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B cells in murine amniotic fluid – a characterization in normal pregnancy and preterm birth

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Introduction

The amniotic fluid – as the medium surrounding the fetus, it is holding a crucial role in the maintenance and development of a successful pregnancy. While providing mechanical protection to the fetus, it also offers considerable immunological defense. In fact, it is known that the amniotic fluid plays a significant role in the innate immune system, as many of its corresponding substances show substantial antimicrobial function. Also, components of the adaptive immune system, including B cells, have been described within the amniotic fluid. An increase of immune cells in the amniotic fluid in cases of intra-amniotic infection indicates their involvement in inflammation-related pathologies of pregnancy. However, especially B cells in the amniotic fluid have not yet been thoroughly investigated.

The aim of this work is a deeper examination of the B-lymphocytes within the amniotic fluid. Based on the analysis of surface molecules this includes their phenotype, origin and function. In the long term this could substantiate our understanding of intraamniotic inflammation and or infection, which are casually linked with preterm birth, fetal inflammatory response syndrome and fetal morbidity.
This, in turn, could pave the way for potential diagnostic methods and treatments.

Methods

For all experiments 8-12-weeks-old pregnant mice were sacrificed at day 14 of pregnancy. The amniotic fluid was collected and specific cell subsets were isolated using MACS cell separation. Cells were then co-cultured with a bone marrow stromal cell line and stimulated *in vitro*.
The analysis of the population distribution and cytokine production was performed by flow cytometry. To analyze IgM-levels in the supernatant of the co culture, ELISA was used. Statistical analysis was performed using GraphPad Prism© software.

Results

The amniotic fluid contains different developmental stages of B cells, which most likely are of fetal origin. This is supported by the expression of paternal surface markers. An extensive proliferation and switch towards a more mature phenotype upon co-culture shows that the immature subsets of amniotic fluid B cells are able to expand and mature *in vitro.* Amniotic

fluid B cells spontaneously produce IgM and show functional adaption upon *in vitro* stimulation as evidenced by the increase of cell activation markers.

Conclusion

For the first time a deep investigation of B-cells within the amniotic fluid was performed, covering phenotype and cell functionality. This work shows that there is a B cell compartment within the amniotic fluid, which, to a certain extent, is able to mature and gain functionality when exposed to external stimuli. This supports the hypothesis of the amniotic fluid as crucial immunological line of defense against inflammatory and infectious challenges during pregnancy.

Abbreviations

AF	Amniotic Fluid
Ag	Antigen
APC	Allophycocyanin
APC-Cy ™ 7	APC combined with a cyanine dye
Bregs	Regulatory B cells
Be cells	Effector B cells
°C	Degrees Celsius
CD	Cluster determinant
CLP	Common lymphoid progenitor
DC	Dendritic cell
dpp	day post plug
ddH_2O	Distilled water
EDTA	Ethylenediaminetetraacetic acid
FACS	Fluorescence activated cell sorting
FBS	Fetal Bovine Serum
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
G	Acceleration of gravity
HRP	Horseradish-peroxidase
HAS	Heat stable antigen
HSC	Hematopoietic stem cell
ILC	Innate lymphoid cell
LPS	Lipopolysaccharide
MACS	Magnetic cell separation
MIAC	Microbial invasion of the amniotic cavity
MLP	Multilineage progenitor
NK cells	Natural killer cells
PBS	Phosphate buffered saline
PE	R-phycoerythrin
PE-Cy ™ 7	Pe combined with a cyanine dye
Pen/Strep	Penicilline/Streptomycine
PerCP-Cy™5.5	PerCP combined with a cyanine dye

PMA	Phorbol-12-myristate-13-acetate
P-Sp	Para-aortic splanchnopleura
SPF	Specific pathogen free
SSC	Side scatter
TMB	Tetramethylbenzidine
TSLP	Thymic stromal lymphopoietin
WHO	Worl Health Organization
YS	Yolk sac
ZSFV	Zentrale Service- und Forschungseinrichtung für Versuchstiere

Figures

1 Introduction

1.1 Immunology of the amniotic fluid

During pregnancy, the amniotic cavity is filled with a clear aqueous liquid, the amniotic fluid (AF). It not only protects the embryo/fetus from physical trauma, but also prevents adhesions of embryo and amnion, enables fetal movement and builds a hydrostatic wedge that supports the opening of the cervical canal during labour [1]. As the medium surrounding the fetus, the amniotic fluid also is involved in clinical intraamniotic infection and or inflammation which is causally linked with preterm labor and birth [2]. While the risk factors for preterm labor and birth are versatile and complex, infection is assumed to be the most common risk [3], especially for extreme preterm birth, as of week 28 [4]. Given that 15 million pregnancies worldwide are complicated by preterm birth each year, it still represents one of the leading causes of fetal and maternal morbidity and mortality [5]. However, the complex pathogenesis of preterm birth is still only poorly understood which in turn results in limited preventive and curative options.

The reaction to most infection processes within the human body involves the innate (unspecific) and the adaptive (acquired) immune system. Most research findings concerning preterm labor induced by inflammation has been focused on the innate limb of the immune system. However there has been increasing evidence on the involvement of the adaptive immune system in this pregnancy complication [3].

Both parts of the immune system contain a cellular and a humoral part. Mast cells, dendritic cells, neutrophil granulocytes, natural killer (NK) cells and macrophages represent the cellular part of the innate immune system. NK cells and macrophages already are a known part of the amniotic fluid. As well as antimicrobial molecules such as α-defensins (HNP1-3), calprotectin (MRP 8/14) and bactericidal/permeability-increasing protein (BPI), which represent the humoral part of the innate immune system.

The cellular part of the adaptive system is built of T and B lymphocytes, while the antibodies (immunoglobulins), produced by activated B lymphocytes (plasma cells), represent the humoral part [6].

