

Characterization of murine amniotic fluid B cells in normal pregnancy and in preterm birth

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Abstract

The amniotic fluid provides mechanical protection and immune defense against pathogens to the fetus. Indeed, components of the innate and adaptive immunity, including B cells, have been described in the amniotic fluid. However, limited information concerning phenotype and functionality of amniotic fluid B cells is available. Hence, we aimed to perform a full phenotypical and functional characterization of amniotic fluid B cells in normal pregnancy and in a mouse model of preterm birth. Phenotypic analysis depicted the presence of two populations of amniotic fluid B cells: an immature population, resembling B1 progenitor cells and a more mature population. Further isolation and *in vitro* co-culture with a bone marrow stroma cell line demonstrated the capacity of the immature B cells to mature. This was further supported by spontaneous production of IgM, a feature of the B1 B cell sub-population. An additional *in vitro* stimulation with lipopolysaccharide induced the activation of amniotic fluid B cells as well as the production of pro and anti-inflammatory cytokines. Furthermore, amniotic fluid B cells were expanded in the acute phase of LPS-induced preterm birth. Overall our data add new insight not only on the phenotype and developmental stage of the amniotic fluid B1 B cells but especially on their functionality. This provides important information for a better understanding of their role within the amniotic fluid as immunological protective barrier, especially with regard to intraamniotic infection and preterm birth.

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Introduction

The amniotic sac is a placental structure of fetal origin that contains a regulated amount of fluid, the amniotic fluid. It is forming a mechanical protection to the fetus against physical trauma and provides nutrients and factors required for fetal growth (Underwood *et al.* 2005). In addition, it represents a critical immune barrier that protects the fetus against pathogens. Indeed, it is known that the AF contains leukocytes as well as proteins implicated in fetal host defense (Galask & Snyder 1970, Schlievert *et al.* 1976, 1977, Tafari *et al.* 1977, Larsen *et al.* 1979, Niemelä *et al.* 1989, Pierce *et al.* 2016). It has also been shown that immune cells, including B cells are constantly present within the amniotic fluid during all stages of pregnancy (Gomez-Lopez *et al.* 2018b). In women undergoing intraamniotic inflammation, caused either by intraamniotic infection, external signals or cellular stress, the numbers of leukocytes as well as the concentration of inflammatory mediators, such as cytokines and antimicrobial peptides, are significantly increased in the AF (Romero *et al.* 2011, Gomez-Lopez

et al. 2018b). In this regard, it has been shown that the numbers of immune cells are significantly higher in the AF of pregnant women suffering from intraamniotic infections, which implicates that the innate as well as the adaptive immune system are involved in the pathogenesis of preterm birth (Gomez-Lopez *et al.* 2016, 2017, Arenas-Hernandez *et al.* 2019, Leng *et al.* 2019).

B cells are pleiotropic cells of the adaptive arm of the immune system with the unique ability to give rise to fully differentiated immunoglobulin-producing cell (Pieper *et al.* 2013). In addition to this critical function, B cells can also present antigens to T cells and produce a wide range of cytokines (Lund 2008). Based on their functionality, localization and developmental origin, B cells can be divided into B1 and B2 B cells (Montecino-Rodriguez & Dorshkind 2012). While B2 B cells are continuously produced during the postnatal life from precursors located in the bone marrow, B1 B cells arise mainly during the embryonic life from progenitor cells initially located in the extra-embryonic membranes and then in the embryonic liver (Montecino-Rodriguez &

Dorshkind 2012). During their development B1 B cells undergo various changes of surface antigen expression. Roughly it has been described that during the process of maturation an upregulation of CD45R(B220) and sIgM takes place so that in this context there are two phenotypes that are to be distinguished. The one of CD19⁺B220^{low/neg} B cell progenitors and a more mature CD19⁺B220⁺sIgM⁺ phenotype (Montecino-Rodriguez & Dorshkind 2012).

