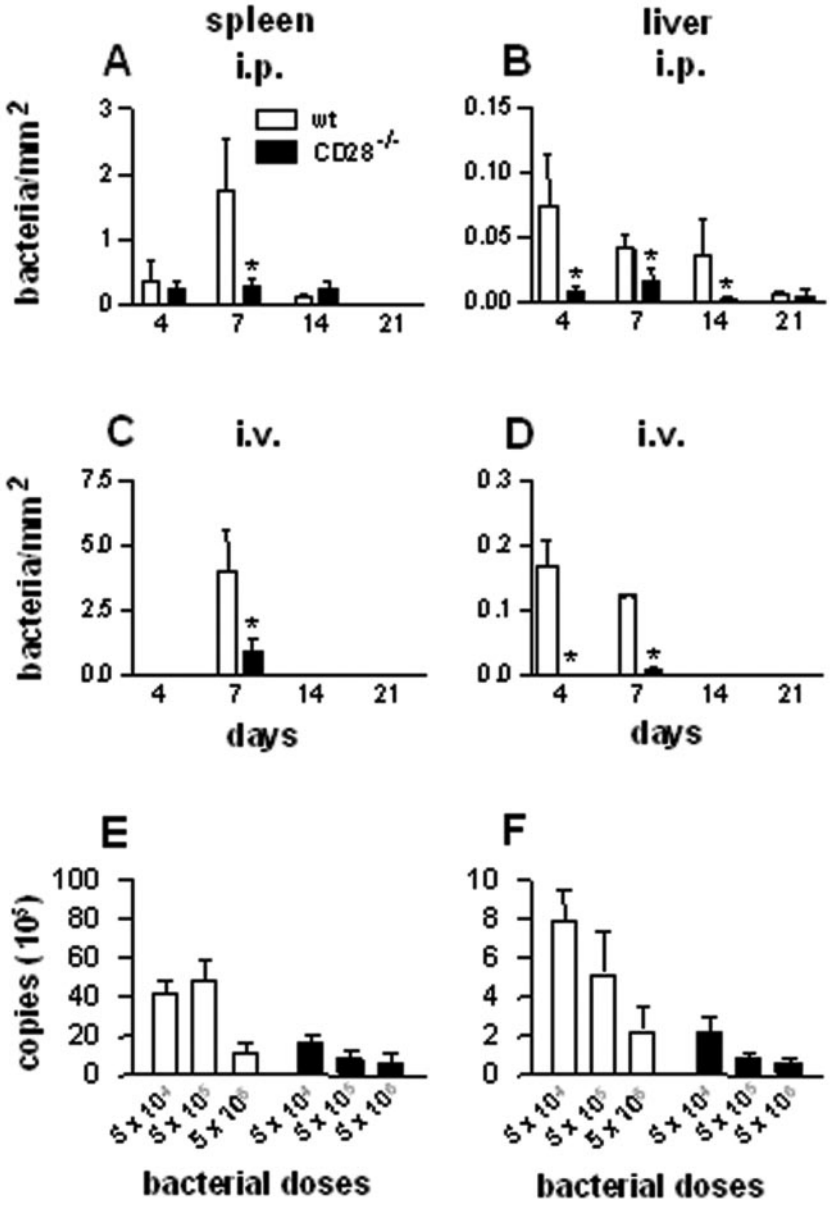
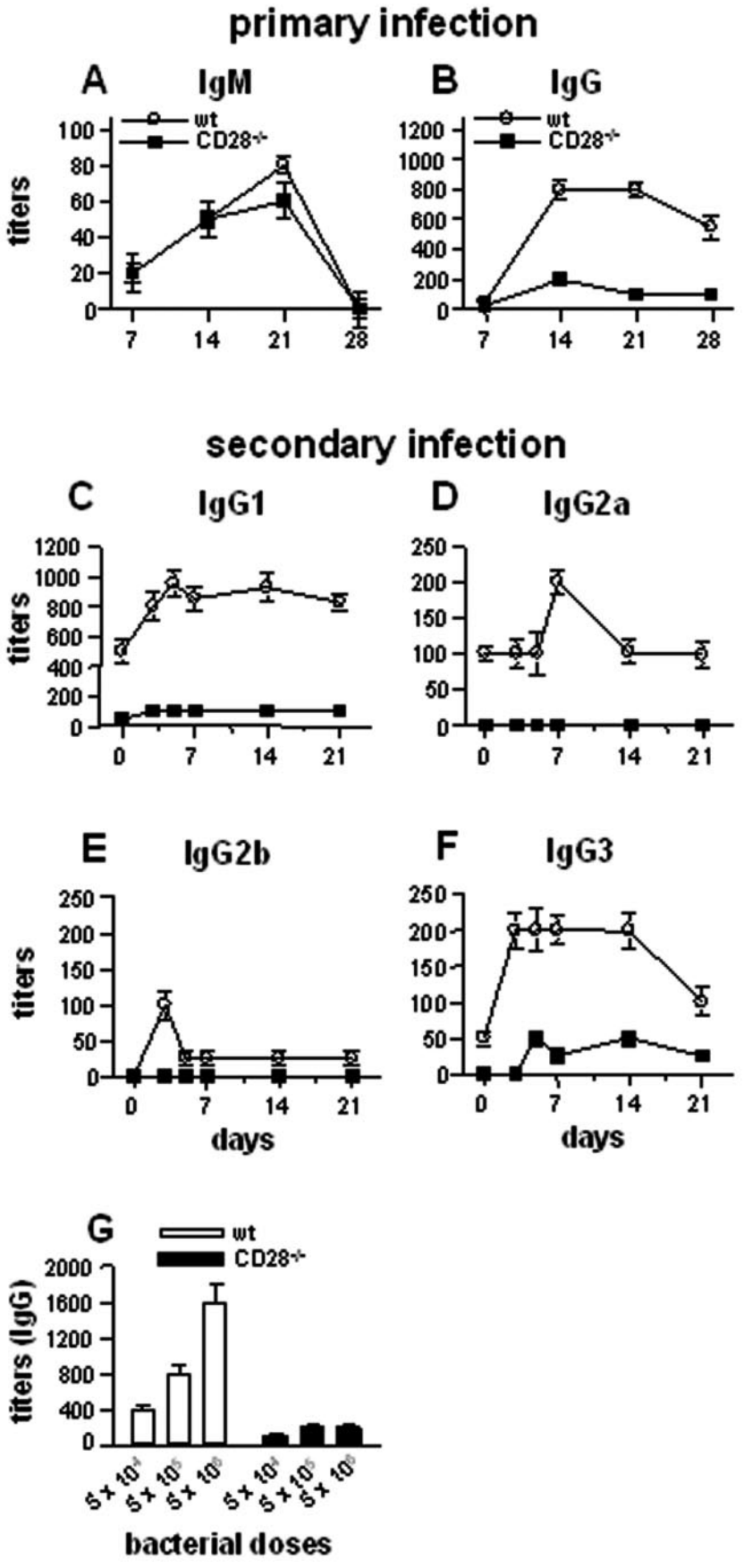