During the past decade, the presence of the adaptive immune system within the fetal-maternal-interface, including the AF, has received increasing scientific attention. Studies investigating the immunophenotype of leukocytes within human AF showed that there are indeed various populations of immune cells present in normal human pregnancy [7]. Among these,

T cells and innate Lymphoid cells (ILCs) were present as well as NK cells and B cells. T cells showed their highest numbers between week 15 to 30 while B cell numbers were low from week 15 to 20. They showed an increase after week 20 and remained stable afterwards. Overall, B cells as well as T cells and NK cells showed a significant increase in the AF of women with intraamniotic infection/inflammation indicating that these cells are part of a fetal host response to microbial invasion of the amniotic cavity (MIAC) [8].

1.2 B cells – development, phenotype and function

Although their main function is the production of antibodies, B lymphocytes also play an important role in orchestrating the immune response. Antigen- presentation and thus initiating a T cell response as well as cytokine-production are just examples for their multifunctionality [9]. Today it is known that B cells display a rich diversity. In mice two B cell populations can be distinguished: A B-1 and a B-2 cell population.

B cell development in general is considered to initiate during embryogenesis. The so-called lineage model proposes B-1 and B-2 cells as two different lineages originating from different precursors [10].

B-2 cells mainly derive from the bone marrow during postnatal life and represent the main B cell population in lymphoid tissues. They are considered the “mediators” of the adaptive immune system. The correlating precursor cells are considered to derive from the aorta-gonad-mesonephros, generating a population of hematopoietic stem cells (HSC) within the fetal liver. These cells then seed the bone marrow in the course of fetal development. The most common opinions depict the development of B-cells as uniform progress including three steps related to the functional rearrangement of gene segments encoding the heavy chain (H-chain) and the light chain (L-chain) of immunoglobulins. First of all there is a rearrangement concerning the D (diversity)- and J (joining)- segment of the H-chain (DH- and JH- rearrangement) within the so-called Pro-B cells. This is followed by an additional rearrangement assembling an upstream V (variable) region to the D-J-segment and another rearrangement of the μ-H-chain segment that transfers the cell to the Pre-B cell stage. After one to two cell divisions and the rearrangement of the κ- and λ- (light-) chain gene segments, an IgM molecule is generated by the combination with the μ-chain. This marks the progression to the stage of immature B cells that leave the bone marrow and continue their maturation within secondary lymphoid tissue such as human spleen and lymph nodes, generating the so-called Marginal-zone- (MZ) and Follicular-zone- (FO) B-cells [11]. These sublineages

are able to pass through Ig class switching and to differentiate into memory cells. The functional end point of B-cell development is the differentiation into immunoglobulin-producing plasma cells [12].

B-1 cells represent a much smaller subpopulation. They mainly originate in different fetal tissues, such as fetal liver and omentum and are abundantly present within serous cavities. The main effects of B1 cells are assigned to innate immune responses [13],[14]. The development of B-1 B cells is depicted to start from B-1 specific CLPs, generating B-1 progenitors and sequentially maturing following the above given scheme. The mature B-1 cells are finally migrating into serous cavities and differentiate into B-1a and B-1b subsets [13].
The discovery of B-1 cell progenitors that are autonomously generated in the fetal Yolk Sac (YS) and para-aortic splanchnopleura (P-Sp) at a pre-HSC-stage before the presence of B-2 cell progenitors [15], led to the establishment of a layered three-phase developmental program of B-1 cell maturation. According to this working-model, the first phase originates from the fetal YS and P-Sp approximately at day 9.0 to 9.5 of pregnancy before the first appearance of HSCs at day 10.5. Corresponding precursors of these B-1 progenitor cells are not yet identified and it remains unclear whether they are developing into B-1 cells that later represent a part of the B-1 population in adults.
The second phase initiates within the fetal liver and bone marrow. B-1 progenitors are generated by HSCs and develop into mature B-1 cells. Even though the development of B-2 cells is also initiated during this phase, B-1 cell production clearly is predominant. Subsequently it shows a peak during late embryogenesis and declines directly before delivery. Whether both B cell subsets originate from the same stem cell or distinct B-1 and B-2 specified HSCs, remains unknown.
However within the third phase, which is located in the bone marrow, mainly B-2 cells are generated. The production of B-1 cells can still be observed during this phase but shows substantially lower intensity than in phase two. [13]. Against this Background some authors propose to use B-1 when referring to fetal development and progeny and B-2 when describing adult development within the bone marrow [16].

