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CD1d-restricted NKT cells modulate placental and uterine leukocyte populations during chlamydial infection in mice

Mohamed Habbeddine[a,b,c], **Philippe Verbeke**[a,1], **Christiane Delarbre**[a], **René Moutier**[a], **Stéphane Prieto**[a,1], **David M. Ojcius**[d], and **Colette Kanellopoulos-Langevin**[a,*]

[a]Laboratory of Inflammation, Gestation and Autoimmunity, Jacques Monod Institute, CNRS and University Paris-Diderot, 15 rue Hélène Brion, 75205 Paris Cedex 13, France

[b]Centre d'Immunologie de Marseille-Luminy (CIML), Aix-Marseille University UM2, France

[c]INSERM U1104 and CNRS UMR7280, Marseille, France

[d]Health Sciences Research Institute and School of Natural Sciences, University of California, 5200 North Lake Road, Merced, CA 95343, USA

Abstract

Invariant CD1d-restricted natural killer T cells play an important immunoregulatory role and can influence a broad spectrum of immunological responses including against bacterial infections. They are present at the fetal–maternal interface and although it has been reported that experimental systemic iNKT cell activation can induce mouse abortion, their role during pregnancy remain poorly understood. In the present work, using a physiological *Chlamydia muridarum* infection model, we have shown that, in vaginally infected pregnant mice, *C. muridarum* is cleared similarly in C57BL/6 wild type (WT) and CD1d$^{-/-}$ mice. We have also shown that infected- as well as uninfected-CD1d$^{-/-}$ mice have the same litter size as WT counterparts. Thus, CD1d-restricted cells are required neither for the resolution of chlamydial infection of the lower-genital tract, nor for the maintenance of reproductive capacity. However, unexpected differences in T cell populations were observed in uninfected pregnant females, as CD1d$^{-/-}$ placentas contained significantly higher percentages of CD4^{+} and CD8^{+} T cells than WT counterparts. However, infection triggered a significant decrease in the percentages of CD4^{+} T cells in CD1d$^{-/-}$ mice. In infected WT pregnant mice, the numbers of uterine CD4^{+} and CD8^{+} T cells, monocytes and granulocytes were greatly increased, changes not observed in infected CD1d$^{-/-}$ mice. An increase in the percentage of CD8^{+} T cells seems independent of CD1d-restricted cells as it occurred in both WT and CD1d$^{-/-}$ mice. Thus, in the steady state, the lack of CD1d-restricted NKT cells affects leukocyte populations only in the placenta. In *Chlamydia*-infected pregnant mice, the immune response against *Chlamydia* is dampened in the uterus. Our results suggest that CD1d-restricted NKT cells play a role in the recruitment or homeostasis of leukocyte populations at the maternal–fetal interface in the presence or absence of *Chlamydia* infection.

*Corresponding author. Present address: INSERM U699, Faculté de Médecine Xavier Bichat, CNRS and Université Paris-Diderot, 16, Rue Henri Huchard, 75018 Paris, France. colette.kanellopoulos@inserm.fr (C. Kanellopoulos-Langevin).
[1]Present address: INSERM U699, Faculté de Médecine Xavier Bichat, CNRS and Université Paris-Diderot, 16, Rue Henri Huchard, 75018 Paris, France.

Keywords

Chlamydia infection; NKT cells; Mouse models; Leukocyte populations; Pregnancy

1. Introduction

Chlamydia trachomatis genital-tract infections lead to infertility or unsuccessful pregnancy in many animal species. Most of the damage due to *Chlamydiae* appears to be unrelated to the presence of the pathogen itself, but is due instead to the inflammation and fibrosis following infection.

In murine pregnancy models, the innate immune system, rather than the adaptive immune system, is responsible for infertility and spontaneous abortions [1,2]. The innate immune system provides rapid defense mechanisms based on recognition of pathogens by macrophages, neutrophils, natural killer (NK) and NKT cells, and secretion of inflammatory cytokines [3], which directly or indirectly lead to damage of Fallopian tubes or the placenta, leading to the demise of the fetus.

With the goal of better understanding the response of the maternal innate immune system to infection, we focused this study on the behavior of placental and uterine leukocytes, and especially CD1d-restricted NKT cells, during *Chlamydia* infection. CD1d-restricted NKT cells can be classified into type 1 NKT cells using an invariant Vα14-Jα18 TCR α chain (CD1d-restricted NKT cells) and type 2 NKT cells with diverse Vα TCR chains [4]. CD1d-restricted NKT cells, which can be activated by α-galactosylceramide (α-GalCer), have been more extensively studied than type 2 NKT cells [5]. However, little is known about the presence or function of uterine NKT cells and, particularly, CD1d-restricted NKT cells during pregnancy.

The activation of iNKT cells by α-GalCer, the ligand of the Vα14-Jα18 TCR chain of CD1d-restricted NKT cells, induced pregnancy loss, while the injection of α-GalCer into CD1d-deficient mice lacking CD1d-restricted NKT cells had no effect on pregnancy [6]. α-GalCer induces CD1d-restricted NKT cell activation and abortion in WT mice through a mechanism involving TNFα, IFNγ and perforin [6,7]. Proinflammatory cytokines such as TNFα, IL-1β and IFNγ appear to be critical mediators in the induction of pregnancy loss [8].

In general, the role of CD1d-restricted NKT cells during bacterial infections is not clear. CD1d-restricted NKT cells played a protective role in host defense against infection by some bacterial pathogens such as *Mycobacterium tuberculosis* and *Pseudomonas aeruginosa* [9,10], but the presence of these cells was detrimental in the case of *Salmonella* and *Listeria* infections [11,12]. In the case of chlamydial infections, CD1d-restricted NKT cells conferred protection against *C. tracho-matis*-induced arthritis [13], or *C. muridarum*-induced genital tract infection [14]; but Bilenki et al. [15] have shown, conversely, that CD1d-restricted NKT cells promote *C. muridarum* lung infection. Moreover, using another chlamydial species, *Chlamydophila pneumoniae*, Joyee et al. [16] reported that CD1d-restricted NKT cells play a crucial role in mediating the protective immune responses against lung infection. Hence, the role of CD1d-restricted NKT cells during chlamydial

infection is still not fully understood. In the experiments by Bilenki et al. [15], *C. muridarum* was inoculated intranasally. We therefore sought to determine whether CD1d-restricted NKT cells may play the same role after intravaginal infection by *C. muridarum*, since the infection pathway, the defense mechanisms of each mucosa and the tissue distribution of CD1d-restricted NKT cells are most likely critical factors in the response to chlamydial infection.