Our group and others have demonstrated that B cell development and function undergo profound modifications during pregnancy, most likely to allow the presence of the semi-allogeneic fetus (Medina *et al.* 1993, Kincade *et al.* 1994, Muzzio *et al.* 2014, Ziegler *et al.* 2018). Moreover, it has also been demonstrated that B cells are critically involved in pregnancy associated pathologies like preeclampsia and preterm birth (Jensen *et al.* 2013, Huang *et al.* 2017, Leng *et al.* 2019). Furthermore, the presence of B cells in reproductive tissues including amniotic fluid has already been described (Huang *et al.* 2017, Gomez-Lopez *et al.* 2018a).

Hence, we aimed in this study to perform a full phenotypical and functional characterization of the amniotic fluid B cells in normal pregnancy in mice as well as in a mouse model of preterm birth.

Material and methods

Animals

Females BALB/c or C57BL/6 (inbred strain) and males C57BL/6 or BALB/c (inbred strain) mice at 8-12 weeks of age were provided by the ZSFV (*Zentrale Service und Forschungseinrichtung für Versuchstiere*) in Greifswald. All mice were maintained in the facilities of the BioTechnikum Greifswald under a 12-h light/12-h darkness cycle and were given *ad libitum* access to food and water. Animal experiments were carried out according to institutional guidelines after ministerial approval (Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei Mecklenburg-Vorpommern (7221.3-1-068/13 to F J)). The experiments were conducted in conformity with the European Communities Council Directive 86/609/EEC.

Virgin BALB/c (H-2D^d) or C57BL/6 (H-2D^b) females were mated 1:1 with C57BL/6 (H-2D^b) or BALB/c (H-2D^d) males respectively until pregnancy was confirmed. The presence of a vaginal plug was designated as day 0 of pregnancy. Pregnant animals were killed at day 14 of pregnancy, individual amniotic sacs were removed from the maternal uterus and the AF collected as shown in Fig. 1. To avoid contamination with maternal cells, only clear, transparent fluids were used for the experiments.

For experiments displayed in Fig. 1, C57BL/6 females were mated with BALB/c males. Pregnant females were systemically challenged (i.p.) at day 15 of pregnancy with a single dose of LPS (10 µg/mice) or PBS as control. Animals were killed 5 h after LPS injection and the AF was collected. The dose of LPS used in this work caused 100% preterm birth (PTB; Huang *et al.* 2017, unpublished data from our laboratory).

Isolation of B cells from AF

AFCD19⁺ cells were magnetically isolated using a commercially available kit (MACS). Briefly, AF was applied onto a 100 µm cell strainer to obtain a single cell suspension. Obtained cells were magnetically labeled with CD19 MicroBeads (Miltenyi Biotec) and then retained in a MACS column placed in a MACS separator. The retained CD19⁺ cells were then eluted and collected as the enriched positively selected cell fraction. Purity in all experiments was up to 90%.

Cell culture and in vitro stimulation

Isolated CD19⁺ AF cells (5000–9000 cells) were placed in a six-well plate that was pre-coated with a bone marrow stromal cell line (OP9) the day before. Cells were co-cultured in 2 ml

