TLR 2 and 4 COOPERATE IN THE CONTROL OF *Brucella microti* INFECTION IN MICE BY CONTROLLING THE GENERATION OF THE CYTOTOXIC T CD8 CELL RESPONSE

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The role of Toll like receptors (TLR) in the control of *B. microti* infection was studied in a mouse model of infection using TLR 2, TLR 4 or TLR 9 deficient mice. In addition, the role of TLR in the maturation of bone marrow derived dendritic cells (BMDC) and their pro-inflammatory cytokines production was studied in vitro. How these facts could affect the ability of Tc cells to inhibit the replication of *B. microti* in bone marrow derived macrophages (BMDM) was also analysed.

MATERIALS AND METHODS

Kinetics of infection. Wild C57BL/10 (B10) and TLR2-/-, TLR4-/-, TLR9-/-, TLR2x4-/- and TLR 2x4x9-/- mice were infected intraperitoneally with 10^5 cfu of *B. microti* and spleen and livers were harvested for bacterial counts and pathological studies at 3, 7, 14 and 21 days post infection (Figure 1, 2 and 3).

***In vitro* studies**. BMDC from B10 and TLR KO mice were infected with a MOI=25 cfu of *B. microti*. After 1.5, 24 and 48h, dendritic cells were lysed, diluted and plated for bacterial counts (Figure 4A). The production of TNFa was quantified in supernatants (Figure 4B).

The expresion of costimulatory molecules for lymphocyte proliferation CD86 (Figure 5A) and CD40(Figure 5B) and of MHC II (Figure 5C) was analyzed by FACS in BMDC stimulated with HK *B. microti* (100:1) or *E. coli* LPS 1µg/ml for 24h.

BMDM from C57BL/10 wild type (wt) mice were infected with a MOI= 25cfu of *B. microti*. Tc cells were obtained by MACS from spleens of wt and TLR deficient mice infected with 10^5 cfu of *B. microti* at 7 days p.i. and added to infected BMDM. Macrophages were lysed, diluted and plated for bacterial counts at 24h p.i. (Figure 6A).

Intracellular expression of the cytotoxic granzyme B, molecule implied in the control of *B. microti* by apoptosis induction (2), was analyzed by flow citometry in MACS-enriched Tc cells from wt and TLR deficient mice (Figure 6B).

Data are represented as Mean±SEM. * p<0.05, **p<0.005, ***p<0.001.

Histopathology studies. Liver and spleen sections from each group of mice were routinely processed for histopathology and stained with H&E. The sections were analyzed to compare the differences in the inflammatory reaction. Liver sections were examined to describe the type and extent of the inflammatory reaction (Figure 7).Spleen sections were analyzed to identify follicle characteristics, cellularity in the sinuses and presence of inflammatory process (Figure 8).

RESULTS

***In vivo*.**

Bacterial load from spleen and liver in TLR2-/-, TLR4-/- and TLR9-/- animals was similar to wt mice while the bacterial clearance was delayed in TLR2x4-/- and TLR 2x4x9-/- mice at 7 and 14 days post-infection. In addition, the bacterial load was similar in TLR2x4-/- and TLR2x4x9-/- mice. (Figures 1, 2 and 3).

***In vitro*.**

We found a dendritic cells maturation impairment in TLR2x4-/- and TLT2x4x9-/- mice as shown by a reduced expression of CD40, CD86, and MHC class II on CD11c+ cells (Figure 5) and production of TNFα (Figure 4B) that could affect the proliferation of specific *B. microti* T cells.

When comparing the ability of ex vivo Tc cells from *B. microti* infected wt, TLR2-/-, TLR4-/-, TLR9-/- TLR 2x4-/- and TLR 2x4x9-/- mice to control *B. microti* replication in BMDMs, the bacterial growth was less reduced when Tc cells from TLR2x4 and TLR2x4x9 deficient mice were added, compared to Tc cells (T CD8) from wt, TLR2-/-, TLR4-/- or TLR9-/- mice, despite the similar expression of cytotoxic granzyme B showed (Fig 6).

Histopathology.

The hepatic lesions in all of the KO mice were similar. The granulomas were larger than in the B10 mice, well demarcated and occasionally associated to individual cell necrosis or large areas of necrosis. In some TLR 2x4x9-/- animals intravascular thrombosis was found.

The hepatic inflammatory reaction in the B10 mice was less severe than in any of the TLR KO. The inflammatory process is dispersed and ill defined. There are small scattered aggregates of inflammatory cells with not well defined granulomas at 7 days pi and 14 days pi. There was no necrosis or thrombosis found in any of the section examined.