FIG. 2. *C. burnetii* loads for CD28$^{-/-}$ and wt mice. (A to D) Wild-type and CD28$^{-/-}$ mice were infected i.p. (A and B) or i.v. (C and D) with 5×10^5 *C. burnetii* organisms and killed at different times. Bacteria were revealed by immunostaining and were numerated by image analysis. The results are expressed as the number of bacteria per square millimeter of spleen (A and C) and liver (B and D) and are the means ± SD from five mice/time point. ∗, $P < 0.05$; represents the difference between CD28$^{-/-}$ mice and wt mice. (E and F) Wild-type and CD28$^{-/-}$ mice were infected i.p. with increasing doses of *C. burnetii* organisms and killed at day 7. The bacterial DNA copy numbers in the spleen (E) and the liver (F) were determined by qPCR.

tected after 14 days, and their titers remained high thereafter (Fig. 3B). During secondary infection, the titers of specific IgG increased to reach a plateau after 5 days (Fig. 3C). For CD28$^{-/-}$ mice, the titers of specific IgG were significantly lower than those of wt mice at each time point during primary (Fig. 3B) and secondary (Fig. 3C) infection. The differences in the titers of specific IgG in wt and CD28$^{-/-}$ mice did not depend on the doses of infecting *C. burnetii* organisms. Indeed, the titers of specific IgG varied with the doses of infecting organisms for wt mice but remained low when *C. burnetii* inoculum increased for CD28$^{-/-}$ mice (Fig. 3G). The study of specific subclasses of IgG was performed with wt and CD28$^{-/-}$ mice during secondary infection, since their titers were below detection threshold during primary infection (data not shown). With wt mice, specific IgG1 were not detected. Specific IgG2a were detected from day 0 to day 14, with a sharp peak at day 7 (Fig. 3D). Specific IgG2b levels were maximal at day 3 and decreased thereafter (Fig. 3E). Specific IgG3 were detected at day 0, and their levels increased to reach a plateau from day 3 to day 14 and decreased thereafter (Fig. 3F). For CD28$^{-/-}$ mice, specific IgG1 (data not shown), IgG2a (Fig. 3D), and IgG2b (Fig. 3E) were never detected. Only specific IgG3 levels


primary infection
A
IgM
B
IgG
wt
CD28-/-
titers
secondary infection
C
IgG1
D
IgG2a
E
IgG2b
F
IgG3
days
G
titers (IgG)
bacterial doses

