Transboundary and Emerging Diseases

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ORIGINAL ARTICLE

Detection of Natural Killer T Cells in Mice Infected with *Rickettsia conorii*

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Summary

Little information is available regarding the role of natural killer T (NKT) cells during the early stage of *Rickettsia conorii* infection. Herein, C3H/HeN mice were infected with the Malish 7 strain of *R. conorii*. Splenocytes from these mice were analysed in the early stage of the infection by flow cytometry and compared with uninfected controls. Our results showed an increase in NKT cells in infected mice. Additionally, NKT interleukin (IL)-17$^+$ cells increased three days after infection, together with a concurrent decrease in the relative amount of NKT interferon (IFN)-γ$^+$ cells. We also confirmed a higher amount of NK IFN-γ$^+$ cells in infected mice. Taken together, our data showed that NKT cells producing Il-17 increased during the early stage of rickettsial infection. These results suggest a connection between IL-17$^+$ NKT cells and vasculitis, which is the main clinical symptom of rickettsiosis.

Introduction

The order Rickettsiales is constituted by a variety of gram-negative bacteria that belongs to the *Rickettsia*, *Orientia*, *Ehrlichia*, *Anaplasma* or *Neorickettsia* genera (Uchiyama, 2012). They are vector-borne pathogens and are able to cause a broad spectrum of symptoms. *Rickettsia conorii* belongs to the spotted fever group (SFG) *Rickettsia*, it is transmitted by the tick *Rhipicephalus sanguineus*, and in spite of the efforts of the scientific community, it is still highly prevalent (Serban et al., 2009; Papa et al., 2010; Podsiadły et al., 2011; Alexandre et al., 2011; Bolaños-Rivero et al., 2011; Colomba et al., 2011; Baltadzhiev and Popivanova, 2012).

The pathogenic mechanism of this bacterium was studied in detail (Walker et al., 2003). *Rickettsia* mainly targets endothelial cells: some bacterial surface proteins (Omp-A and Omp-B) interact with a protein-dependent host receptor thus allowing the penetration of bacteria into non-professional phagocytes. *Rickettsia* is then able to actively evade the host defences avoiding the contact with lysosomal enzymes lysing phagosome membrane prior to its fusion with the cellular lysosome. The phagosomal escape is a complex mechanism that involves many proteins endowed for phospholipase A_2 activity (Balraj et al., 2009). The cell undergoes a potent ROS activation, together with a depletion of glutathione and increased levels of catalase, being unable to sustain the ROS-induced damage (Hong et al., 1998). *Rickettsia* is then able to spread from cell to cell without passing through the intracellular space. It thus disseminates to vascular endothelial cells and, as a logical effect, it causes endothelial dysfunction and activation, acute phase responses, increased vascular permeability, oedema and alteration in coagulation (Mansueto et al., 2012).

Host defence against rickettsial infection involves both innate and cellular immunity. The role of Toll-like receptor (TLR)-4 during an early immune response following a *Rickettsia conorii* challenge was clearly demonstrated (Jordan et al., 2009). Dendritic cells (DCs) bearing an activated

TLR-4 induce the activation of NK cells, through an IL-12 signal. Consequently, there is an increase in IFN-γ and TNF-α production. Both cytokines stimulate endothelial nitric oxide synthetase (NOS)2, thus limiting the infection (Jordan et al., 2009).

After the first lane of defence, cell-mediated immunity is thought to play a crucial role. Thanks to cytokine activation, the CD8 cytotoxic T-lymphocyte activity is essential for the clearance of intracellular bacteria (Walker et al., 2001).

The immune response that follows *Rickettsia* spreading is termed as 'rickettsial vasculitis'. A reduction in circulating CD4 T cells and a perivascular infiltration by CD4 and CD8 T lymphocytes, together with B cells and macrophages, was demonstrated (Mansueto et al., 2012).

The aim of this work was to determine the role of NKT cells in the course of infection with *R. conorii*. We describe an increase in NKT during the early stage of infection; these cells result $IL17^+$ but not $IFN\gamma^+$.