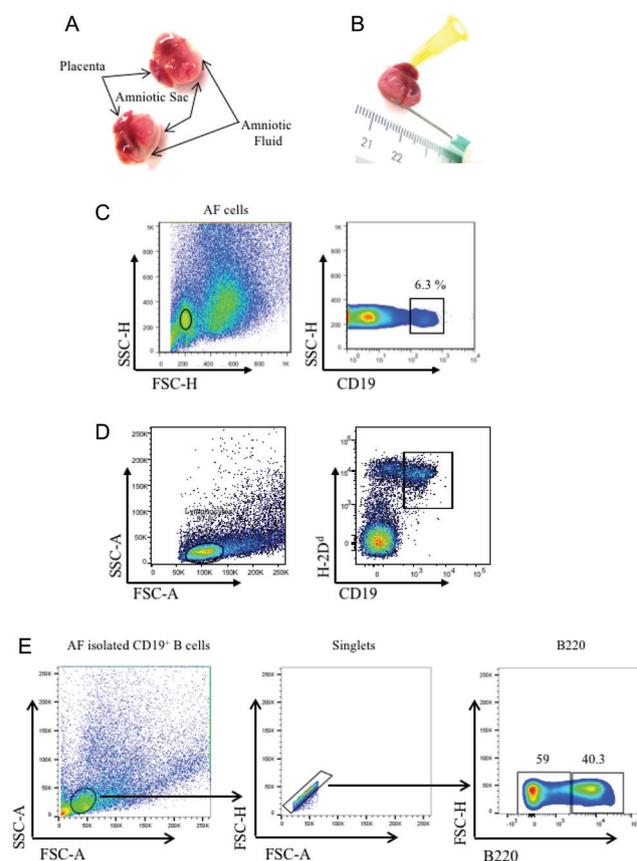


Figure 1 Isolation and characterization of AF cells. (A) Murine feto-maternal unit after removal from the maternal uterus, showing the placenta and the amniotic sac containing the AF. (B) Collection of the AF with a 0.6 × 25 mm syringe (green). (C) Gating strategy for the flow cytometric analysis of CD19⁺ AF-B cells. (D) Expression analysis of paternal MHC I molecule (H-2D^d) within CD19⁺ AF-B cells. (E) Pure magnetically isolated CD19⁺ AF-B cells were stained with fluorochrome-conjugated antibodies against CD45R (B220) and analyzed by flow cytometry. Lymphocytes were identified by their scatter properties (FSC-A × SSC-A plot or FSC-H × SSC-H) and then doublets were excluded by gating on FSC-A × FSC-H. Fluorescence minus one (FMO) was used as control. Data is representative of at least eight independent animals.

of culture medium per well (α -MEM-medium, 10% FBS, 1% Pen/Strep, 5×10^{-5} M β -Mercaptoethanol, 50 units/mL IL-7 and 10 ng/mL flt-3 ligand) (Yoshimoto *et al.* 2011) for 10 days at 37°C in steam-saturated atmosphere (5% CO₂). The medium was changed every second day. In same experiments, after 10 days in culture, cells were further stimulated with LPS (10 μ g/mL; 0111:B4) for 48 h, with or without the addition of Phorbol-12-myristate-13-acetate (PMA), Ionomycin and Brefeldin A for the last 5 h of culture.

Cell staining and flow cytometry

Before staining and to avoid unspecificity, cells were washed twice in FACS buffer (PBS 1x+BSA 1%+0.1 sodium azide) and incubated with Mouse BD Fc Block™ solution following manufacturer recommendations. Afterward, cells were resuspended in 100 μ L of FACS buffer containing specific extracellular antibodies (B220 PE (RA3-6B2), 0.2 mg/mL; IgM APC (II/41), 0.2 mg/mL; CD19 PE-Cy7 (1D3), 0.2 mg/mL; CD69 PerCP-Cy5.5 (H1.2F3), 0.2 mg/mL; MHCII FITC (2G9), 0.5 mg/mL; H-2D^d Alexa Fluor 647 (32-2-12), 0.5 mg/mL) for 30 min at 4°C. For intracellular protein detection, cells were first stained for extracellular markers as explained above and then treated with BD Cytofix/Cytoperm Fixation and

Permeabilization Solution. Next, cells were incubated for 30 min at 4°C with 100 μ L of FACS buffer containing specific antibodies (TNF- α PE (MP6-XT22), 0.2 mg/mL; IFN- γ APC (XMG1.2), 0.2 mg/mL and IL-10 FITC (JES5-16E3), 0.5 mg/mL). Unless indicated otherwise, all antibodies were purchased from Biolegends, Germany. Data were acquired on BD FACSCanto™ or BD-Accuri C6 Plus™ Flow Cytometers and analyzed by using FlowJo software.

IgM ELISA

Levels of IgM in the supernatants of the co-culture system were assayed by using a commercially available ELISA Kit (Mouse IgM ELISA ‘Ready-Set-Go®’, eBioscience) following the supplier’s recommendations.