To study the involvement of CD1d-restricted NKT cells in pregnancy and chlamydial infection, we compared 4 groups of pregnant mice: C57BL/6 (B6) WT and CD1d-deficient ($CD1d^{-/-}$) pregnant mice, infected or not with *C. muridarum*. We first verified that the course of infection was not affected in non-pregnant B6 mice by CD1d-deficiency or CD1d-restricted NKT cell activation by α-GalCer. Subsequently, we characterized the uterine and placental leukocytes in WT and $CD1d^{-/-}$ pregnant females to determine whether CD1d-restricted NKT cells had any effect on other leukocyte populations during infection with *C. muridarum*.

Our results indicate that although CD1d-restricted NKT cells are not required for the resolution of chlamydial infection of the lower-genital tract, they do play a role in the recruitment or homeostasis of several other uterine and placental leukocyte populations present at the fetal–maternal interface of vaginally-infected mice.

2. Materials and methods

2.1. Animals

C57BL/6JRj (B6) wild type (WT) mice were obtained from the breeding center CERJ Janvier (Le Genest Saint Isle, France) and $CD1d1^{-/-}$ mice (CD1d1 homozygous knock-out mice), back-crossed onto the C57BL/6J genetic background, were obtained from Dr. Van Kaer [17]. Colonies were raised and housed in specific pathogen-free conditions, following institutional guidelines.

2.1.1. Infection of nonpregnant animals with *C. muridarum*—We used five nonpregnant WT B6 and five nonpregnant $CD1d^{-/-}$ seven week-old mice per experiment. The mice were injected subcutaneously with progesterone (2.5 mg of Depo-Provera) 7 days before vaginal infection with 10^6 infectious forming units (IFU) of *C. muridarum* in a final volume of 20 μl.

2.1.2. Injection of nonpregnant mice infected with *C. muridarum* with α-galactosylceramide (α-GalCer)—We injected 10 nonpregnant C57BL/6 mice with progesterone 7 days before infection with 10^6 IFU of *C. muridarum*. Five of these mice were injected 4 times i.p. with 2.5 μg of α-GalCer (a gift from Dr. M. Bonin [18]), on days −2, 0, +3 and +7 after vaginal infection. The other 5 control mice were injected with excipient at the same times.

2.1.3. Infection of pregnant mice with *C. muridarum*—B6 WT and $CD1d^{-/-}$ pregnant mice were infected vaginally with 10^6 IFU of *C. muridarum*, 5.5 days *post coitum*

(dpc) and sacrificed at 14.5 dpc. Uninfected B6 WT and CD1d$^{-/-}$ pregnant mice were used as controls and sacrificed at 14.5 dpc.

2.2. Quantification of infection

The course of infection was monitored by collecting cervico-vaginal swabs for 3 weeks after infection, and quantifying the *Chlamydiae* present on the swabs. We used sterile DACRON polyester tipped applicators from Puritan (Hardwood Products Company LP Guilford, Maine, USA) for the vaginal swabs. Bacterial DNA was extracted from the swabs using the QIAGEN-DNA minikit. The recovered DNA was quantified by real time PCR (qPCR). We used Brilliant SYBR Green QPCR Master Mix and the MX3000P Instrument from Stratagene (Agilent). As a measure of infection, we quantified expression of the *omp* gene coding for the major outer membrane protein (MOMP) of *C. muridarum*. Quantification of the bacteria in the vaginal swabs was calculated using a standard curve of serial numbers of copies of a plasmid containing one insert of MOMP. This insert of 209 bp was obtained by amplification of chlamydial DNA with Pfu DNA polymerase (Stratagene) using two specific primers for MOMP: F, 5′-ACAACATGGAACCCAACGAT-3′; and R, 5′-TCGAT-CAAGCGTGTCTCAAC-3′. This PCR product was then cloned and transformed in TOP10 One shot bacteria using the Zero Blunt TOPO PCR Cloning Kit (Invitrogen).

2.3. Placental and uterine leukocyte preparations

We isolated the leukocytes from the uterus and placenta of B6 WT and CD1d$^{-/-}$ uninfected and *Chlamydia*-infected mice at day 14.5 pc. Each group of mice consisted of 3 mice. The placenta and uterus were cut in small pieces. The uterus was then incubated for 30 min at 37 °C with 1 mg/ml Collagenase A (Roche; Ref. 103586). The two organs were crushed and filtered on 70 μm cell strainers (Cell strainer Falcon BD 352350). The uterine preparation was then submitted to a 30 min digestion at 37 °C with DNAse (DNAse I, Roche, Ref. 10104159001). After centrifugation at 300 g at +4 °C for 15 min, the pellets of placental and uterine cells were dispersed in ACK lysis buffer until total lysis of the erythrocytes, and then centrifuged for 15 min at 300 g at +4 °C. Each cell pellet was resuspended in 6 ml of 80% Percoll, and covered by a layer of the same volume of 40% Percoll. After centrifugation at 1500 g at +4 °C for 30 min, leukocytes located at the interface were collected and washed before the Flow Cytometry analysis.

2.4. Flow cytometry analysis of leukocyte populations in placenta and uterus of mice

The cells were incubated first with a purified anti-mouse CD16/CD32 antibody (BD Biosciences 553142) for 30 min then, washed with PBS containing 4% fetal bovine serum (FBS) and 0.1% azide and centrifuged at 280 g for 5 min. After resuspension, the cells were incubated for 1 h at +4 °C with the following antibodies: PE-anti-mouse CD4^{+} (Southern Biotech); FITC-anti-mouse CD8α (eBioscience); PE-anti-mouse CD11b (eBioscience); PE-Cy7-anti-mouse CD11c (eBioscience); FITC-anti-mouse CD19 (BD Biosciences); biotin-anti-mouse B220/CD45R (BD Biosciences), biotin-anti-mouse Gr-1 (BD Biosciences); PE-anti-mouse NK1.1 (BD Biosciences); and biotin-anti-mouse TCRβ chain (eBio-science). Predetermined optimal concentrations of antibodies are added to the cells and incubated for 1 h on ice. Bio-tinylated antibodies are revealed by Streptavidin-APC (eBio-science). The

cells were washed with PBS containing 4% FBS and 0.1% azide, resuspended in the same buffer containing propidium iodide (to exclude dead cells) and analyzed on a CyAn LX flow cytometer (Dako Cytomation) equipped with 488 nm and 635 nm lasers, at the Institut Jacques Monod's Cytometry Core facility. The cell populations analyzed were gated on the viable lymphoid cell population and on the basis of forward and side scatter criteria. The following cell populations were studied: $CD4^+$ T cells ($CD4^+$ and $TCR\beta^+$), $CD8^+$ T cells ($CD8^+$ and $TCR\beta^+$), NK cells ($NK1.1^+$), NKT cells ($NK1.1^+$ and $TCR\beta^+$), B cells ($CD19^+$ and $B220^+$), monocytic cells ($CD11b^+$ and $Gr1^+$), granulocytes ($CD11b^+$ and $Gr1^{high}$), and dendritic cells (DC, $CD11b^+$, $CD11c^+$ and $Gr1^-$).