Materials and Methods

Rickettsia conorii Malish 7 strain was cultured in VERO cells, isolated and quantified as described elsewhere (Eisemann et al., 1984; Milano et al., 2000). 3×10^5 CFU of this strain were intravenously injected into 5 C3H/HeN mice (Harlan Laboratories, Venray, theNetherlands), 8–10 weeks old. Mice were all maintained in sterile conditions and fed with *ad libitum* water and food. Five mice of the same strain were injected with PBS only and analysed as control group. Mice were sacrificed 3 days after infection, spleens were harvested, mechanically homogenized and splenocytes were isolated by Ficoll gradient.

Murine splenocytes from infected and control group were quantified (trypan-blue staining vital count) and divided into two aliquots: an aliquot was cultured in 24-well Costar plates (1×10^6 cells for each well) in RPMI 1640 medium (GIBCO, Carlsbad, CA, USA) supplemented with 10% foetal calf serum (FCS), penicillin, streptomycin, gentamicin and L-glutamine with and without Ionomycin (50 ng/ml)/phorbol myristate acetate (PMA) (1 μg/ml) as stimulus and analysed 24 h later to test the percentage of DC (CD11c positive subset)/$IL\text{-}12^+$ cells and $IL\text{-}17^+$ or $IFN\text{-}\gamma^+$ NK cells, the other aliquot was cultured in the same medium for 3 days with and without alpha-galactosylceramide (αGalCer) at the concentration of 10 ng/ml as stimulus for the detection of IL-17 or IFNγ producing NKT cells.

Cells were labelled with FITC anti-mouse CD11c (HL3 clone; BD, Franklin Lakes, NJ, USA) for the detection of DCs, FITC anti-mouse NK1.1 (PK136 clone, BD) for the detection of NK cells, CD1d:Ig recombinant fusion protein, mouse DimerX (BD), loaded with αGalCer, for the detection of NKT cells, PE anti-mouse IFN-γ (XMG1.2 clone; BD), PE anti-mouse IL-12 (C15.6 clone; BD), PE anti-mouse IL-17A (TC11-18H10 clone; BD). Flow cytometry analysis of labelled samples was performed by BD FAC-Scan cytometer (BD) and Cell Quest-Pro software (BD, Franklin Lakes, NJ, USA). 10 000 events were acquired for each sample.

All the experiments were repeated at least twice. Statistical analysis of results obtained was performed by Mann–Whitney test and results were assumed significant when P-value < 0.05.

Experiments were carried out in accordance with European Communities Council Directive of 24 November 1986 (86/609/EEC) and with the authorization of the Italian Ministry of health (Decreto Ministeriale N° 101/2006 –A).

Results

The number of dendritic, NK and NKT cells increases in the spleens of *Rickettsia conorii* infected mice

The comparison of spleens obtained from infected and control mice (Fig. 1a) showed a pronounced splenomegaly

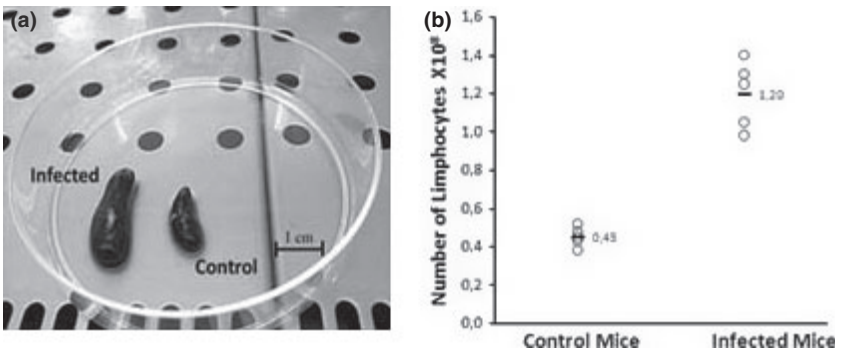

Fig 1. (a) Example of spleens drawn from infected (on the left) and control mice (on the right): the first is about threefold bigger than the second and shows signs of necrosis. (b) The difference in dimensions between spleens of infected and control mice reflects a similar situation in terms of splenocytes counts. The average cell number in spleens of infected mice is threefold higher when compared with those of control mice. This difference resulted significant ($P < 0.05$, analysed by Mann–Whitney test).

in all the infected mice 3 days after infection. The average amount of splenocytes in infected mice resulted a mean of 1.2×10^8. This number was about three times higher if compared with the amount of splenocytes obtained from control mice (4.5×10^7) (Fig. 1b).