Statistical analysis

Data were analyzed with PRISM software (version 5.0, GraphPad). T-test or Mann–Whitney test was applied as appropriated to evaluate differences of means. Significant differences between groups were indicated with asterisks (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

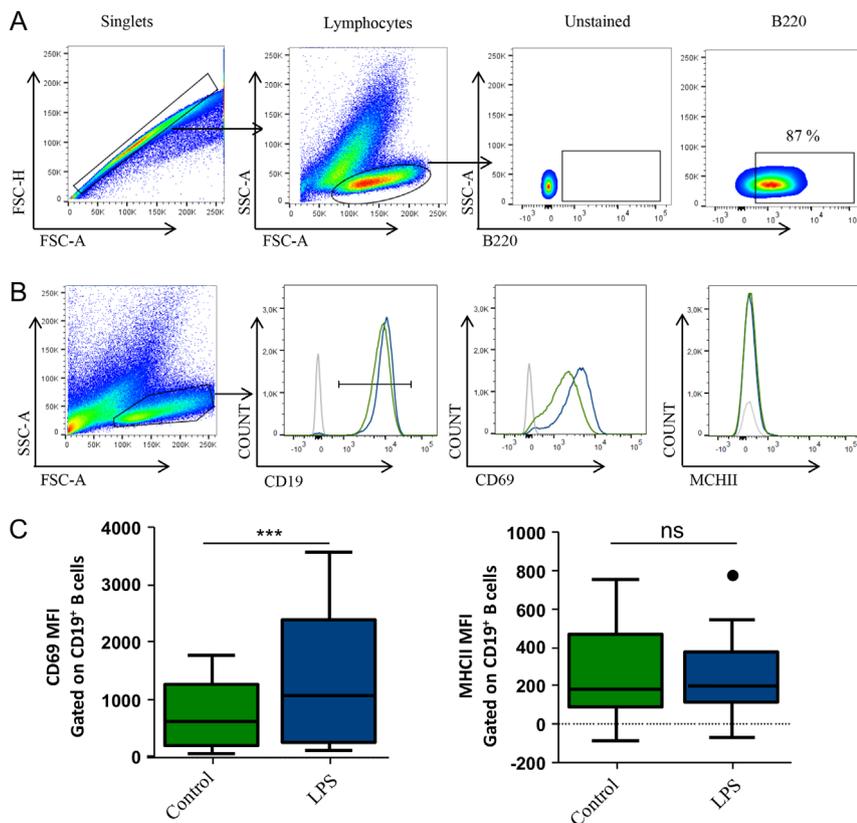


Figure 2 Phenotypic characterization of isolated AF CD19⁺ B cells co-cultured with a bone marrow stromal cell line. (A) Magnetically isolated CD19⁺ AF-B cells were co-cultured with a bone marrow stromal cell line (OP9) for 10 days and then stained with fluorochrome-conjugated antibodies against CD45R (B220). (B) Representative histograms showing gating strategy for the analysis of the expression of B cell activation markers, CD69 and MHCII in AF-isolated CD19⁺ cells after co-culture with OP9 stromal cell line. Pure isolated CD19⁺ AF-B cells were co-cultured with OP9 stromal cells for 10 days and then treated with LPS (10 μ g/mL) for 48 h. Thereafter, cells were stained with fluorochrome-conjugated antibodies against CD19, CD69 and MHCII and analyzed by flow cytometry using a FACSCanto flow cytometer. Gray histograms represent corresponding unstained controls. (C) Box and whiskers graphs showing mean fluorescence intensity (MFI) of CD69 and MHCII in CD19⁺ B cells. Data were collected with FACSCanto flow cytometer and analyzed with FlowJo software. Lymphocytes were identified by their scatter properties (FSC-A \times SSC-A plot) and then doublets were excluded by gating on FSC-A \times FSC-H. Fluorescence minus one (FMO) was used as control. Data were analyzed with PRISM software (version 5.0, GraphPad). Data are shown as mean \pm s.e.m. of five mice per group. *** $P < 0.001$ as analyzed by Mann–Whitney test.