In the TLR KO animals the splenic pathology is characterized by ill-defined small lymphoid follicles and variable degrees of lymphoid depletion. In some severe cases, such as 7-day TLR2x4-/-, and 7-day TLR 2x4x9-/-, the follicles are small or vestigial.

The splenic pathology in the B10 animals is characterized by larger lymphoid nodules than in the TLR KO animals with moderate numbers of lymphocytes, and ill defined but not depleted follicles.

Figure 1. Number of *Brucella* per **spleen** of wt C57BL/10, TLR 2-/-, TLR 4-/-, and TLR 2x4-/- mice infected intraperitoneally with 10^5 cfu of *B. microti* and analysed at 3, 7, 14 and 21 days post-infection. (Mean±SEM). **p<0.005, ***p<0.001.

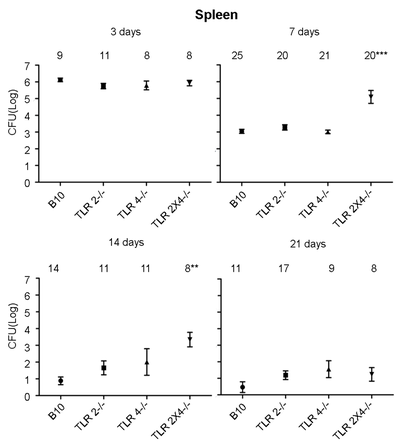

Figure 2. Number of *Brucella* per **liver** of wt C57BL/10, TLR 2-/-, TLR 4-/-, and TLR 2x4-/- mice infected intraperitoneally with 10^5 cfu of *B. microti* and analysed at 3, 7, 14 and 21 days post-infection. (Mean±SEM). **p<0.005, ***p<0.001.

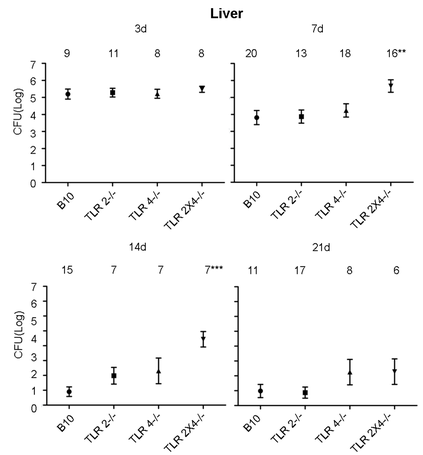

Figure 3. Number of *Brucella* per **spleen (A)** and **liver (B)** of wt C57BL/10, TLR 9-/-, TLR 2x4-/-, and TLR 2x4x9-/- mice infected intraperitoneally with 10^5 cfu of *B. microti* and analysed at 7 and 14 days post-infection. Mean±SEM. **p<0.005, ***p<0.001.

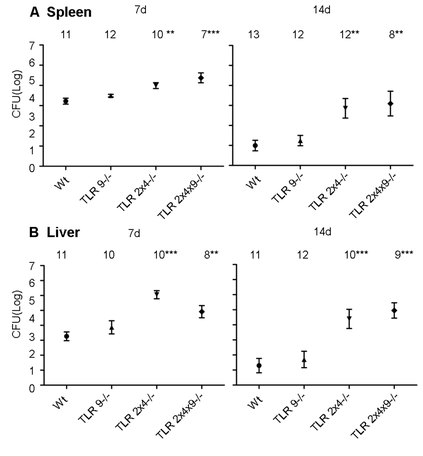

Figure 4.
A: Intracellular multiplication of *B. microti* in BMDC.
B: Production of TNFa in BMDC infected with *B. microti.* Mean±SEM. **p<0.005, ***p<0.001.