We focused our attention on leucocyte subsets involved in innate immunity and, by flow cytometry, we measured the percentage of DCs, NK and NKT cells.

Spleens of untreated C3H/HeN mice contained a mean of 1.37% of NKT cells, while in infected mice, spleens 3.3% of NKT were detected (data not shown). Spleens of C3H/HeN infected mice contained 3.0% of NK cells, while a percentage of 1.5% of NK cells was found in control mice (data not shown).

Dendritic cells ($CD11c^+$ cells) percentage in spleens of infected mice was almost twofold higher than control mice (0.8% and 1.5%, respectively, when the cells were *in vitro* stimulated with Ionomycin/PMA) (data not shown).

Dendritic cells from spleens of *Rickettsia conorii* infected C3H/HeN mice produce a huge amount of IL-12

The analysis of results related to IL-12^+ DCs showed a definite difference between stimulated splenocytes of infected mice and the same group cultured with Ionomycin/PMA, confirming data previously published by Walker et al. (Billings et al., 2001; Jordan et al., 2009). In infected mice, the 0.78% of IL-12^+ DCs have been detected, while in control mice, 0.40% of IL-12^+ DCs were found. This difference enhanced when splenocytes were shortly cultured *in vitro* with Iono/PMA; the percentage of IL-12^+ DCs in control mice were about unchanged (0.42%), while the percentage of IL-12^+ DCs in infected mice increased up to 1.4%.

IFN-γ^+ NK cells and IL-17^+ NKT cells increased during early phases of *Rickettsia conorii* infection

The percentages of IL-17^+ and IFN-γ^+ cells, previously showed by Jordan et al. (2009) among $CD4^+$ cells in experimental rickettsial infection, were analysed in NK and NKT cells. The percentages of NK IL-17^+ (Fig. 2a) were 0.4% and 0.7% in splenocytes of infected and control mice, respectively. These values increased up to 0.65% in splenocytes of infected mice and decreased to 0.47% in splenocytes of control mice when the cells were cultured with Iono/PMA.

The percentage of IFN-γ^+ NK cells, showed in Fig. 2b, was 0.21% in splenocytes of control mice. This percentage increased up to 0.75% in splenocytes of infected mice in the same culture conditions. When splenocytes were cultured with Iono/PMA, we found 0.47% and 0.35% of IFN-γ^+ NK cells in control and infected mice, respectively.

The analysis of results concerning the percentages of IL-17^+ NKT cells in spleens of infected and control mice is represented in Fig. 2c. Splenocytes of C3H/HeN control mice contained an 0.18% of IL-17^+ NKT, while in infected mice, the percentage of IL-17^+ NKT cells increases up to 0.9%. Splenocytes of infected mice cultured with αGalCer showed 0.6% of IL-17^+ NKT cells. This percentage drop to 0.16% in splenocytes of control mice.

Figure 2D showed the analysis of the results concerning the number of IFN-γ^+ NKT cells. In the splenocytes of infected and control mice cultured without αGalCer, these cells were 0.08% and 0.16%, respectively. Splenocytes of infected and control mice cultured with αGalCer showed, respectively, a percentage of 0.12% and 0.2% of IFN-γ^+ NKT cells.