2.5. Statistical analysis

Data were analyzed for statistical significance using the Student's *t* test.

3. Results

3.1. In nonpregnant mice, the course of *C. muridarum* infection is similar in WT and $CD1d^{-/-}$ mice, even after α-GalCer injection

B6 WT and $CD1d^{-/-}$ mice were infected vaginally with 10^6 IFU of *C. muridarum*. The course of infection was followed during 3 weeks by quantification of bacteria in vaginal swabs. The intensity and duration of infection was similar in $CD1d^{-/-}$ and WT mice (Fig. 1A). The differences observed at day 7 between the two groups of 5 mice were not significant due to the large variation in the number of bacteria collected from each group. A peak of infection was observed on day 7, and the level of infection then declined rapidly. At the vaginal level, the infection was resolved after about 3 weeks.

To confirm a lack of involvement of NKT cells in the resolution of vaginal-chlamydial infection, we examined the effect of α-GalCer, which activates CD1d-restricted NKT cells, on the course of infection in WT mice. Five WT mice were injected i.p. 4 times with 2.5 μg α-GalCer on −2, 0, +3, +7 days post-infection, and were compared with 5 control infected mice injected i.p. with PBS only (Fig. 1B). Similarly to the results in Fig. 1A, the differences between the two groups of mice were not significant, with infection peaking on day 7 and resolved after 3 weeks.

3.2. Comparison of gestation outcome and leukocyte populations in the placenta and uterus of uninfected versus *Chlamydia*-infected WT and $CD1d^{-/-}$ pregnant mice

3.2.1. Abortion rate and litter size—WT and $CD1d^{-/-}$ mice were infected 5.5 dpc and sacrificed on 14.5 dpc, i.e. after 9 days of infection. The total number of embryos at 14.5 dpc was similar in uninfected pregnant WT and $CD1d^{-/-}$ mice, with a mean litter size of 8.2 (±0.96) embryos per mouse in B6WT mice versus 8.7 (±0.58) in $CD1d^{-/-}$ females. The percentages of aborted embryos in uninfected females were 5.6% (±9.6) in WT mice and 7.9% (±5.6) in $CD1d^{-/-}$ mice (no significant difference, Fig. 1C).

In infected pregnant WT and $CD1d^{-/-}$ mice, the number of embryos at 14.5 dpc was comparable (8 ± 2.39 versus 7.7 ± 2.43, respectively). In infected mice, the percentages of aborted/abnormal embryos were not significantly different in the two groups as we observed

great variations between animals (50.7% ± 35.8 for WT mice versus 38.9% ± 24.5 for $CD1d^{-/-}$ mice).

In conclusion, no significant differences were observed between *Chlamydia*-infected WT and $CD1d^{-/-}$ mice in their gestation course and number of progeny.

3.2.2. Placenta

3.2.2.1. Placental leukocytes in uninfected pregnant mice: The following results were expressed per placenta to avoid the variations related to the number of fetal-placental units per mouse. The average number of total leukocytes per placenta recovered from 3 uninfected animals was 2.5×10^5 cells ± 6.5×10^4 in WT mice (24 placenta total) and $1.3 \times 10^5 \pm 2.5 \times 10^4$ cells in $CD1d^{-/-}$ mice (24 placentas total), which is not significantly different (Fig. 2A, white bars). In the placentas of both strains of uninfected-mice, the most abundant cell populations (in percentage and number) were granulocytes, which represented about 30% of the total leukocyte population (Fig. 2B, C). Monocytic cells represented about 18%, and B lymphocytes are around 15% of the whole placental leukocyte population of uninfected WT and $CD1d^{-/-}$ mice (Fig. 2C). The percentage of dendritic cells (DCs) was about 4%, which was also similar in WT and $CD1d^{-/-}$ placentas (Fig. 2B, C). Likewise, NK cells represented 1% and NKT cells only 0.1% of the placental leukocyte populations of uninfected mice. Interestingly, the percentage of $CD8^+$T cells was significantly higher in $CD1d^{-/-}$ (3.8%) than in WT placenta (2.3%) ($p < 0.005$). The percentage of $CD4^+$ T cells was also significantly higher in placenta of $CD1d^{-/-}$ (7.3%) than in placenta of WT (3.7%) uninfected mice ($p < 0.001$) (Fig. 2B). Total numbers of all placental leukocytes populations were not significantly different between uninfected WT and $CD1d^{-/-}$ mice.

Thus, the main difference between placental leukocytes in $CD1d^{-/-}$ versus B6 WT uninfected mice were the significantly higher percentages of $CD4^+$ and $CD8^+$ T cells in $CD1d^{-/-}$ animals.

Thus, the comparison of placental leukocyte populations in WT versus $CD1d^{-/-}$ uninfected pregnant mice suggests that placental $CD4^+$ and $CD8^+$ T cells are negatively regulated by the presence of CD1d-restricted NKT cells.

3.2.2.2. Placental leukocytes in *Chlamydia*-infected pregnant mice: In the placentas of infected WT mice, the total number of leukocytes was not significantly different ($1.4 \times 10^5 \pm 2.9 \times 10^4$ cells per placenta, total of 19 placentas) from the B6WT uninfected controls ($2.5 \times 10^5 \pm 6.5 \times 10^4$ cells) (Fig. 2A, gray bars). In the placenta of infected $CD1d^{-/-}$ mice, the number of leukocytes ($1.4 \times 10^5 \pm 3.7 \times 10^4$ cells per placenta) was similar to the $CD1d^{-/-}$ uninfected control ($1.3 \times 10^5 \pm 2.5 \times 10^4$ cells per placenta, total of 19 placentas) (Fig. 2A, bars $Cd1d^{-/-}$). Comparing numbers of placental leukocyte populations of infected mice with those of control uninfected mice, it appeared that no cell population number was affected significantly by chlamydial infection in WT or $CD1d^{-/-}$ mice (Fig. 2D, E). Similarly, considering the percentages of the different placental leukocyte populations (Fig. 2B, C), we did not find significant alterations in infected WT or $CD1d^{-/-}$ mice compared to non-infected counterparts, with the exception of the percentage of placental $CD4^+$ T cells in $CD1d^{-/-}$ infected mice, which was significantly decreased when compared to uninfected

mice of the same strain (from 7.3% to 5%, $p < 0.005$) (Fig. 2B). Comparing the percentages of the different placental populations in WT versus CD1d$^{-/-}$ infected mice, we found a significant increase ($p < 0.005$) of the percentage of B cells in CD1d$^{-/-}$ (14.6%) versus WT (10.8%) infected mice, and a significant decrease of the percentage of granulocytes in CD1d$^{-/-}$ (24%) versus WT infected mice (35.5%, $p < 0.005$) (Fig. 2C).