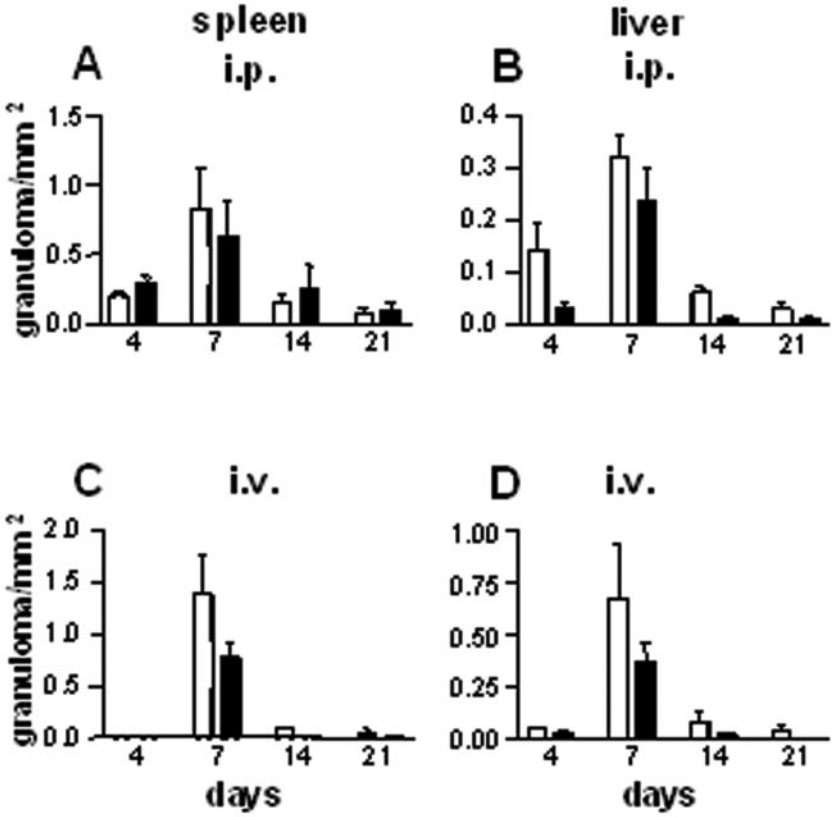


FIG. 4. Granuloma expression in CD28$^{-/-}$ and wt mice. Wild-type (open bars) and CD28$^{-/-}$ (filled bars) mice were infected i.p. (A and B) or i.v. (C and D) with 5×10^5 *C. burnetii* organisms and killed at different times. Granulomas were revealed by histochemical analysis. Their numbers in the spleen (A and C) and the liver (B and D) were determined microscopically. The results are expressed as the number of granulomas per square millimeter of tissue and are the means ± SD from five mice/time point.

were found 3 days after reinfection, and they remained low until day 21 (Fig. 3F). Taken together, these results show that the lack of CD28 affects IgG responses during primary infection and most markedly during secondary infection by *C. burnetii*.

Role of CD28 in granulomatous response to *C. burnetii* infection. As granuloma formation reflects host ability to mount a protective immune response, we investigated granuloma formation in *C. burnetii*-infected mice. In wt and CD28$^{-/-}$ mice, granulomas were mainly composed of macrophages, lymphocytes, and few polymorphonuclear leukocytes and were detected in liver lobules, portobiliary spaces, and splenic red pulp (Fig. 1B). Granulomas were detected in the spleens of wt and CD28$^{-/-}$ mice 4 days after i.p. infection; their numbers became maximal at day 7 and steadily decreased thereafter to be undetectable after 21 days (Fig. 4A). In the liver, granulomas were detected after 4 days; they reached maximum numbers at day 7, and their numbers markedly decreased thereafter to remain residual after 21 days in wt and CD28$^{-/-}$ mice (Fig. 4B). For wt and CD28$^{-/-}$ mice infected i.v., granulomas were detected in the spleen (Fig. 4C) and the liver (Fig. 4D) at day 7, and their numbers dropped thereafter. With secondary infection, a low number of granulomas was detected in spleen and liver and there was no difference between wt and CD28$^{-/-}$ mice (data not shown). These results show that lack of CD28 has no effect on granuloma formation in primary and secondary infections.