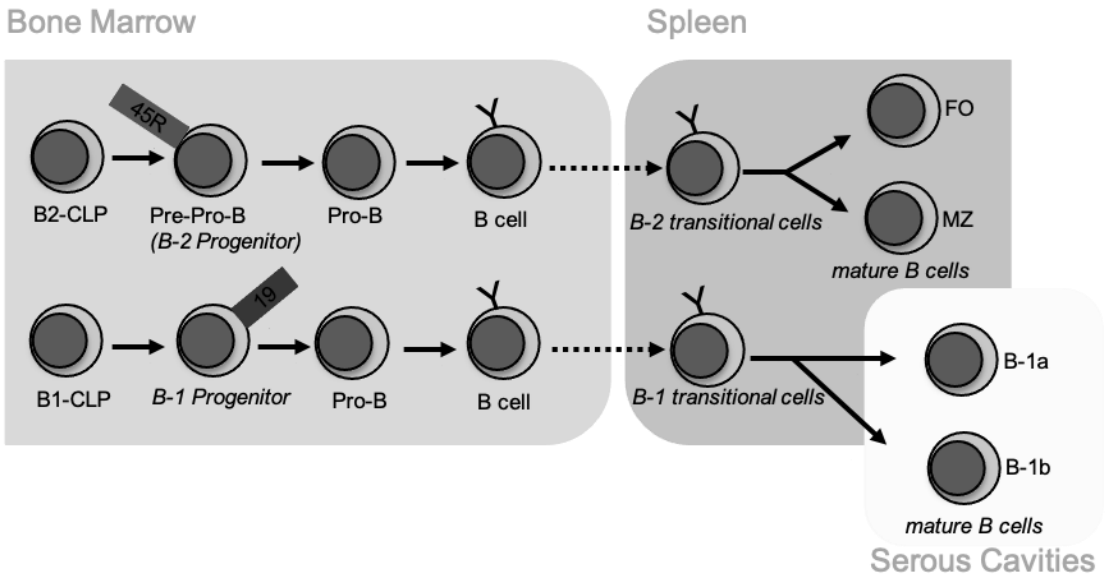

Figure 1- B-1 and B-2 development modified from Encarnacion Montecino-Rodriguez & Dorshkind 2012, page14 [13]
The development of B-2 cells starts in the bone marrow after birth. From CLPs cells mature through Pre-Pro, Pro-B, and Pre-B (not shown) stages into immature sIgM^{+} cells. Pre-Pro- cells are referred to as B-2 progenitors. sIgM^{+} cells migrate to the spleen where they mature through B-2 transitional cells into follicular (FO) or marginal (MZ) zone B cells.
B-1 cells derive from specialized B-1 CLPs that follow the above given Pre-Pro, Pro-B, and Pre-B (not shown) stages and then also mature into immature sIgM^{+} B-1 cells. After going through transitional B-1 cell stages in the spleen, mature B-1 cells migrate into serous cavities and acquire the B-1a or B-1b phenotype.

B-lymphocytes can be distinguished by their surface molecules. The B lineage development originates from hematopoietic stem cells (HSCs). These are stem cells expressing CD93/AA4.1, a marker for early B cell stages in bone marrow, adult spleen and fetal liver [17]. It is present from early progenitors through immature B cell stages [16]. Moreover HSCs express CD43 a marker also expressed for example on Pro-B cells, B-1 cells and plasma cells [18].

The following stage of development is characterized by the definite restriction to B lineage, which is signified by the expression of CD45R(B220). The according subsets including Pre-Pro-B or “fraction A” [16] stages can be recognized as CD45R(B220)$^{+}$CD43^{+} and HSA/CD24 ^{low}. HSA (heat stable antigen) or CD24 is a membranous protein that is typically acquired by hematopoietic progenitors in the context of commitment to the B-lineage [19].
Cells of this phenotype are also referred to as “B-2-progenitors” by Montecino *et al.* [13]. “B-1-progenitors” show a AA4.1/CD93^{+}, CD45R(B220)$^{-\text{ or lo,}}$ CD19^{+} phenotype. This progenitor

is able to differentiate into two distinct B-1a ($sIgM^+CD11b^+CD5^+$) and B-1b ($sIgM^+$ $CD11b^+$ $CD5^-$) subsets [20] but does not generate B-2 cells.

Independently, CD19 is described as marker for more advanced B cell stages, which is recognizable on “fraction B” and “fraction C” cells within the Pro-B cell subset. These cells show a $CD45R(B220)^+CD19^+CD43^+$ and $HSA/CD24^+$ or $HSA/CD24^{++}$ (“fraction C”) phenotype [16],[21]. With advancing maturation, $HSA/CD24^{hi}$, $CD45R(B220)^+$, $CD19^+$, $CD43^+$ (“fraction C”) and $HSA/CD24^{hi}CD45R(B220)^+CD19^+CD43^-$ (“fraction D”) cells evolve, summarized as Pre-B cells. Finally, immature and mature B cells arise, that are recognized as $CD45R(B220)^+CD19^+CD43^-HSA/CD24^-$ and $sIgM^+$, while AA4.1/CD93 is no longer expressed [16],[21].

	HSC	Pre-Pro-B Fraction A B2 Prog	B1 Prog.*	Fraction B	Fraction C	Fraction D	Immature/ mature B cells	B1-a*	B1-b*
CD93	+	+	+	+	+	+	-		
CD43	+	+	+	+	+	-	-		
CD45R (B220)		+	-/low	+	+	+	+	+	+
CD24		low		+	++	++	-		
CD19			+	+	+	+	+	+	+
sIgM							+	+	+
CD11b								+	+
CD5								+	-

Figure 4 - Chart of B lineage development showing cell surface phenotype. Modified from Hardy, Hakayawa 2001, page 597 [16]
B1-a and B1-b subsets are generated from B1-Progenitor cells.

CD69 is a surface-antigen which has been proposed as a marker of early activation. On murine B-cells, CD69 expression can be induced by the exposure to lipopolysaccharides (LPS) [22]. It is assumed that CD69 plays an important role in intracellular signaling in the majority of cells. Accordingly, a number of intracellular signals and a variety of resulting cellular end responses have been identified in different murine and human cell lineages upon crosslinking of CD69 [23].

An important function of the B cells, apart from antibody production, is the presentation of antigens to specific T-cells [20]. This involves the so-called Major Histocompatibility Complex (MHC).

The MHC represents a set of genes that code for MHC molecules, a group of cell surface proteins whose primary role it is to identify foreign peptide fragments and display them to specialized immune cells which is then followed by the initiation of an immune response [24]. There are two main subgroups of MHC molecules to be distinguished. MHC Class I molecules that are present on the surfaces of all nucleated cells with the exception of sperm cells and certain neurons and MHC Class II molecules which are expressed on antigen-presenting cells such as macrophages, dendritic cells and lymphocytes, including B cells [25]. It is assumed that the internalization, processing and display of foreign proteins by B-cells via MHC molecules is crucial for T-cell activation and the initiation of a T-cell immune response.

In this regard, immature B-cells mainly react to antigens, such as lipopolysaccharides, that are T-cell-independent and set off quick antibody responses independent of MHC class II and corresponding T-helper-cells. Mature B-cells on the other hand, are predominantly found in the spleen's lymphoid follicles and in lymph nodes where they face and react to antigens that are T-cell- and thus MHC II -dependent and bound to dendritic-cells (DCs). This leads to their proliferation and differentiation into memory or plasma cells [24]. These processes indicate the important role of MHC II for B cell proliferation, activation and differentiation [26].

Apart from these functional aspects, in mammalian pregnancy, the fetus expresses maternal and paternal MHC I molecules [27]. The latter of which can be used as confirmation for the fetal origin of cells in an experimental context.