Thus, in the absence of CD1d-restricted NKT cells, and in response to a chlamydial infection, placental B cell percentages increased significantly, while placental granulocyte frequency decreased.

3.2.3. Uterus

3.2.3.1. Uterine leukocytes in uninfected pregnant mice: The average number of total leukocytes recovered from the whole uterus of 3 uninfected pregnant females was $8.3 \times 10^5 \pm 8.8 \times 10^4$ cells in WT mice ($n = 24$ implantation sites) and $9.4 \times 10^5 \pm 9.4 \times 10^4$ cells in CD1d$^{-/-}$ mice ($n = 24$ implantation sites) (Fig. 3A, white bars). Likewise, no significant difference in any leukocyte sub-population (in percentage or number) could be observed between WT and CD1d$^{-/-}$ uninfected pregnant mice (Fig. 3B–E).

In WT and CD1d$^{-/-}$ mice, dendritic cells (DCs) (Fig. 3B, D) and monocytic cells (Fig. 3C, E) were the most abundant cells in the uterus (from 20 to 27% of the total uterine leukocytes). The percentages of NK cells were similar in WT and CD1d$^{-/-}$ mice and represented about 10% of uterine leukocytes (Fig. 3B). The percentage of uterine granulocytes was comparable also in both types of mice: 2.2% in CD1d$^{-/-}$ mice and 4.3% in WT mice, a drastic difference from the placental population (Fig. 2C). CD4$^+$ and CD8$^+$ T cells represented about 5% of uterine leukocytes in both groups of mice, while NKT and B cells were minor leukocyte populations of the uterus (from 1% to 2% of total cells, Fig. 3B, C).

In conclusion, no significant difference in any leukocyte population (in percentage or number) could be observed between WT and CD1d$^{-/-}$ uninfected pregnant mice (Fig. 3B–E).

3.2.3.2. Uterine leukocytes in *Chlamydia*-infected pregnant mice: In the uterus of *Chlamydia*-infected WT mice, the total number of leukocytes was 2.6 fold higher ($2.2 \times 10^6 \pm 4 \times 10^5$ cells, $n = 19$ implantation sites) than in uninfected mice ($8.3 \times 10^5 \pm 8.8 \times 10^4$ cells, $p < 0.05$, $n = 24$ implantation sites). In contrast, in the uterus of infected CD1d$^{-/-}$ mice ($n = 19$ implantation sites), the total number of leukocytes did not change, compared to the uterus of CD1d$^{-/-}$ uninfected mice ($n = 24$ implantation sites), ($9 \times 10^5 \pm 5 \times 10^5$ cells versus $9.4 \times 10^5 \pm 9.4 \times 10^4$ cells, respectively) (Fig. 3A).

In *Chlamydia*-infected WT mice, the percentages of uterine monocytic cells and CD8$^+$ T cells were drastically increased (Fig. 3B, C, $p < 0.001$) compared to uninfected WT mice. Conversely, the percentage of uterine NK was significantly diminished (Fig. 3B, C, $p < 0.05$) during infection in WT mice. DC followed the same decreasing trend, but the difference was not statistically significant ($p = 0.058$) due to the variability from mouse to mouse. Moreover, the total number of uterine leukocyte populations was enhanced during

infection in WT mice, but only the numbers of $CD4^+$ T cells ($p < 0.001$), $CD8^+$ T cells ($p < 0.05$), monocytic cells and granulocytes ($p < 0.05$) were significantly augmented: 3, 10.4, 4.7 and 2.7 fold, respectively (Fig. 3D, E).

In $CD1d^{-/-}$ mice, chlamydial infection had little effect on the different leukocyte populations of the uterus. Their numbers were similar to those of uninfected controls. Comparing percentages, only the percentage of $CD8^+$ T cells increased 2.6 fold during chlamydial infection (Fig. 3B and C, $p < 0.05$). When we compared uterine leukocyte populations of WT versus $CD1d^{-/-}$ infected pregnant mice, we found that percentages of uterine monocytic cells and granulocytes ($p < 0.005$), and numbers of granulocytes ($p < 0.001$) were much lower in $CD1d^{-/-}$ mice (Fig. 3C, E).

Thus, our results in the uterus of infected pregnant females again suggest a role for Cd1d-restricted NKT cells in the recruitment of other leukocyte populations ($CD4^+$ and $CD8^+$ T cells, monocytes and granulocytes) or the control of their homeostasis.

3.3. Comparison of the effects of a *C. muridarum* infection on placental and uterine leukocyte populations in WT and $CD1d^{-/-}$ pregnant mice (comparison within each strain of the effect of infection compared to no infection)

Using the data from Figs. 2 and 3, we plotted the ratios between leukocyte population cell numbers in infected versus uninfected pregnant mice in WT or $CD1d^{-/-}$ mice. We chose to compare cell numbers, as a more accurate parameter of the individual fate of the distinct leukocyte populations. The results are presented in Fig. 4.

As shown in Fig. 4A, in placenta from WT mice (white bars), *Chlamydia* infection caused the number of $CD8^+$ T cells to increase by 90%, while all other leukocyte populations were marginally decreased. In contrast, $CD1d^{-/-}$ mice (black bars) presented a much more drastic increase (226%) in the number of placental $CD8^+$ T cells, as well as a doubling of placental NKT cells, possibly in compensation for the lack of Cd1d-restricted NKT cells. All other population numbers remained unaffected.

Fig. 4B presents the results of a similar analysis performed on the uterine cell populations from the same mice. In WT mice, the total leukocyte number increased by 164%, and 4 different populations also increased in number during *Chlamydia*-infection: granulocytes (by 176%), $CD4^+$ T cells (210%), monocytic cells (372%) and $CD8^+$ T cells (944%). In contrast, in $CD1d^{-/-}$ infected-mice, only $CD8^+$ T cell number increased (by 179% only), compared to $CD1d^{-/-}$ uninfected mice.

In conclusion, placental leukocyte populations were only marginally affected by *Chlamydia*-infection in either strain of mice, except for a net tendency to increasing numbers of $CD8^+$ T cells (+90% chez les WT et +226% chez les $CD1d^{-/-}$ mice), and an augmentation of NKT cells (+104%) only in $CD1d^{-/-}$ mice.

In the uterus, the immune response against *Chlamydia* seen in WT mice is drastically dampened in the absence of CD1d: no increase in total leukocyte numbers, granulocytes, $CD4^+$ T cells, NKT cells and monocytic cells, and a smaller increase of $CD8^+$ T cell number

only. This strongly suggests a possible role for CD1d-restricted NKT cells in the control, recruitment or homeostasis of several other leukocyte populations at the maternal–fetal interface during *Chlamydia* infection.