Role of CD28 in cytokine production during *C. burnetii* infection. IFN-γ, TNF, and IL-10, known to be involved in host response to *C. burnetii* (12, 15, 16, 21), were measured in supernatants from *C. burnetii*-stimulated spleen cells and peritoneal macrophages from i.p. infected mice. IFN-γ was detected in splenocyte supernatants at day 4 postinfection, and its amounts moderately increased to reach the highest values at day 14 with both wt and CD28$^{-/-}$ mice (Fig. 5A). A similar pattern of IFN-γ production was observed with splenocytes

FIG. 3. Ab response in CD28$^{-/-}$ and wt mice. (A to F) Wild-type and CD28$^{-/-}$ mice were infected i.p. with 5×10^5 *C. burnetii* organisms (primary infection) and reinfected after 30 days (secondary infection). Serum was collected at different times of infection or reinfection. The presence of IgM, IgG, and IgG subclasses directed against *C. burnetii* was assessed by immunofluorescence using inactivated *C. burnetii*. The results as titers are expressed as means ± SD from five mice/time point. (G) Wild-type and CD28$^{-/-}$ mice were infected i.p. with increasing doses of *C. burnetii* organisms and killed at day 14. The presence of specific IgG was assessed by immunofluorescence. The results as titers are expressed as means ± SD from five mice.

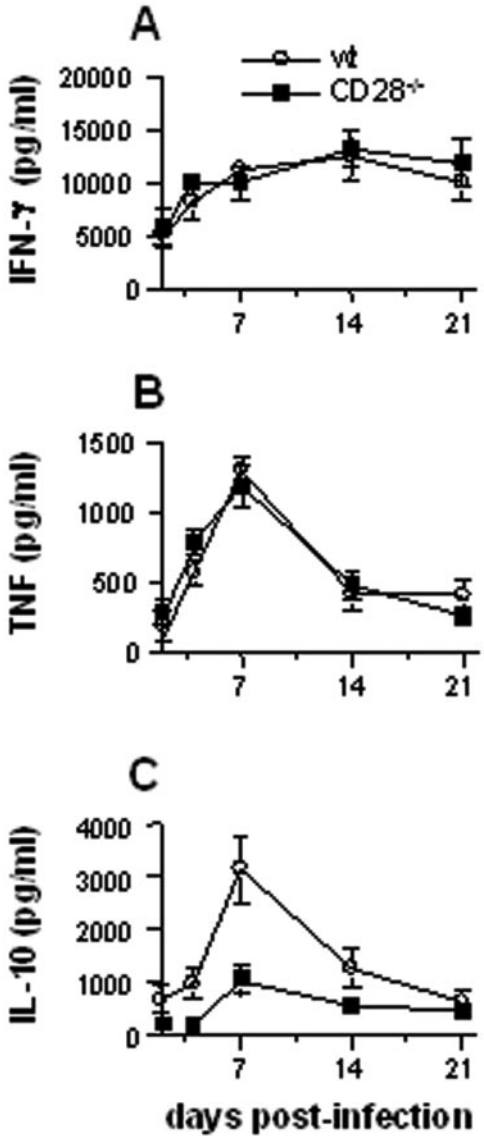


FIG. 5. Cytokine production in CD28$^{-/-}$ and wt mice. Splenocytes (A) and peritoneal macrophages (B and C) from wt and CD28$^{-/-}$ mice were isolated from uninfected (day 0) and *C. burnetii*-infected mice. Cells were incubated with inactivated *C. burnetii* (10:1 bacterium-to-cell ratio) for 24 h. The amounts of IFN-γ (A), TNF (B), and IL-10 (C) contained in cell supernatants were determined by immunoassays. Results are expressed as pg/ml and represent the means ± SD from three experiments.

from wt and CD28$^{-/-}$ mice infected i.v. (data not shown). TNF was detected in macrophage supernatants at day 4; its amounts reached maximum values at day 7 and steadily decreased thereafter for wt and CD28$^{-/-}$ mice infected i.p. (Fig. 5B). After i.v. infection, the TNF amounts were high at day 4 and decreased thereafter for both types of mice (data not shown). In contrast with IFN-γ and TNF, IL-10 amounts released by peritoneal macrophages were different for wt and CD28$^{-/-}$ mice. Indeed, they were high (520 ± 180 pg/ml) at day 0, reached a peak value (about 3,000 pg/ml) at day 7, and markedly decreased thereafter for wt mice. For CD28$^{-/-}$ mice, they were low (less than 50 pg/ml) at day 0 and increased moderately (about 1,000 pg/ml) at day 7 before returning to low values. IL-10 amounts were significantly lower ($P < 0.01$) for CD28$^{-/-}$ mice than for wt mice at days 4, 7, and 14 (Fig. 5C). Similar results were obtained when an i.v. route was used instead of an i.p. route (data not shown). We also investigated the production of Th2 cytokines such as IL-5. IL-5 was not detected in supernatants from wt and CD28$^{-/-}$ spleen cells (data not shown). Taken together, these results show decreased production of IL-10 in CD28$^{-/-}$ mice.