As indicated above the main function of B cells is the production of antibodies (immunoglobulins). One of them is IgM, which, in its pentameric form is the first class of antibodies to be produced upon antigen-contact [28]. Therefore, it is predominant in the early stages of an immune response and the first class to be produced by neonates. The forming and expression of IgM marks the transition into the immature B cell stage in which cells exit into circulation and migrate to the spleen in order to complete their maturation [11]. This makes IgM a crucial phenotypic marker in the assessment of B cells and their development and maturation.

In addition to their multiple functions within the immune system, B cells are also able to produce cytokines. Cytokines are a diverse group of proteins and glycoproteins that play an important role in the regulation of immune responses. Although they are not effector molecules themselves, they function as chemical mediators between cells, regulating immunological defense against pathogens and inflammatory reactions as well as cellular growth and differentiation. This gives B cells the ability to amplify and suppress immune responses independently of the production of antibodies. [29]. With regard to this function, two types of B cells can be distinguished based on the cytokines they produce. Regulatory B cells (Bregs), that produce IL-10 and Transforming growth factor β-1 (TGFβ-1) and effector B (Be)cells. Be-cells can be further divided into Be-1 cells, producing IFN- γ, IL-12 and TNF-α and Be-2-cells, producing IL-2, IL-4, IL-6 and TNF-α. Which group the Be cells grow into is determined by the T-cells by which they are primed [30].

Taking all these aspects of B cell biology into account the aim of this work was a basic investigation and characterization of B cells, their phenotype and function, within murine amniotic fluid and in the context of preterm birth.

2 Material and methods

2.1 Animals

Female BALB/cAnN Crl (H-2D^{d}) and male C57BL/6 (H-2D^{b}) mice at 8-12 weeks of age were provided by the ZSFV (*Zentrale Service- und Forschungseinrichtung für Versuchstiere*) in Greifswald. BALB/cAnN (H-2D^{d}) and C57BL/6 (H-2D^{b}) mice are both considered general multipurpose mouse models. Both express the same major histocompatibility (MHC) gene cluster „H2“ [31],[32].

This cluster represents a set of genes that code for MHC molecules, a group of cell surface proteins whose primary role it is to identify foreign peptide fragments and display them to specialized immune cells which is then followed by the initiation of an immune response [24]. The matching of MHC antigens is well known to have a positive effect on graft acceptance for example in organ transplantation [33].

Due to this the above given mating strategy represents a model for normal pregnancy outcome.

In order to confirm the fetal origin of the amniotic cells examined, the expression of paternal MHC I was analyzed. For this, an opposite breeding strategy with BALB/cAnN Crl (H-2D^{d}) males and C57BL/6 (H-2D^{b}) females was used. The animals were also provided by the ZSFV (*Zentrale Service- und Forschungseinrichtung für Versuchstiere*) in Greifswald at 8-12 weeks of age.

All experiments were carried out according to institutional guidelines after ministerial approval [Reviewing board institution: *Landesverwaltungsamt Sachsen-Anhalt* (2-1019 to Federico Jensen) and *Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei Mecklenburg Vorpommern* (7221.3-1-068/13-1) to Federico Jensen and Damián Muzzio (11.11.2014)]. All experiments were conducted in conformity with the European Communities Council Directive 86/609/EEC.
The animals were kept in open cages under specific pathogen free (SPF) conditions and in a 12-hour light cycle. Temperature and humidity were kept at 20 ± 2 °C and 60 ± 20 % while chow and water (acidified, pH 2,5) were applied *ad libitum*.
Virgin BALB/cAnN Crl (H-2D^{d}) or C57BL/6 (H-2D^{b}) females were mated 1:1 with BALB/cAnN Crl (H-2D^{d}) or C57BL/6 (H-2D^{b}) males. Animals were inspected for vaginal plugs two times a day. The presence of a plug was designated as day 0 of pregnancy/ day 0 post plug (dpp). Pregnant animals were sacrificed at day 14 post plug. The amniotic fluid was extracted and stored on ice until further processing. To display pregnancy disturbances a systemic LPS challenge (10 µg/mouse) to pregnant females was applied on day 15 post plug, causing up to 100 % preterm births [34]. Animals were sacrificed 5 h after injection. As control PBS injections were applied. The amniotic fluid was extracted and processed as explained above [35].

2.2 MACS

In order to isolate specific cell populations from the murine amniotic fluid, magnetic activated cell sorting (MACS) was used, which is a widespread technique for cell separation [36],[37]. Based on the immunspecific reaction of a target cell and a magnetic-bead-coupled-antibody it allows an enrichment or a depletion of a desired cell population from a complex cell suspension. The magnetically labeled cells are separated by applying a magnetic field [38] which is a mild but highly effective isolation technique [39]. For this work commercially available kits and magnetic columns (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) were

used. $CD19^+$ B cells were isolated from amniotic fluid using a $CD19^+$ MicroBeads isolation kit and then transferred to cell culture.

2.3 Cell culture

To ensure and support the growth of $CD19^+$ cells and most of all, sensitive precursor cells extracted from the AF, OP9 stromal cells were used for co-culture. These cells provide an anti-apoptotic environment for both hemogenic precursors and hematopoietic progenitors and increase their proliferation [40].

For the experiments displayed, an OP9 Embryonic Stem Cell Macrophage (Mouse Mus musculus) Strain (C57BL/6 x C3H) F2-*op/op* was used.

Cells were cultured in 75 cm^2 flasks at 37 °C in a steam-saturated atmosphere (5 % CO_2). The culture medium consisted of Alpha Medium (500 ml, w 2,0 g/L $NaHCO_3$ w/o L-Glutamine w/o Nucleosides Tested for endotoxin), 20 % FBS and 1 % antibiotics.

For co-culture with isolated $CD19^+$ cells, OP9 stromal cells were sowed in 6-Well-plates. The above given medium was supplied with 5 x 10^{-5} M β-Mercaptoethanol, 50 units/ml IL-7 and 10 ng/ml flt-3 ligand [15] and the magnetic isolated $CD19^+$ cells (see above) were distributed in 2 ml of this medium per provided well. The cells were then added to the wells in which OP9 stromal cells had been sown the day before.