These data clearly indicate that, although WT and CD1d$^{-/-}$ pregnant mice respond in a similar fashion both clinically and macroscopically to intra-vaginal *Chlamydia* infections, the cellular responses within the placenta and the uterus are clearly different in the two strains of mice.

3.4. Comparison of the placental and uterine leukocyte populations in CD1d$^{-/-}$ versus WT pregnant mice, in the presence or absence of a vaginal *C. muridarum* infection (comparison of the two strains, without or with infection)

We then asked how each leukocyte population, in the placenta or uterus, was affected by the absence of CD1d-restricted NKT cells, in the absence or presence of a *Chlamydia* infection. We thus calculated the ratios of the mean of each leukocyte population number from CD1d$^{-/-}$ versus WT mice, infected or not with *C. muridarum*. Results are shown in Fig. 5.

Fig. 5A presents ratios of the means of placental leukocyte numbers from CD1d$^{-/-}$ versus WT pregnant mice. In the absence of infection (white bars), we found that nearly all placental leukocyte populations decreased by 50% at most in CD1d$^{-/-}$ mice, excepted CD4^{+} T cell numbers, which did not change. The total numbers of leukocytes, NK cells, NKT cells, monocytes, DC and granulocytes diminished by at least 40%. The same comparison performed in *Chlamydia*-infected mice (black bars) revealed that CD1d$^{-/-}$ mice had higher absolute numbers than WT mice of the following cells in the placenta: NK (about 17%), DC (23%), B cells (35%), CD8^{+} T cells (45%) and NKT cells (92%); but they had 36% fewer granulocytes than WT control mice.

In the uterus of CD1d$^{-/-}$ versus WT mice, the data are again quite different from the placenta (Fig. 5B). In the absence of infection (white bars), in CD1d$^{-/-}$ compared to WT mice, the uterine populations of CD8^{+} T cells, CD4^{+} T cells and B cells increased by 80, 60, and 40%, respectively, and granulocytes diminished by nearly 40%. During *Chlamydia* infection (black bars), strikingly, all CD1d$^{-/-}$ cell types were diminished by at least 40%, and granulocytes by up to 80% ($p < 0.01$) compared to cells collected in uterus from WT mice.

Thus, Fig. 5 shows that the lack of CD1d-restricted NKT cells appears to affect the leukocyte numbers in the placenta and uterus of pregnant mice, but in a mirror image fashion when: 1-the two organs at the fetal–maternal interface are compared and 2- the mice are infected or not.

4. Discussion

Our results indicate that neither CD1d-restricted NKT cells nor the CD1d molecule are essential for the resolution of a vaginal infection with *C. muridarum*. The infection was eliminated in the lower genital tract after 3 weeks in both groups of mice. Joyee et al. [16,19] reported that the activation of iNKT cells by α–GalCer reduced *C. pneumoniae*

infection but enhanced *C. muridarum* infection. Conversely, in our experiments, the activation and expansion of iNKT cells by α-GalCer [20–22] in WT mice had no effect on the resolution of infection. Moreover, uninfected or *Chlamydia*-infected WT and CD1d$^{-/-}$ mice presented no difference in their gestation course or number of progeny.

The number of NKT cells was extremely low in the placenta, representing only 0.1% of the total population of leukocytes. It is possible that some of them are derived from minor blood contaminations during tissue preparation. In the uterus, their number was higher, representing about 1.5% of the total leukocytes per uterus in WT and CD1d$^{-/-}$ mice. It was not possible to estimate the percentages of CD1d-restricted type 1 and type 2 NKT cells [23] in these organs from these results. The recruitment of CD1d-restricted NKT cells in the placenta and uterus is in agreement with the experiments of Apostolou et al. [24] and Mempel et al. [25], showing that iNKT cells migrated and accumulated at inflammatory sites resulting from the presence of bacterial components.

In CD1d$^{-/-}$ mice, most placental leukocyte populations were not modified significantly by chlamydial infection, with the exception of a significant decrease in CD4^{+} T cells. The decrease of the CD4^{+} T cells in the placenta of infected CD1d$^{-/-}$ mice could be related to the lack of CD1d-restricted NKT cells in these animals. In the placentas of uninfected CD1d$^{-/-}$ mice, the percentages of CD4^{+} T and CD8^{+} T cells were significantly larger, compared to WT mice, although they showed comparable total leukocyte levels. Moreover, percentages of placental granulocytes were significantly reduced in infected CD1d$^{-/-}$ mice compared to infected WT animals. These observations again suggest that CD1d-restricted NKT cells could play a role in the regulation of CD4^{+} and CD8^{+} T cells and granulocytes in the placenta.

In the uterus of uninfected pregnant mice, the total numbers of leukocytes and percentages or numbers of the different uterine populations were similar in the WT and CD1d$^{-/-}$ mice, implying that the presence of CD1d-restricted NKT cells in the WT pregnant uterus had no effect on the distribution or frequency of other leukocytes in the uterus.

Conversely, when pregnant mice were infected with *Chlamydia*, CD1d-restricted NKT cells were most probably activated and could influence other leukocyte populations. Type 1 NKT cells (CD1d-restricted NKT cells) were probably more involved in chlamydial infection than type 2 NKT cells. Indeed, these two types of cells are activated by glycolipids presented by CD1d molecules, but display distinct functional characteristics [26,27].

The uterine CD1d-restricted NKT cells seemed to react to chlamydial infection and to interact with other leukocytes, since the modifications of the uterine leukocyte populations were much more striking in infected WT mice than in infected CD1d$^{-/-}$ mice. In fact, in the uterus of infected – compared to the uterus of non-infected – WT mice, the numbers of CD4^{+} T cells, CD8^{+} T cells, monocytic cells and granulocytes were enhanced, the percentages of NK cells decreased, and the percentages of monocytic cells and CD8^{+} T cells increased. The large increase in the percentage of CD8^{+} T cells in the uterus of WT-infected mice was also observed in the uterus of CD1d$^{-/-}$ infected mice, but on a much reduced scale (944% vs 179%), suggesting that this increase was at least partially dependent on CD1d-

restricted NKT cells. It was interesting to notice that this modification was the only one found in the uterus of $CD1d^{-/-}$ mice related to infection. This result was in agreement with the experiments of Roan et al. [28], who found that $CD8^+$ T cells migrated into the genital mucosa of *Chlamydia*-infected mice. Our study shows that this recruitment appears to be conserved also in $CD1^{-/-}$ mice.

During infection, the CD1d-restricted NKT cells were likely activated by glycolipids derived from the *Chlamydiae*, especially in the uterus, since they induced modifications of the other leukocyte populations. The consequences of chlamydial infection were larger and more numerous in the uterus than in the placenta. We therefore conclude that, during chlamydial infection, uterine leukocytes interact directly or indirectly with placental leukocytes.