Results

The amniotic fluid contains B cells in different stages of their development

We began isolating amniotic fluid cells from the amniotic sac of pregnant mice on day 14 of pregnancy (Fig. 1A and B) and analyzed the presence of CD19⁺ B cells by flow cytometry. As shown in Fig. 1C, 6–13% of the AF lymphocytes were positive for the B cell antigen CD19 (Fig. 1C). In addition, using a C57BL/6 (female) × BALB/c (male) breeding strategy, we analyzed the expression of H-2D^d (BALB/c MHC I) in AF-CD19⁺ cells. As shown in Fig. 1D, the vast majority of the AF-CD19⁺ cells expressed paternal MHC I antigen. Next, in order to perform a more detailed characterization of these cells, we proceeded to magnetically isolate CD19⁺ cells from AF of pregnant mice and perform a phenotypic characterization. The expression analysis of CD45R (B220) in isolated CD19⁺ AF cells allowed the distinction of two well-defined populations. A B220 expressing CD19⁺ population of B cells (CD19⁺B220⁺; Fig. 1D) and a second population, which was either negative or expressed very low levels of B220

(CD19⁺B220^{lo-neg}; Fig. 1D). The latter have already been described as that of fetal B-1 B cell progenitors (Montecino-Rodriguez *et al.* 2006).

In summary, these results demonstrate the presence, in the AF, of B cells in different stages of their development which additionally express paternal MHC I.

Immature amniotic fluid B cells expand and mature *in vitro*

To examine the capacity of AF-B cells to mature *in vitro*, we next magnetically isolated CD19⁺ B cells from the AF of pregnant mice and co-cultured them with a bone marrow stromal cell line (OP9), which is known to support immature B cell maturation (Montecino-Rodriguez *et al.* 2006, Ji *et al.* 2008). After 10 days in culture, we observed an extensive proliferation of the CD19⁺ AF-B cells co-cultured with the OP9 cells (data not showed). Interestingly, phenotype analysis showed that after 10 days in culture, the vast majority of the AF CD19⁺ B cells expressed B220 (Fig. 2A).

Overall, these results show that immature CD19⁺ AF-B cells are able to phenotypically mature *in vitro*.