This co-culture was again incubated at 37 °C in steam-saturated atmosphere (5 % CO_2) for 10 days. The medium was changed every second day.

After 10 days in culture a 24 or 48 h-stimulation with LPS0111:B4 was performed. LPS is a characteristic component of the cell walls of gram negative bacteria, known as an stimulant of murine B cell proliferation and mitogen for B cells in mice [34]. This stimulation was supplemented by the addition of Phorbol-12-myristate-13-acetate, Ionomycine and Brefeldin A for the last 5 h. Phorbol-12-myristate-13-acetate is an activator of proteinkinase C and therefore known as mitogen for B-cells. Ionomycine as a calcium ionophore, leads to an enhancement of intracellular cytokine production [41] and Brefeldin A, an inhibitor of cellular protein transport, prevents cytokine excretion [42]. After this, cells were washed twice in FACS buffer (1 % BSA, 0,1 % NaN_3, 1x Phosphate buffered saline (PBS) (9.55 g/L)) and transferred to cell-staining for flow cytometry.

2.4 Flow cytometry/ Fluorescence activated cell sorting

One widely used method for cell characterization is the detection of the cell's unique expression of surface markers (clusters of differentiation). By detecting one of them or a specific pattern, different cell types can be sorted, analyzed and quantified. The technique of flow cytometry or fluorescence activated cell sorting (FACS) makes use of these cell-specific patterns and the specificity of the antibody-antigen reaction. Cells are being labeled with fluorochrome-coupled antibodies specific to their surface-antigens and then exposed to a laser pulse. This excites the fluorochromes bound to the cells and the so emitted light is captured. By analyzing the emission spectra this allows the differentiation of various cells within a cell suspension.

Molecule	Label	Clone
CD19	PE-Cy ™7	1D3
B220	PE	RA3-6B2
B220	PE-Cy ™7	RA3-6B2
CD24	FITC	M1/69
CD43	PE	S7
CD93	APC	AA4.1
IgM	APC-Cy ™ 7	RMM-1
IgM	APC	IL/41
CD69	PerCP-Cy™ 5.5	H1.2F3
MHC II (H-2D^d)	FITC	2G9
H-2D^d	Alexa Fluor 647	32-2-12
CD5	PE-Cy ™7	53-7.3
TNF-α	PE	MP6-XT22
IFN-γ	APC	XMG1.2
IL-10	FITC	JES5-16E3
Il-17	PerCP-Cy™ 5.5	eBio17B7

Figure 7 - Chart showing the fluorochrome-coupled antibodies used for flow cytometry analysis

For flow cytometry analysis single cell suspensions obtained from the co-culture of AF cells were stained with specific antibodies (Figure 5) following standard protocols. To avoid unspecific binding, cells were treated with Mouse BD Fc Block™ and subjected to repeated washing steps in FACS buffer before antibody staining. Unstained cells were used as negative control samples.

The analysis of cytokine production included an additional intracellular staining for which cells were treated with a permeabilization solution (BD Cytofix/Cytoperm™) before antibody staining. In order to keep cells in a permeabilized state BD Perm/Wash buffer™ was used for all correlating washing steps.

Data was acquired on a BD FACS Canto™ or BD-Accuri C6 Plus™ flow cytometer using BD FACSDiva Software Version 6.1.3 (2009) and analyzed with FlowJo VX analysis software (Tree Star Inc.).

Lymphocytes were identified by their scatter properties (FSC-A × SSC-A plot). A doublet exclusion was performed by plotting forward scatter (FSC) height against FSC area. Within the single cell or lymphocyte population cells were then analyzed for the different fluorophores used in the different experiments respectively.

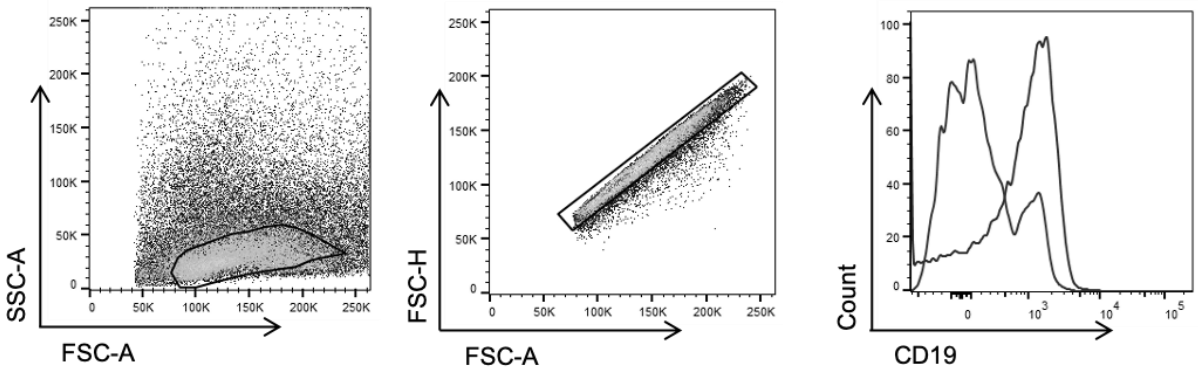

Figure 8 - CD19 analysis on AF cells
Representative pseudocolor Dotplots and histograms depicting the gating strategy for $CD19^{+}$ cells in the amniotic fluid of pregnant (14 dpp) female mice. Histograms are shown for stained samples (right peak) and negative controls from unstained samples (left peak).

2.5 ELISA

To quantify levels of IgM in the supernatants of the co-culture, the antibody-based verification procedure, Enzyme-linked immunosorbent assay (ELISA), more precisely the so-called

sandwich-ELISA was performed. This procedure is very sensitive, as the analyte is bound between two primary antibodies and then detected through color reaction.
For this essay an eBioscience ELISA-kit (“Ready-Set-Go®”) was used according to the manufacturers instructions.