As both $CD4^+$ T cells and NK cells have previously been shown to play a role in clearing chlamydial infection, the CD1d-dependent loss of CD1d-restricted NKT cells seems to be functionally compensated by an increase in $CD4^+$ and $CD8^+$ T cells, suggesting an interaction between activated uterine leukocytes and CD1d-restricted NKT cells.

The absence of CD1d-restricted NKT cells in *Chlamydia*-infected pregnant mice resulted in non significant variations in the numbers of the different placental leukocyte populations compared to B6 WT mice; while in the uterus, the immune response against *Chlamydia* seen in $Cd1d^{-/-}$ mice was dampened compared to WT mice: there was no increase in total leukocyte numbers, granulocytes, $CD4^+$ T cells, and monocytic cells, and a much reduced augmentation only of the percentage of $CD8^+$ T cells.

Taken together, these results suggest a possible role for CD1d-restricted NKT cells in the control, recruitment or homeostasis of leukocyte populations at the maternal–fetal interface during *Chlamydia* infection.

Acknowledgments

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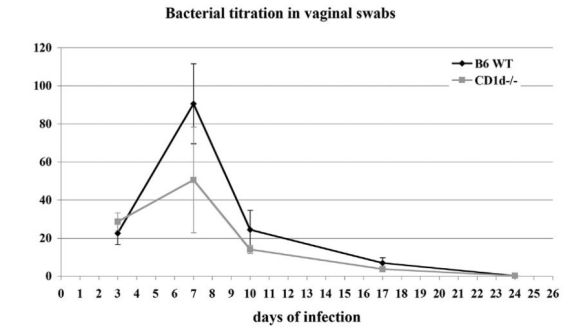
A.



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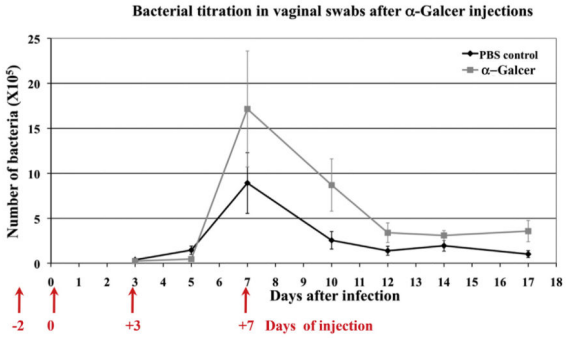

C. **Comparison of reproductive capacities in B6 WT vs CD1d-/- mice infected or not by *C.muridarum***

Crosses / Status	B6WTxB6WT		CD1d-/-xCD1d-/-	
	Average litter size	% abortion	Average litter size	% abortion
Non-infected	8.25 (± 0.96)	5.6 (± 9.6)	8.67(± 0.58)	7.9(±5.6)^{NS}
Infected	8 (± 2.39)	50.7 (± 35.8)	7.71 (± 2.43)	38.9 (±24.5)^{NS}

Fig. 1.

Comparable time course and level of chlamydial infection in WT and CD1d$^{-/-}$ non-pregnant mice. Bacterial DNA of *C. muridarum* was prepared from vaginal swabs and was quantified by qPCR for copies of a plasmid containing one insert of DNA coding the major outer membrane protein (MOMP) of *C. muridarum*, as described in Materials and methods section. (A) The number of bacteria (±standard error of the mean) was measured for 24 days after vaginal infection of WT (black line) and CD1d$^{-/-}$ (gray line) mice with 10^6 IFU of bacteria. (B) The number of bacteria (±standard error of the mean) was measured for 17 days after vaginal infection of WT (black line) and CD1d$^{-/-}$ (gray line) mice with 10^6 IFU of bacteria and after 4 i.p. injections of 2.5 μg of α-GalCer, or PBS as a control, at −2, 0, +3 and +7 days post-infection. Arrows: days of α-GalCer or PBS injections (cf. Materials and Methods). Data are represented as mean ± sem of $n = 5$ mice per group. (C) Comparison of reproductive capacities (litter size and abortion rate) of B6 WT vs CD1d$^{-/-}$ mice infected or not by C. muridarum. No statistically significant differences were observed between the groups (4 uninfected mice per group, 7 to 8 infected mice per group). These numbers of mice correspond to 33 different placenta and corresponding uterine implantation sites in uninfected B6 mice and 26 in uninfected CD1d$^{-/-}$ mice, while in infected mice, 64 placenta and implantation sites were analyzed in B6 mice and 54 in CD1d$^{-/-}$ infected mice. NS: non-significant compared to corresponding B6WT crosses.