Discussion and Conclusions

The immune response against *R. conorii* infection requires the recruitment of a number of subsets of both innate and adaptive immunity. Many studies on NK or DCs for innate immunity and on CD4 Th1 T cells or cytotoxic CD8 T cells clarified the role of these subsets in the context of *R. conorii* infection. Considering the ability of NKT cells to rapidly produce a large amount of cytokines and their role as bridge between innate and adaptive immunity, we wanted to assess if this subset plays a role during the early stages of *R. conorii* infection.

All the infected mice, sacrificed 3 days after infection, showed a pronounced splenomegaly. The size of spleens from infected mice was increased when compared with the spleens of controls and this ratio is equal to that found among the mean number of cells obtained from the spleens of infected mice and those obtained from the spleens of controls. This aspect well reflects a massive increase in different cell subsets arrival in the spleen during experimental rickettsial infection.

Results regarding NK and DC cells confirmed the involvement of these subsets in the immune response against *R. conorii* infection as previously showed by Walker et al. The increase in IFN-γ in NK cells and the production of IL12 in DC from infected mice both cultured with Iono/PMA and without any stimulus suggest that these cells are markers of innate immune response activation which usually prelude to efficient adaptive immune response.

Dendritic cell are activated by LPS of *R. conorii* via TLR4 and it was shown to be an essential step for early activation of NK cells against *R. conorii* infection and subsequently T cell recruitment. The threefold increase in IL-12^+ DC in spleens of infected mice enhances the activation of NK cells and leads to the recruitment of CD4 T cells (Jordan et al., 2008). On the other side, IFN-γ production by NK cells