DISCUSSION

Converging results from animal models of *C. burnetii* infection and patients with Q fever demonstrate the major role of T-cell-mediated adaptive immunity in protection against *C. burnetii* (1, 21, 22, 26, 32). Here, we reported that CD28 favors *C. burnetii* infection. Indeed, the lack of CD28 resulted in the decrease of *C. burnetii* load in spleen and liver independently of the route of infection (i.p. or i.v.) and the type of infection (primary versus secondary infection). Although *C. burnetii* infection depended on the size of bacterial inoculum in wt mice, increasing *C. burnetii* inoculum did not induce bacterial replication in CD28$^{-/-}$ mice. The role of CD28 in *C. burnetii* infection is distinct from several infectious models. Indeed, *S. enterica* serovar Typhimurium load is increased in the livers of CD28$^{-/-}$ mice at early times following i.v. infection, and the organisms persist in livers and spleens of CD28$^{-/-}$ mice, thus establishing chronic infection (33). Similarly, during primary and secondary i.v. infection with *L. monocytogenes*, the lack of CD28 is associated with elevated bacterial loads at initial times postinfection (34). The control of *Mycobacterium bovis* growth and the clearance of lymphocytic choriomeningitis virus are maintained in the absence of CD28 (2, 19). Our findings may be related to *L. major* infection of CD28$^{-/-}$ mice that control the infection more efficiently than wt mice (11).

The role of CD28 in the generation of Abs specific for *C. burnetii* strengthens the statement that Abs are not associated with protection. Indeed, the titers of specific IgG and IgG subclasses were decreased during primary and secondary infection of CD28$^{-/-}$ mice. This confirms that Abs directed against *C. burnetii* are not protective since their decrease in CD28$^{-/-}$ mice is associated with increased bacterial clearance. The alteration of humoral response in CD28-deficient mice has been previously reported. In *S. enterica* serovar Typhimurium-infected CD28$^{-/-}$ mice, specific IgG3 levels were reduced and IgG1 and IgG2a were undetectable (33). This is related to the direct role of CD28 in Ab response, since CD28$^{-/-}$ mice fail to develop germinal centers and have decreased levels of Ab isotypes (28). Under our conditions, we did not find decreased formation of the germinal centers in the draining lymph nodes (data not shown).

The decreased *C. burnetii* burden within the spleens and the livers of CD28$^{-/-}$ mice was not associated with alteration of the granuloma formation, which reflects the efficient cell-mediated immune response towards *C. burnetii*. This finding is consistent with the report of Hogan et al., in which granulomas were found to be formed during *M. bovis* infection despite the absence of CD28 (19). The lack of CD28 or its ligands, CD80

and CD86, has no effect on granuloma formation in response to *Schistosoma mansoni*, a model of Th2 granuloma (18, 25). IFN-γ and TNF are two cytokines known to be involved in the formation of granuloma (24). In accordance with the lack of effect of CD28 on granuloma formation, we found that the production of IFN-γ and TNF was not affected in *C. burnetii*-infected $CD28^{-/-}$ mice. Our results are distinct from other reported data. The production of IFN-γ by *S. enterica* serovar Typhimurium-stimulated splenocytes is impaired in $CD28^{-/-}$ mice (33). It is also depressed in response to influenza virus (6) and *T. cruzi* (35). The frequency of IFN-γ-producing cells was decreased in $CD28^{-/-}$ mice infected with *L. monocytogenes* (34). In contrast to these reports, our results agree with two other reports. $CD86^{-/-}$ mice, in which CD28 signaling is partly affected, exhibit an increased IFN-γ production and a higher resistance to *L. major* (9). Mice lacking CD28 display increased IFN-γ expression during *Pneumocystis carinii* infection (5). Sustained production of IFN-γ may account for granuloma formation and for the clearance of *C. burnetii*, since the response to *C. burnetii* infection was not associated with changes in T-cell polarization. Although it has been reported that CD28 signaling is linked to the initiation of Th2 differentiation (3), we did not detect any changes of IL-4 and IL-5 production in the presence or the absence of CD28, suggesting that a Th1/Th2 shift does not occur in *C. burnetii* infection.