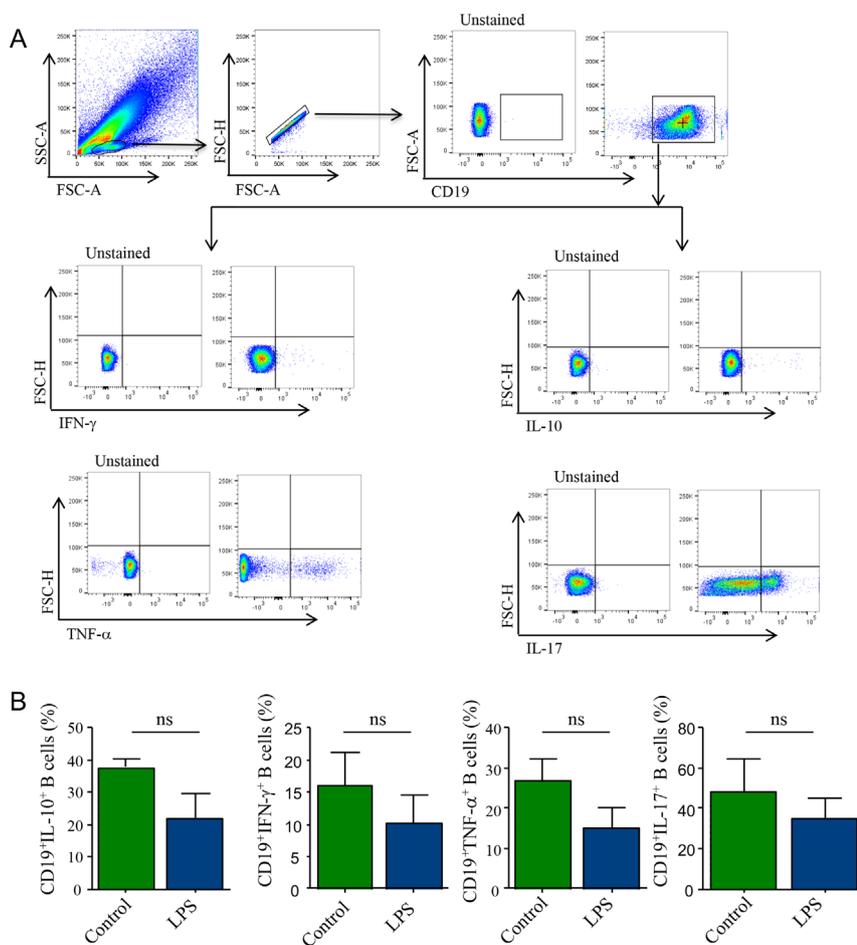


Figure 3 Amniotic fluid B cells produce pro as well as anti-inflammatory cytokines. (A) Representative pseudocolor plots showing gating strategy used to analyze intracellular cytokine production by AF-B cells. Pure isolated CD19⁺ AF-B cells were co-cultured with OP9 stromal cells for 10 days and then treated with LPS (10 μg/mL) for 48 h. Thereafter, cells were stained with fluorochrome-conjugated antibodies against CD19, TNF-α, IFN-γ, IL-17A and IL-10. Data were collected with FACSCanto flow cytometer and analyzed with FlowJo software. Lymphocytes are identified by their scatter properties (FSC-A × SSC-A plot) and then doublets were excluded by gating on FSC-A × FSC-H. Data are shown as mean ± S.E.M. of five mice per group. No statistically significant differences were observed among the groups as analyzed by Student's *t*-test.

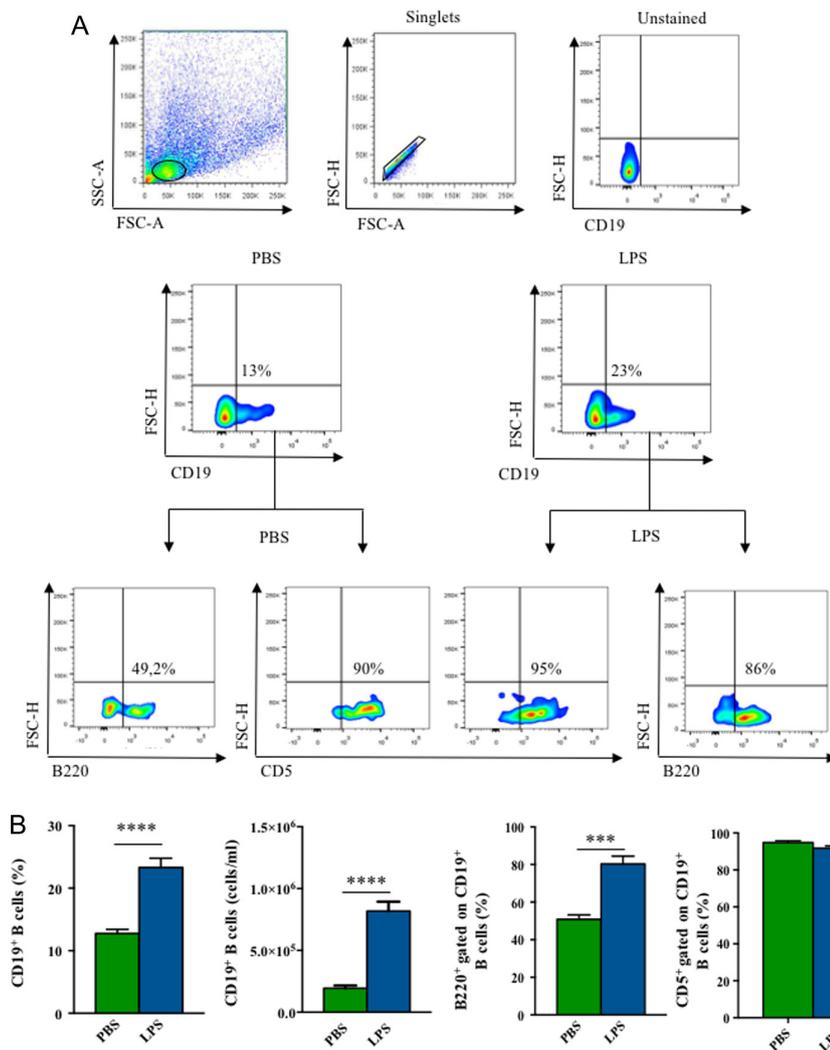


Figure 5 B cells are augmented in the amniotic fluid during acute phase of LPS-induced PTB. Pregnant mice were injected (i.p.) with LPS (10 µg/mice) on day 16 of pregnancy and killed 5 h later. (A) Representative pseudocolor plots showing gating strategy used to analyze different B cell subpopulations in the AF during acute phase of LPS-induced PTB. (B) Bar graphs show percentages and total numbers of CD19⁺ B cells as well as percentages of CD19-gated B220 and CD19-gated CD5 B1 B cells in the AF during acute phase of LPS-induced PTB. Data are shown as mean ± S.E.M. of six mice per group. *** $P < 0.001$; **** $P < 0.0001$ as analyzed by Student's *t*-test.

postnatal life (reviewed in Montecino-Rodriguez & Dorshkind 2012).