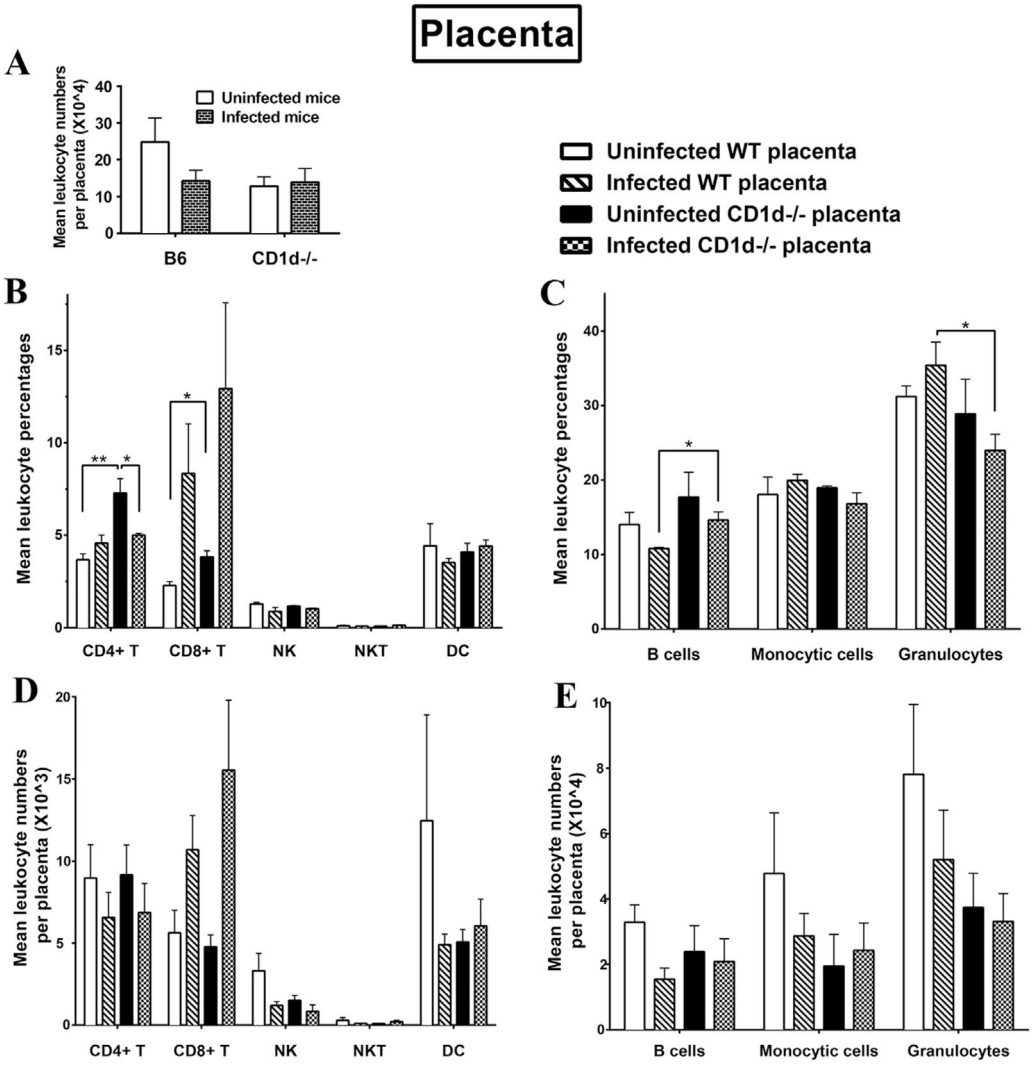

Fig. 2.

Modulation of placental leukocyte populations by a *C. muridarum*. infection in WT and CD1d$^{-/-}$ pregnant mice. Total leukocyte numbers per placenta (A) (±standard error of the mean), in infected (gray bars) and uninfected (white bars) WT or CD1d$^{-/-}$ pregnant mice (day 14.5 pc), determined by flow cytometry analysis, as described in Materials and methods section. Flow cytometry analysis of placental leukocyte subpopulations (CD4^{+} T cells, CD8^{+} T cells, NK, NKT) cells and DC (B and D), and B cells, monocytic cells, granulocytes (C and E), in percentages (B, C) or numbers (D, E) (±standard error of the mean), in the placenta of infected and uninfected B6 WT or CD1d$^{-/-}$ pregnant mice. Placental leukocyte population percentages are in B and C, numbers are in D and E. Note the scale differences for the 2 groups of leukocyte populations. Placentas were from: WT uninfected mice (white bars), or WT infected mice (hatched bars), or CD1d$^{-/-}$ uninfected mice (black bars), or CD1d$^{-/-}$ infected mice (checked bars). *$p < 0.05$, **$p < 0.001$ Data are represented as mean ± sem of $n = 3$ mice per group.

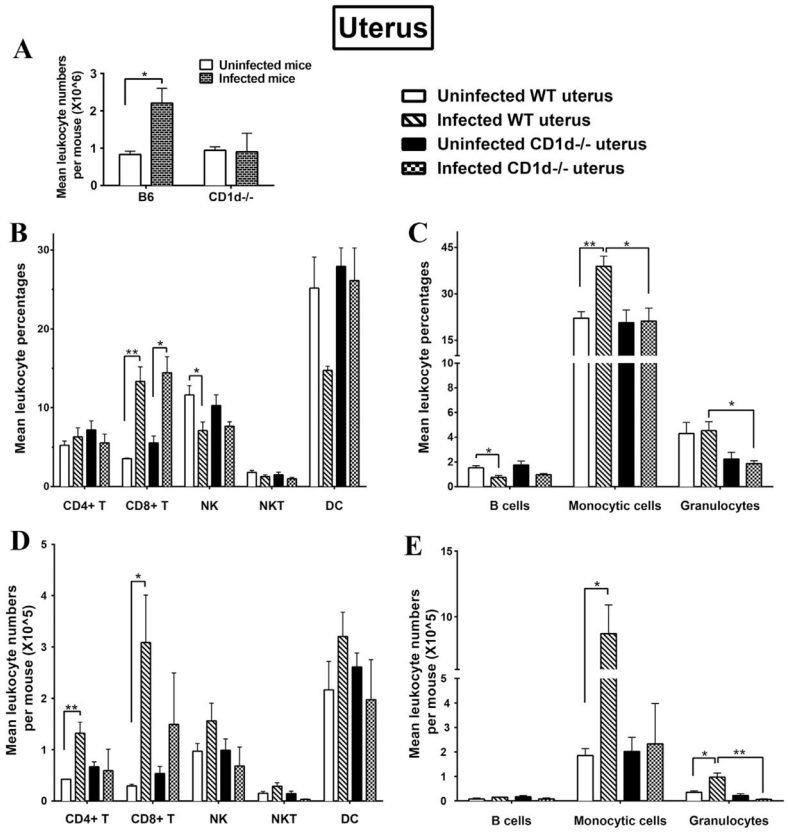

Fig. 3.

Modulation of uterine leukocyte populations by a *C. muridarum*. infection in WT and CD1d$^{-/-}$ pregnant mice. Total leukocyte numbers in the uterus (A) (±standard error of the mean) of infected (black bars) and uninfected (white bars) WT or CD1d$^{-/-}$ pregnant mice (day 14.5 pc), determined by flow cytometry analysis, as described in Materials and methods section. Flow cytometry analysis of uterine leukocyte subpopulations (CD4^{+} T cells, CD8^{+} T cells, NK, NKT cells and DC) (B and D), and B cells, monocytic cells, granulocytes (C and E), in percentages (B, C) or numbers (D, E) (±standard error of the mean), in the uterus of infected and uninfected B6 WT or CD1d$^{-/-}$ pregnant mice. Uterine leukocyte population percentages are in B and C, numbers are in D and E. Note the scale differences for the 2 groups of leukocyte populations. Uterus was from: WT uninfected mice (white bars), or WT infected mice (hatched bars), or CD1d$^{-/-}$ uninfected mice (black bars), or CD1d$^{-/-}$ infected mice (checked bars). *$p < 0.05$, **$p < 0.001$ Data are represented as mean ± sem of $n = 3$ mice per group.