The lack of CD28 was associated with a decreased IL-10 production by peritoneal macrophages. This suggests that CD28 limits the development of an efficient microbicidal response to *C. burnetii* by inducing IL-10 and, consequently, by impairing Th1 immune response. We have previously shown that IL-10 promotes bacterial replication and is associated with microbicidal defect of chronic Q fever (15). Two different reports are in agreement with our results that CD28 acts through IL-10. $CD28^{-/-}$ mice control the lesions induced by *L. major*, whereas wt mice develop progressive nonhealing infections; their resistance to *L. major* is characterized by reduced number of parasites, increased IFN-γ expression, and decreased expression of IL-4 and IL-10 (11). In the murine model of septic shock, the administration of agonistic anti-CD28 Abs prevents animal death via the induction of IL-10; the protective effect of CD28 ligation is prevented with anti-IL-10 Abs (41). It is likely that the lack of CD28 affects *C. burnetii* clearance by altering IL-10-producing cell subsets. Indeed, it has been reported that the population of regulatory T cells expressing CD4 and CD25 is severely depleted in $CD28^{-/-}$ mice (37). Macrophage function may also be affected by the alteration of the CD28/B7 pathway. Alteration of CD28-CD80/CD86 interactions leads to the generation of alternatively activated macrophages able to suppress mixed lymphocyte reaction (40). Finally, it is likely that decreased IL-10 production associated with maintained IFN-γ production improves microbicidal competence of macrophages by relieving the inhibitory effect of IL-10.

In this report, we studied the role of CD28 costimulation in *C. burnetii* infection. CD28 initiates an activation program, which leads to decreased microbicidal competence of macrophages via the modulation of IL-10 production. This finding suggests another role for CD28 that is usually associated with development of T-cell-mediated immune protection. CD28 may be involved in the persistence of *C. burnetii* infection despite the development of efficient cell-mediated immune response.

ACKNOWLEDGMENTS

We thank Jean-Christophe Orsoni and Patrick Gibier for taking care of the $CD28^{-/-}$ mice at the animal facilities of the Institut de Cancérologie de Marseille.

This work was supported by a grant "Equipe labelisée 2001" from the Ligue Nationale contre le Cancer (to J. A. Nunès and D. Olive).