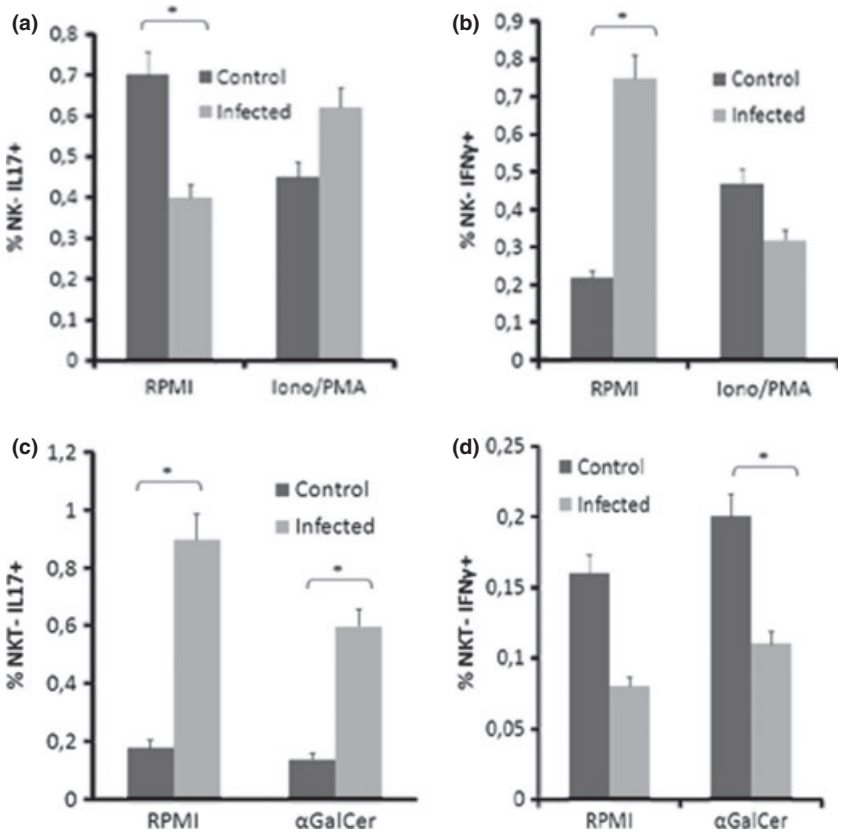

Fig 2. (a) Percentages of NK IL-17$^+$ are higher in unstimulated splenocytes of control mice than in splenocytes of infected mice and this difference resulted significant ($P < 0.05$). No significant difference was detected with Iono/PMA as stimulus; (b) A significant difference in the percentage of NK IFN-γ^+ cells was detected comparing unstimulated splenocytes of infected mice with controls. The use of Iono/PMA as stimulus results in a down-regulation of NK IFN-γ^+ cells both in infected and control mice without any significant difference among the two groups. (c) NKT cells from spleens of infected mice produce significant higher amounts of IL-17 cytokine if compared with NKT cells from spleens of control mice both with or without αGalCer stimulus. (d) The percentages of IFN-γ^+ NKT cells show an opposite trend with respect to IL-17$^+$ NKT cells: this subset is always poorly represented in splenocytes of infected mice. In control mice, a small percentage of IFN-γ^+ NKT was found, but higher than the percentage found in infected mice when splenocytes were cultured without αGalCer stimulus, and significant higher when cells were stimulated with αGalCer. Each difference of subsets percentage between infected and control mice was considered significant when *P*-value is <0.05 (Mann–Whitney test).

is another important stimulus for CD4 and CD8T cells activation. NK cells play thus a double role as effector cells of innate immunity (Billings et al., 2001) and, together with DC, as primer of adaptive immunity. In our experiment, no production of IL-17 by NK cells after *R. conorii* infection was measured.

The activation of secondary response in C3H/HeN mice, in which TLR4 plays a central role, leads to the onset of Th1 and of Th17subsets, and the last was seen starting from day 5 after infection even if significant levels of IL-17 were measured in sera starting from day 3 after infection (Jordan et al., 2008).This temporal gap could be explained by NKT flow cytometry analysis. The results obtained, particularly the huge increase in IL-17$^+$ NKTcells number in spleens of C3H/HeN infected mice, compared with the percentage of NKT IL-17$^+$cells in spleens of control mice, seems to be consistent with an NKT cells involvement during the early phase of *Rickettsia* infection. The percentage of IL-17-producing NKT cells in the spleens of infected mice is quite high both in αGalCer stimulated and in unstimulated cells. The threefold increase in percentage of IL-17$^+$ NKT cells detected in infected mice compared with control mice let us suppose that NKT cells probably play an important role as IL-17 producers and could be considered as an activation marker of NKT cells in *Rickettsia* infection and could transactivate CD4$^+$IL17$^+$ cells previously showed (Jordan et al., 2009).

The percentage of IFN-γ^+ NKT cells that were detected in the spleens of control mice was not relevant, but also in the spleens of infected mice, the percentage of these cells was very low both with and without αGalCer stimulus. The activation of NKT cells is often characterized by a huge production of IFN-γ (Sireci et al., 2007), but the high plasticity of these cells in terms of number of cytokines

produced combined with the *Rickettsia* infection and the cytokine milieu produced by DCs and NK cells *in vivo* could shifted the cytokine production towards IL-17, consequently, low levels of IFN-γ were produced by NKT cells. We could hypothesize a crosstalk between NK and NKT cells for which the last were addressed towards the production of IL-17 through an activation signal constituted by the IFN-γ produced by NK cells, but other experiments are needed to demonstrate this phenomenon.

As regards IL-17, it should be considered that it could be involved in pathogenic behaviour in infectious as well as in autoimmune diseases (Stevenson et al., 2008; Pierer et al., 2011; Kezic et al., 2012; Marijnissen et al., 2012). In our model of *rickettsia* infection, the presence of a Th17 subset is more related to a protective rather than a pathogenic role (Mansueto et al., 2012) , but very little information is known about IL-17$^+$ NKT cells and the role of this subset in the immune response against *rickettsia* should be deeper investigated. There is the possibility that an early production of IL-17 by NKT cells could help the onset of the immune response. Moreover in other disease models (autoimmune diseases), IL-17-producing cell subsets are involved in pathogenesis of vasculitis (Bajwa et al., 2009; Keino et al., 2011). As vasculitis is the main clinical symptom of rickettsiosis, we could also hypothesize a pathogenic role of early producing IL-17$^+$ NKT cells.

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Conflicts of interest

The authors have not declared any potential conflicts of interest.

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