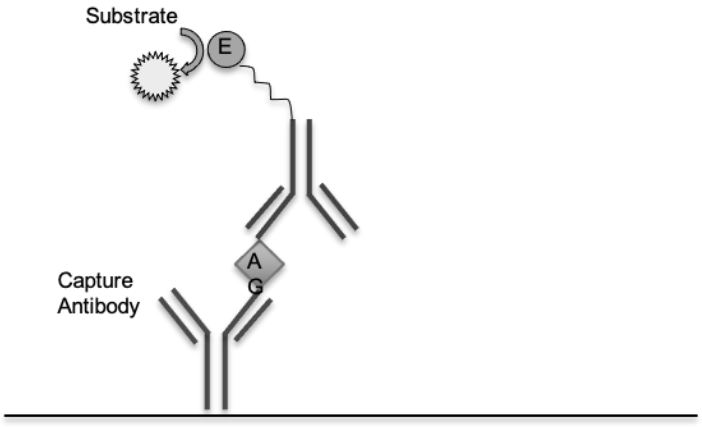

Figure 9 - Principle of a “Sandwich-ELISA”
The analyte is bound between two primary antibodies and then detected through color reaction.
AG Antigen, E Enzyme

A provided 96-well-plate was coated with capture antibody. This antibody was used to later bind the antigen, IgM. Blocking buffer (Assay buffer A diluted 1:10 in deionized water) was used in order to block free protein binding sites, so that background activity of unspecific adsorbed enzyme-linked antibodies or rather antigen molecules was minimized [43]. A standard and the samples extracted from cell culture supernatants were applied to the wells, inducing the antigen-binding to the capture antibody. Remaining unbound antigens were removed during repeated washing steps and the detection antibody, binding specifically to the antigen was applied. The specific bindings of capture antibody, antigen and detection antibody were then verified by an enzyme linked to the detection antibody converting an added substrate to a chromatic product. In this case horseradish peroxidase (HRP) was the enzyme catalyzing the oxidation of primarily colorless Tetramethylbenzidine (TMB), which lead to a yellowing of the substrate.
The enzymatic reaction was stopped by adding stop solution (1 M H_2SO_4). The intensity of the yellowing, directly proportional to the quantity of bound antigen/IgM, was investigated via absorptiometry in a microplate fluorometer.

3 Results

3.1 Murine amniotic fluid contains B cells in different developmental stages

The primary step in the investigation of amniotic fluid cells with the goal of identifying and characterizing lymphocytes, was the analysis of the B cell antigen CD19 and its expression. Revealing a rate of 6-13 % $CD19^+$ AF cells this confirmed the presence of B cells within the amniotic fluid. An additional analysis of H-$2D^d$ (BALB/c MHCI) expression on $CD19^+$ AF cells determined the fetal origin of these cells which to a large extent expressed paternal MHCI antigen.

Based on the expression of CD45R(B220) on $CD19^+$ AF cells two main populations were distinguished. One $CD19^+CD45R(B220)^+$ population and one that expressed no or only low levels of CD45R(B220) ($CD19^+CD45R(B220)^{lo\text{-}neg}$) which, based on the definition of Montecino-Rodriguez et. al. [44] can be described as B-1 B cell progenitor [35]. Some cells of the $CD19^+CD45R(B220)^+$ population additionally express CD43 and CD93 while CD24 and IgM were expressed by an even greater number (Fig. 4).

In summary, these results confirm that murine amniotic fluid contains B cells of fetal origin and in different developmental stages [35]. Additional figures, a supplementary description and used gating strategies can be found in the cited publication.