Interestingly, almost half of the AF-B cells described in this work showed a phenotype which resembles that of the B1 B cell progenitors (CD19⁺B220^{low/neg}) previously described (Montecino-Rodriguez *et al.* 2006, Yoshimoto *et al.* 2011). Remarkably, these authors also demonstrated, as we did here, that CD19⁺B220^{low/neg} B1 progenitor cells achieve a mature phenotype (CD19⁺B220⁺) upon *in vitro* co-culture with a bone marrow stromal cell line (Yoshimoto *et al.* 2011).

The fact that the AF-B cells spontaneously produced and released IgM *in vitro*, strongly suggests that these cells most likely belong to the B1 B cell subset. Underlining this, we also showed that the vast majority of AF-B cells express the B1 B cell marker CD5 (Montecino-Rodriguez & Dorshkind 2012).

Overall, the results obtained in our work strongly suggest that the AF contains B1 progenitors as well as B1 B cells in advanced stages of their development.

Besides providing nutrients and growth factors as well as conferring mechanical protection to the fetus during pregnancy, the amniotic fluid represents a critical immunological barrier against invading pathogens (Davis *et al.* 1983, Schmidt 1992). Indeed, it has been recently demonstrated that numbers of AF immune cells are increased upon maternal infection or inflammation (Gomez-Lopez *et al.* 2018b). In keeping with this, using a mouse model of LPS-induced preterm birth, we showed here that percentages as well as total numbers of CD19⁺ B cells were increased in the acute phase of preterm birth. Moreover, we additionally showed that the increase observed in total B cell percentages in the AF upon maternal challenge with LPS was conducted by an increase in the percentages of CD19⁺B220⁺ mature B1 B cells. These results reinforce the idea of AF-B1 B cells as critical immune component of the fetus's defense against maternal infections. Indeed, B1 B cells are considered innate-like immune cells that mediate the first line of defense against pathogens (Baumgarth 2011). They respond rapidly to bacteria or

bacterial components like LPS and differentiate into IgM-producing cells (Nguyen *et al.* 2015). In addition, B1 B cells are the major source of IgM natural antibodies which are spontaneously produced in the absence of foreign antigens and play a critical role in providing immune protection from pathogens (Berland & Wortis 2002, Baumgarth 2011). Interestingly, we showed here that B1 B cells not only proliferate in the AF of pregnant mice that were systemically challenged with LPS but also spontaneously produce IgM *in vitro*.

In summary, we provided evidences in this work demonstrating the presence of B1 B cells, most likely of embryonic origin, in different stages of their development, in the amniotic fluid of pregnant mice. These AF-B1 B cells have the potential to grow and mature *in vitro* as well as show activation upon LPS stimulation and spontaneously produce IgM. This gives new insight into the role of B1 B cells within the AF as immunologically active barrier. In addition to this, the significant increase of B1 B cells, especially of their mature immunoglobulin-producing phenotype, in LPS-induced preterm birth suggests the importance of these cells in pathological pregnancies.

Even though further works are needed, this paves the way for deeper investigations of the function and protective capacity of the AF-B1 B cells in the context of pregnancy.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

B I, J L and M D performed experiments, analyzed data and contributed to draft the manuscript; V N, E J, M F, Z K, M K and V M S performed experiments; Z M contributed with reagents; J F conceived the working hypothesis, designed the experiments, supervised the work, wrote the manuscript and provided financial supports.

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The results in this paper, apart from those displayed in Fig. 6, are part of the dissertation of Imke Bommer in order to obtain the degree Dr. med. at the University of Greifswald, Germany.

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