REFERENCES

1. **Andoh, M., T. Naganawa, A. Hotta, T. Yamaguchi, H. Fukushi, T. Masegi, and K. Hirai.** 2003. SCID mouse model for lethal Q fever. Infect. Immun. **71:**4717–4723.
2. **Andreasen, S. O., J. E. Christensen, O. Marker, and A. R. Thomsen.** 2000. Role of CD40 ligand and CD28 in induction and maintenance of antiviral $CD8^+$ effector T cell responses. J. Immunol. **164:**3689–3697.
3. **Andres, P. G., K. C. Howland, A. Nirula, L. P. Kane, L. Barron, D. Dresnek, A. Sadra, J. Imboden, A. Weiss, and A. K. Abbas.** 2004. Distinct regions in the CD28 cytoplasmic domain are required for T helper type 2 differentiation. Nat. Immunol. **5:**435–442.
4. **Barton, G. M., and R. Medzhitov.** 2002. Control of adaptive immune responses by Toll-like receptors. Curr. Opin. Immunol. **14:**380–383.
5. **Beck, J. M., M. B. Blackmon, C. M. Rose, S. L. Kimzey, A. M. Preston, and J. M. Green.** 2003. T cell costimulatory molecule function determines susceptibility to infection with *Pneumocystis carinii* in mice. J. Immunol. **171:**1969–1977.
6. **Bertram, E. M., A. Tafuri, A. Shahinian, V. S. Chan, L. Hunziker, M. Recher, P. S. Ohashi, T. W. Mak, and T. H. Watts.** 2002. Role of ICOS versus CD28 in antiviral immunity. Eur. J. Immunol. **32:**3376–3385.
7. **Brouqui, P., and D. Raoult.** 2001. Endocarditis due to rare and fastidious bacteria. Clin. Microbiol. Rev. **14:**177–207.
8. **Brown, D. R., J. M. Green, N. H. Moskowitz, M. Davis, C. B. Thompson, and S. L. Reiner.** 1996. Limited role of CD28-mediated signals in T helper subset differentiation. J. Exp. Med. **184:**803–810.
9. **Brown, J. A., R. J. Greenwald, S. Scott, A. N. Schweitzer, A. R. Satoskar, C. Chung, L. R. Schopf, D. van der Woude, J. P. Sypek, and A. H. Sharpe.** 2002. T helper differentiation in resistant and susceptible B7-deficient mice infected with *Leishmania major*. Eur. J. Immunol. **32:**1764–1772.
10. **Capo, C., F. Zugun, A. Stein, G. Tardei, H. Lepidi, D. Raoult, and J.-L. Mege.** 1996. Upregulation of tumor necrosis factor alpha and interleukin-1β in Q fever endocarditis. Infect. Immun. **64:**1638–1642.
11. **Compton, H. L., and J. P. Farrell.** 2002. CD28 costimulation and parasite dose combine to influence the susceptibility of BALB/c mice to infection with *Leishmania major*. J. Immunol. **168:**1302–1308.
12. **Dellacasagrande, J., C. Capo, D. Raoult, and J.-L. Mege.** 1999. IFN-γ-mediated control of *Coxiella burnetii* survival in monocytes: the role of cell apoptosis and TNF. J. Immunol. **162:**2259–2265.
13. **Fenollar, F., P.-E. Fournier, M. P. Carrieri, G. Habib, T. Messana, and D. Raoult.** 2001. Risks factors and prevention of Q fever endocarditis. Clin. Infect. Dis. **33:**312–316.
14. **Fenollar, F., P.-E. Fournier, D. Raoult, R. Gérolami, H. Lepidi, and C. Poyart.** 2002. Quantitative detection of *Tropheryma whipplei* DNA by real-time PCR. J. Clin. Microbiol. **40:**1119–1120.
15. **Ghigo, E., C. Capo, D. Raoult, and J.-L. Mege.** 2001. Interleukin-10 stimulates *Coxiella burnetii* replication in human monocytes through tumor necrosis factor down-modulation: role in microbicidal defect of Q fever. Infect. Immun. **69:**2345–2352.
16. **Ghigo, E., C. Capo, C.-H. Tung, D. Raoult, J.-P. Gorvel, and J.-L. Mege.** 2002. *Coxiella burnetii* survival in THP-1 monocytes involves the impairment of phagosome maturation: IFN-γ mediates its restoration and bacterial killing. J. Immunol. **169:**4488–4495.
17. **Harris, R. J., P. A. Storm, A. Lloyd, M. Arens, and B. P. Marmion.** 2000. Long-term persistence of *Coxiella burnetii* in the host after primary Q fever. Epidemiol. Infect. **124:**543–549.
18. **Hernandez, H. J., A. H. Sharpe, and M. J. Stadecker.** 1999. Experimental murine schistosomiasis in the absence of B7 costimulatory molecules: reversal of elicited T cell cytokine profile and partial inhibition of egg granuloma formation. J. Immunol. **162:**2884–2889.
19. **Hogan, L. H., W. Markofski, A. Bock, B. Barger, J. D. Morrissey, and M. Sandor.** 2001. *Mycobacterium bovis* BCG-induced granuloma formation depends on gamma interferon and CD40 ligand but does not require CD28. Infect. Immun. **69:**2596–2603.
20. **Honstettre, A., E. Ghigo, A. Moynault, C. Capo, R. Toman, S. Akira, O. Takeuchi, H. Lepidi, D. Raoult, and J.-L. Mege.** 2004. Lipopolysaccharide from *Coxiella burnetii* is involved in bacterial phagocytosis, filamentous actin reorganization, and inflammatory responses through Toll-like receptor 4. J. Immunol. **172:**3695–3703.
21. **Honstettre, A., G. Imbert, E. Ghigo, F. Gouriet, C. Capo, D. Raoult, and J.-L. Mege.** 2003. Dysregulation of cytokines in acute Q fever: role of interleu-