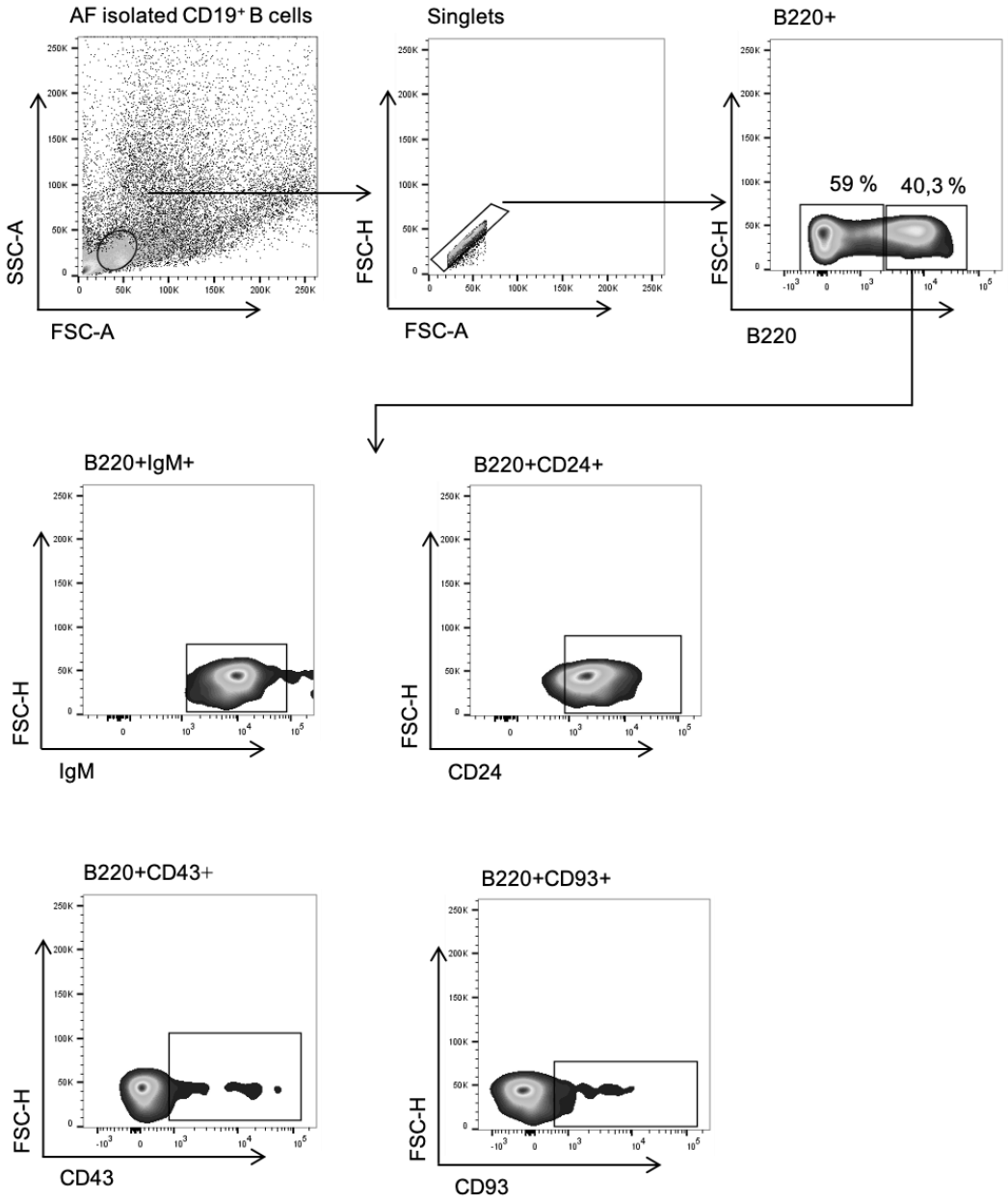

Figure 12 - Representative pseudocolor Dotplots depicting the gating strategy for the phenotype analysis of CD19⁺ amniotic fluid cells
Magnetically isolated CD19⁺ AF cells were stained with fluorochrome coupled antibodies against CD45R(B220), IgM, CD24, CD43 and CD93. and analyzed by flow cytometry. Lymphocytes were identified by their scatter properties (FSC-A × SSC-A plot) 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.

3.2 Amniotic fluid B cells proliferate and maturate *in vitro*

A 10-day co-culture of CD19$^+$ amniotic fluid cells with a bone marrow stromal cell line (OP9) revealed a visually detectable increase in the number of CD19$^+$ cells originating from the amniotic fluid. A phenotypic analysis of these cells after an additional stimulation with LPS showed that now up to 80 % of CD19$^+$ AF cells express CD45R(B220) [35]. When further analyzed for other surface markers, low numbers of these cells show IgM expression and a mild expression can be found for CD93 while CD24 and CD43 are expressed by the highest number of cells. The stimulation with LPS for 24 h leads to a visible but not statistically significant increase in the expression of CD45R(B220), CD24, CD43 and CD93 while a stimulation for 48 h has almost no or even slightly opposite effects. Overall these results indicate that CD19$^+$ AF B cells are able to proliferate and phenotypically maturate *in vitro* [35]. Additional figures, a supplementary description and used gating strategies can be found in the cited publication.

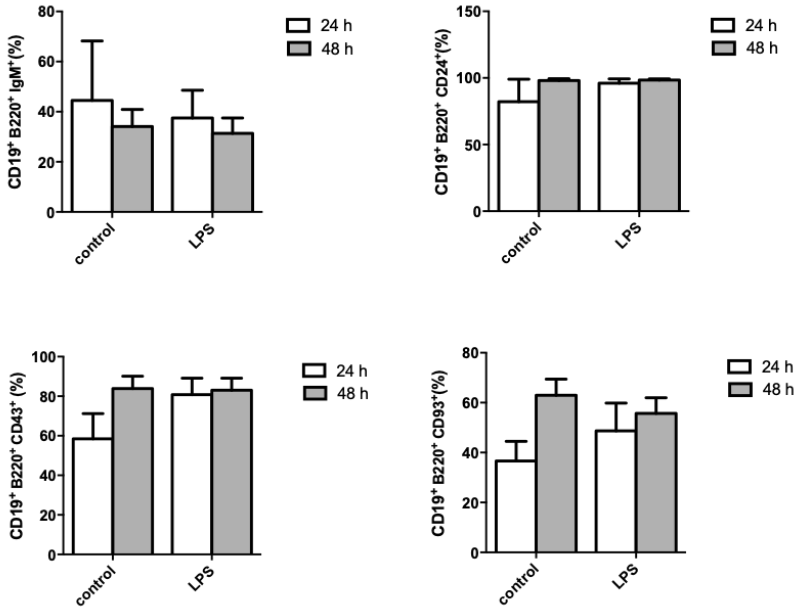

Figure 13 - Phenotypic characterization of isolated AF CD19$^+$ B cells after co-culture with a bone marrow stromal cell line and LPS stimulation
Pure isolated CD19$^+$ AF B cells were co-cultured with OP9 stromal cells for 10 days and then treated with LPS (10 µg/ml) for 24 or 48 h. Thereafter cells were stained with fluorochrome coupled antibodies against IgM, CD24, CD43 and CD93. Data were analyzed with PRISM software (version 5.0 GraphPad). Data are shown as Mean ± S.E.M. Graph bars showing the percentage of IgM, CD24, CD43 and CD93 expression on B220$^+$ gated cells. No statistically significant differences were observed among the groups as analyzed by two-way ANOVA and Bonferroni post-hoc-test.

3.3 Amniotic fluid B cells are activated upon LPS treatment *in vitro*

When co-cultured and LPS-stimulated $CD19^+$ amniotic fluid cells were analyzed for the expression of activation marker CD69 and MHCII results revealed a significant increase of CD69 expression. However, no difference was observed in MHCII expression. In addition to this the analysis of intra-cellular pro-inflammatory (TNF-α, IFN-γ, IL-17A) as well as anti-inflammatory (IL-10) cytokine production by $CD19^+$ amniotic fluid cells also showed no differences related to LPS stimulation. Altogether these results show that an *in vitro* LPS stimulation of AF B cells leads to an activation of these cells but has no significant impact on cytokine production [35]. Figures, a supplementary description and used gating strategies can be found in the cited publication.