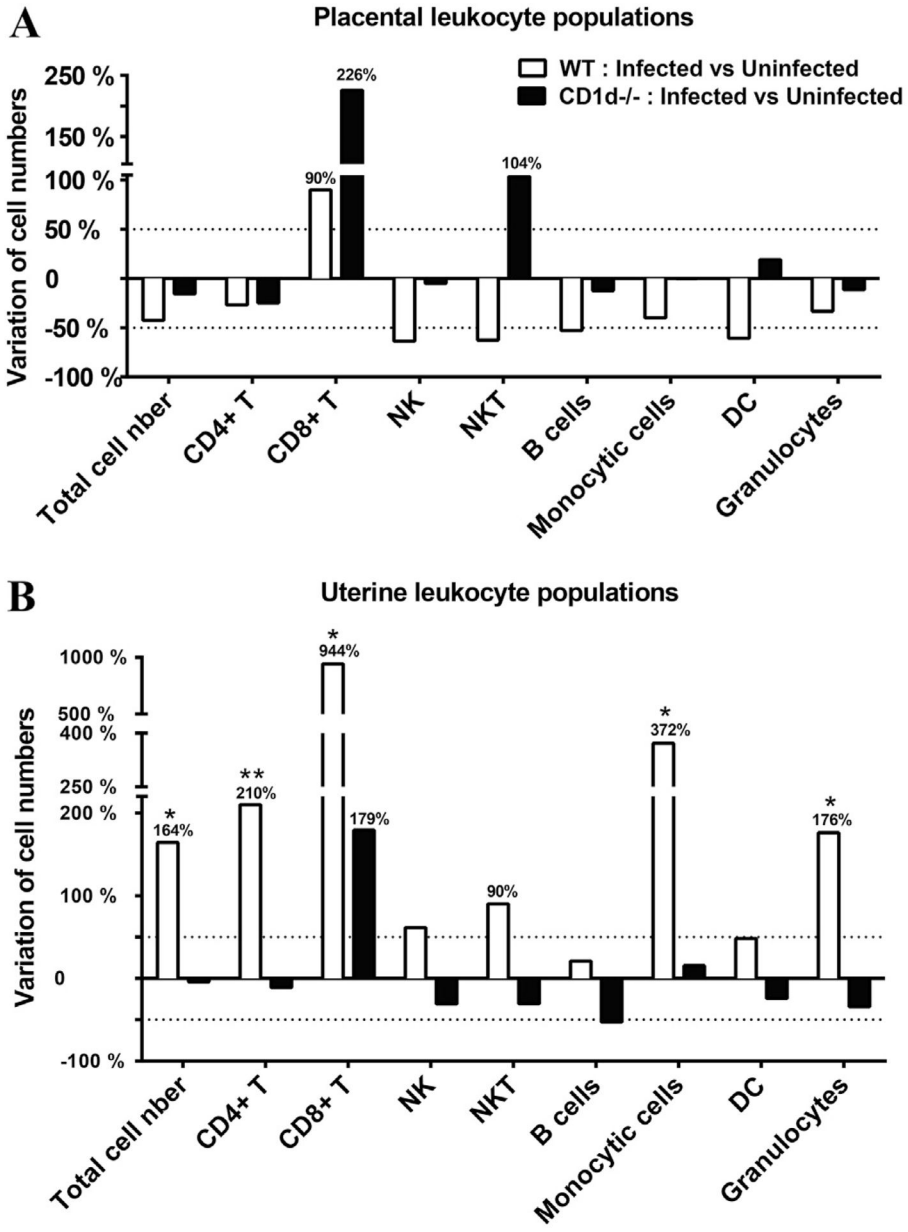

Fig. 4.

Variation of placental and uterine leukocyte population numbers during a *C. muridarum*. infection, in WT or CD1d$^{-/-}$ pregnant mice. Data from Figs. 2 and 3 were used to compare the effects of a *C. muridarum* infection on placental and uterine leukocyte populations, in WT versus CD1d$^{-/-}$ pregnant mice. A. Ratios (expressed as percentages) of the means of placental leukocyte population numbers in infected versus uninfected WT mice (white bars), or in infected versus uninfected CD1d$^{-/-}$ mice (black bars). B. Similar presentation of data as in A, but with leukocyte populations from the uterus of the same animals.

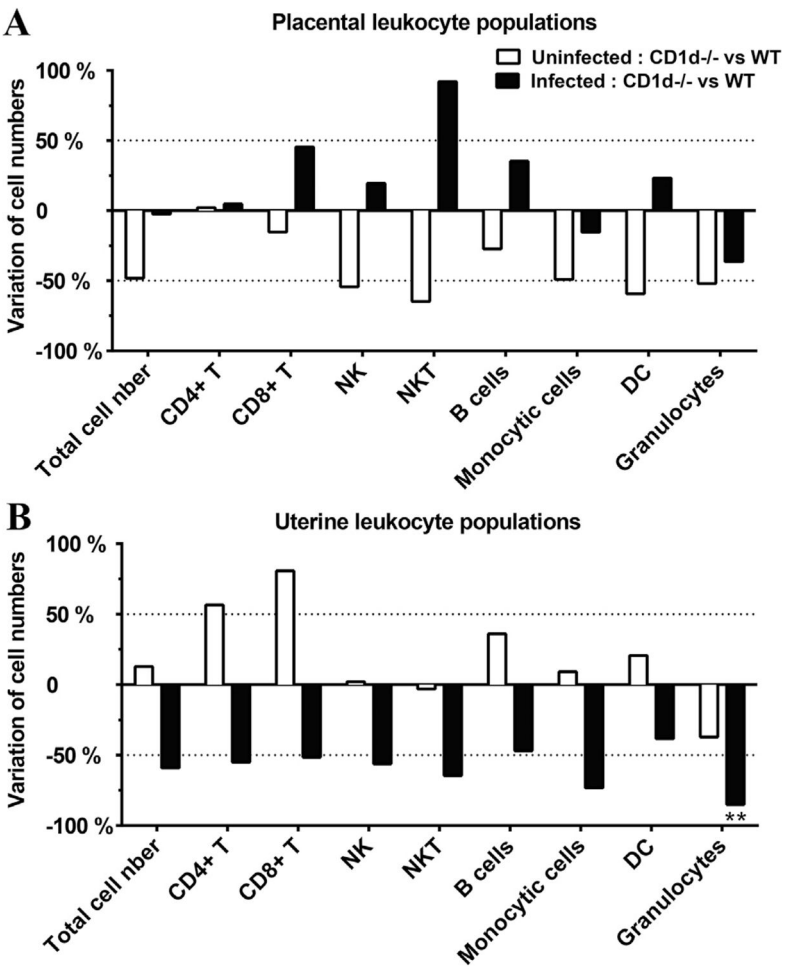

Fig. 5.

Variation of placental and uterine leukocyte population numbers in CD1d$^{-/-}$ versus WT mice, in the presence or absence of a *C. muridarum*. infection. Data from Figs. 2 and 3 were used to compare the placental and uterine leukocyte populations in CD1d$^{-/-}$ versus WT pregnant mice (day 14.5 pc), in the presence or absence of a vaginal *C. muridarum* infection. A. Ratios (expressed as percentages) of the means of placental leukocyte population numbers in uninfected CD1d$^{-/-}$ mice versus WT mice (white bars), or in infected CD1d$^{-/-}$ mice versus WT mice (black bars). B. Similar presentation of data as in A, but with leukocyte populations from the uterus of the same animals.