kin-10 and tumor necrosis factor in chronic evolution of Q fever. J. Infect. Dis. **187:**956–962.
22. **Izzo, A. A., and B. P. Marmion.** 1993. Variation in interferon-γ responses to *Coxiella burnetii* antigens with lymphocytes from vaccinated or naturally infected subjects. Clin. Exp. Immunol. **94:**507–515.
23. **Izzo, A. A., B. P. Marmion, and D. A. Worswick.** 1988. Markers of cell-mediated immunity after vaccination with an inactivated, whole-cell Q fever vaccine. J. Infect. Dis. **157:**781–789.
24. **Kaufmann, S. H. E.** 1999. Immunity to intracellular bacteria, p. 1335–1371. *In* W. E. Paul (ed.), Fundamental immunology. Lippincott, Philadelphia, Pa.
25. **King, C. L., J. Xianli, C. H. June, R. Abe, and K. P. Lee.** 1996. CD28-deficient mice generate an impaired Th2 response to *Schistosoma mansoni* infection. Eur. J. Immunol. **26:**2448–2455.
26. **Koster, F. T., J. C. Williams, and J. S. Goodwin.** 1985. Cellular immunity in Q fever: specific lymphocyte unresponsiveness in Q fever endocarditis. J. Infect. Dis. **152:**1283–1289.
27. **La Scola, B., H. Lepidi, and D. Raoult.** 1997. Pathologic changes during acute Q fever: influence of the route of infection and inoculum size in infected guinea pigs. Infect. Immun. **65:**2443–2447.
28. **Lenschow, D. J., T. L. Walunas, and J. A. Bluestone.** 1996. CD28/B7 system of T cell costimulation. Annu. Rev. Immunol. **14:**233–258.
29. **Lepidi, H., J. E. Bunnell, M. E. Martin, J. E. Madigan, S. Stuen, and J. S. Dumler.** 2000. Comparative pathology, and immunohistology associated with clinical illness after *Ehrlichia phagocytophila*-group infections. Am. J. Trop. Med. Hyg. **62:**29–37.
30. **Linsley, P. S., and J. A. Ledbetter.** 1993. The role of the CD28 receptor during T cell responses to antigen. Annu. Rev. Immunol. **11:**191–212.
31. **Maurin, M., and D. Raoult.** 1999. Q fever. Clin. Microbiol. Rev. **12:**518–553.
32. **Mege, J.-L., M. Maurin, C. Capo, and D. Raoult.** 1997. *Coxiella burnetii*: the 'query' fever bacterium. A model of immune subversion by a strictly intracellular microorganism. FEMS Microbiol. Rev. **19:**209–217.
33. **Mittrucker, H. W., A. Kohler, T. W. Mak, and S. H. Kaufmann.** 1999. Critical role of CD28 in protective immunity against *Salmonella typhimurium*. J. Immunol. **163:**6769–6776.
34. **Mittrucker, H. W., M. Kursar, A. Kohler, R. Hurwitz, and S. H. Kaufmann.** 2001. Role of CD28 for the generation and expansion of antigen-specific $CD8^+$ T lymphocytes during infection with *Listeria monocytogenes*. J. Immunol. **167:**5620–5627.
35. **Miyahira, Y., M. Katae, S. Kobayashi, T. Takeuchi, Y. Fukuchi, R. Abe, K. Okumura, H. Yagita, and T. Aoki.** 2003. Critical contribution of CD28-CD80/CD86 costimulatory pathway to protection from *Trypanosoma cruzi* infection. Infect. Immun. **71:**3131–3137.
36. **Raoult, D., T. Marrie, and J.-L. Mege.** 2005. Natural history and pathophysiology of Q fever. Lancet Infect. Dis. **5:**219–226.
37. **Salomon, B., D. J. Lenschow, L. Rhee, N. Ashourian, B. Singh, A. Sharpe, and J. A. Bluestone.** 2000. B7/CD28 costimulation is essential for the homeostasis of the $CD4^+CD25^+$ immunoregulatory T cells that control autoimmune diabetes. Immunity **12:**431–440.
38. **Shahinian, A., K. Pfeffer, K. P. Lee, T. M. Kundig, K. Kishihara, A. Wakeham, K. Kawai, P. S. Ohashi, C. B. Thompson, and T. W. Mak.** 1993. Differential T cell costimulatory requirements in CD28-deficient mice. Science **261:**609–612.
39. **Stein, A., H. Lepidi, J.-L. Mege, T. J. Marrie, and D. Raoult.** 2000. Repeated pregnancies in BALB/c mice infected with *Coxiella burnetii* cause disseminated infection, resulting in stillbirth and endocarditis. J. Infect. Dis. **181:** 188–194.
40. **Tzachanis, D., A. Berezovskaya, L. M. Nadler, and V. A. Boussiotis.** 2002. Blockade of B7/CD28 in mixed lymphocyte reaction cultures results in the generation of alternatively activated macrophages, which suppress T-cell responses. Blood **99:**1465–1473.
41. **Wang, R., Q. Fang, L. Zhang, L. Radvany, A. Sharma, N. Noben-Trauth, G. B. Mills, and Y. Shi.** 1997. CD28 ligation prevents bacterial toxin-induced septic shock in mice by inducing IL-10 expression. J. Immunol. **158:**2856–2861.
42. **Zhang, G. Q., H. To, T. Yamaguchi, H. Fukushi, and K. Hirai.** 1997. Differentiation of *Coxiella burnetii* by sequence analysis of the gene (com1) encoding a 27-kDa outer membrane protein. Microbiol. Immunol. **41:**871–877.

Editor: J. L. Flynn