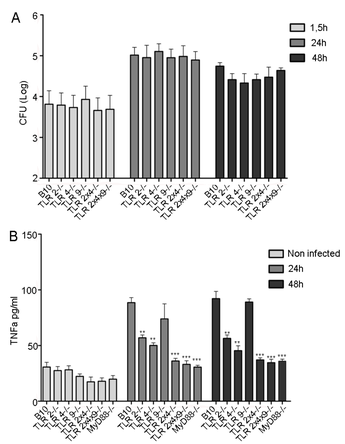

Figure 7

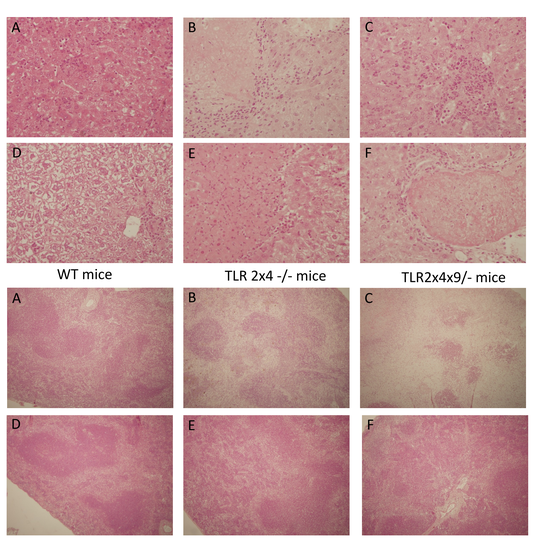

Figure 8

Figure 7. Liver H&E sections (400x). Representative granulomas and inflammatory reaction seen in the most affected groups. A: Multifocal inflammation. B: Focal necrosis. C: Multifocal inflammatory nodules. D: No significant lesions. E: Focal necrosis. F: Occasional thrombi.

Figure 8. Spleen H&E sections (100x). Representative changes seen in the most affected groups. A: Ill defined follicles. B: Sparse lymphoid follicles and lymphoid depletion. C: Vestigial lymphoid follicle. D: Ill defined follicles. E: Sparse lymphoid follicles and lymphoid depletion. F: Small sparse lymphoid follicles.

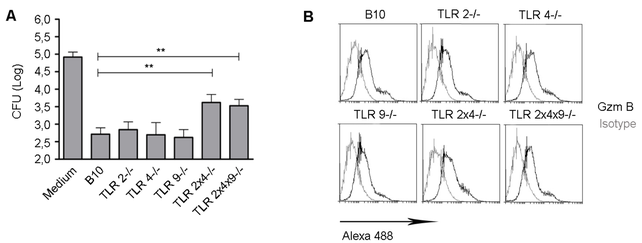

Figure 6.
A. Ability of *B. microti* specific Tc cells (T CD8) from B10, TLR 2-/-, TLR 4-/-, TLR 9-/- ,TLR 2x4-/- and TLR 2x4x9-/- mice to control bacterial replication in BMDM. Mean±SEM **p<0.005
B. Intracellular expression of cytotoxic granzyme B in MACS-enriched Tc cells from wt and deficient mice analyzed by flow citometry.

CONCLUSIONS

1- Mice lacking in both TLR 2 and TLR 4 receptors present a higher susceptibility to infection by *B. microti*, suggesting that in vivo TLR 2 and TLR 4 cooperate to control *B. microti* infection.

2- Our results indicate that dendritic cells deficient in both TLR 2 and TLR 4 showed a decreased expression of MHC II, CD40, CD86 and production of pro-inflammatory cytokines when compared with wt BMDC.

3- Tc cells from both TLR 2 and TLR 4 deficient mice showed a reduced capacity to diminish *B. microti* replication in BMDM and this could be caused by the generation of less specific *B. microti* Tc cells due to dendritic cells maturation impairment.

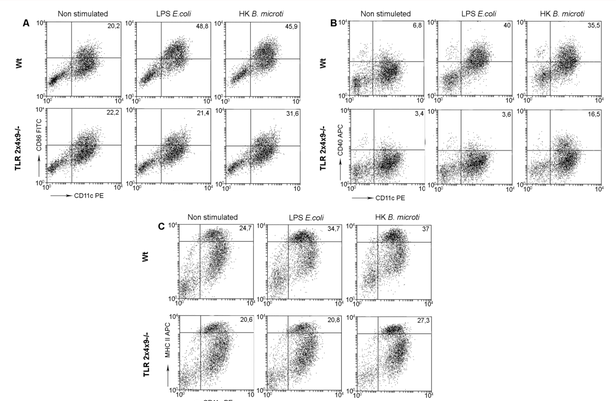

Figure 5. BMDC maturation induced by HK *B. microti* (100:1) or *E. coli* LPS (1µg/ml). Wt and 2x4x9-/- cells were analysed for expression of surface CD 86 (**A**), CD40 (**B**) or MHC II (**C**) on CD11+ cells .

REFERENCES

1. Jiménez de Bagüés MP et al. J. Infect. Dis. 2010; 202 (1):3-10.
2. Arias M, et al. Cell Reports. Jul 2014; 8, 1–10

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