3.4 Amniotic fluid B cells produce IgM *in vitro*

Addressing the B cells capacity to maturate into differentiated plasma cells able to produce antibodies, the supernatants of co-cultures stimulated with LPS were analyzed for IgM. Results revealed that already in unstimulated control samples cells produced detectable quantities of IgM. LPS stimulation however did not significantly alter the IgM production of AF B cells. These results indicate that AF B cells are capable of spontaneous IgM production *in vitro* [35]. Figures and a supplementary description can be found in the cited publication.

3.5 The number of amniotic fluid B cells increases in the acute phase of LPS induced preterm birth

The phenotype analysis of AF B cells in a mouse model of LPS-induced preterm birth revealed a significant increase of $CD19^+$ AF cells in total numbers as well as percentages upon LPS treatment and when compared to PBS-treated controls.

A significant increase could also be observed for $CD19^+CD45R(B220)^+$ amniotic fluid cells. Remarkably, the majority of AF B cells expressed the B-1 B cell marker CD5 regardless of the group they belonged to. Overall, these results indicate that the number and phenotype of AF B cells is significantly affected by the presence of bacterial components in maternal circulation during pregnancy [35]. Figures, supplementary description and used gating strategies can be found in the cited publication.

4 Discussion

More than one in ten babies are born premature worldwide every year and approximately one million of them die every year due to complications of preterm birth. This makes prematurity the main cause of death in children under the age of five. And the numbers are still increasing [45]. Although medical advancement has improved the survival of premature children, it increased the prevalence of disabilities in this group [46]. The socioeconomic impact of preterm birth can only be estimated and cost the United states of America billions of dollars every year [47]. With more than 60 % of all preterm births, numbers are the highest in Africa and South Asia. But also, the United States rank 6th among the ten countries with the highest preterm birth rates worldwide [45].Overall, the risk for preterm birth is higher (12 %) in countries with lower incomes than in those with higher incomes (9 %). Also, within countries, low-income settings are associated with higher preterm birth rates. A huge inequality can also be seen with regard to survival rates. While in high-income countries extreme prematurity (before 28 weeks) results in a 10 % death-rate, in low-income countries more than 90 % of extremely premature babies die [45].

Preterm labor, which ultimately results in preterm birth, is a syndrome caused by different pathologic processes. One of them is inflammation and or infection within the fetal-maternal interface, which includes the amniotic fluid.

The amniotic fluid is the medium surrounding the embryo and fetus throughout pregnancy. It is providing room for growth and movement and represents an important line of protection. Not only physically but also in terms of antimicrobial and anti-inflammatory functions. This makes the AF an important piece of the puzzle that is the pathogenesis of preterm labor and birth. These antimicrobial and anti-inflammatory functions have been part of a more detailed investigation during the past years.

Having used amniotic fluid as a diagnostic medium in humans for decades, mostly in the field of genetics, it became apparent that it contains antimicrobial peptides [48] as well as pro- and anti-inflammatory cytokines [49]. Moreover it was shown by Ditadi *et al.* [50] that the human and murine amniotic fluid contains hematopoietic stem cells with multilineage potential, also able to give rise to T and B cells.

It was known that cells of the adaptive immune system, and thus B cells, are part of the human fetal-maternal-interface. They are present not only in the placental bed, in which they were first described, but also in the decidua basalis and the decidua parietalis [3]. Leng *et al.* even described the presence of B-1 B cells, which were increased in the decidual tissues

of women undergoing preterm birth, especially in combination with chorioamniotic inflammation [3]. However, data on cells of the adaptive immune system and especially B cells within the AF was still scarce.

Today, B cells are considered a fixed component of the human amniotic fluid [7],[8] and the fact that AF B cells are increased in pregnancies with intraamniotic infection and or inflammation strongly suggests an important role in the immune response to bacterial colonization of the amniotic cavity [8],[51]. A study by Frascoli *et al.* published in 2018 [52] even suggests a fetal response against microbial challenges within the amniotic cavity involving fetal lymphocytes. Analyzing cord blood of preterm infants, they were able to show that the environment of the fetus, characterized by inflammation during preterm labor, results in a fetal inflammatory response. They assume that this then leads to a cascade inducing an early activation of fetal dendritic cells and T cells and their priming against maternal antigens. This in turn results in the stimulation of uterine contractility, promoting preterm labor and eventually preterm birth.

In concordance with this, it was now confirmed, that the murine amniotic fluid contains B cells of different developmental stages whose numbers increased in the acute phase of LPS induced preterm birth [35]. However, while B cells in other tissues of the fetal-maternal-interface were of maternal origin, it needed to be determined whether these AF cells were the product of maternal blood cell contamination or of fetal origin, as well.

In humans B cell generation occurs *in situ* in the fetal omentum and liver as early as 8 weeks of gestation [53]. At 12 weeks of gestation B cells detected in fetal liver and spleen increasingly express the fetal B-lineage marker CD5 [54] [55] which led to the suggestion that B cells found in the human amniotic fluid during this period could also be of fetal origin. [8]. Indeed, a large part of AF B cells in a murine model of LPS triggered preterm birth also show CD5 expression [35]. Together with the fact that the majority of immune AF B cells express paternal MHCI antigen [35] this reinforces the assumption that AF B cells are indeed part of a fetal host response against microbial challenges within the amniotic cavity.

As mentioned above B cells were found in the murine AF at different stages of development [35]. One of the stages correspond to $CD19^{+}CD45R(B220)^{lo\text{-}neg}$ cells, defined as B-1 B cell progenitor by Montecino-Rodriguez *et al.* [44]. Following the definition of B cell developmental pathways set by Hardy *et al.,* an also detected $CD19^{+}CD45R(B220)^{+}$ phenotype could be interpreted as that of a Pro-B (Fraction B/C) cell subset. Low numbers of these cells express AA4.1/CD93 and CD43, surface markers that are present from the HSC stage but are not expressed by immature and mature B cells [16]. A higher number of cells expressed CD24

which is a marker expressed in high levels by Fraction C and Fraction D cells [16]. This reinforces the assumption that $CD19^+CD45R(B220)^+$ cells belong to Pro- or Pre- B cell subsets.

A detected IgM expression on $CD19^+CD45R(B220)^+$ cells [35], suggests the presence of another phenotype, which seems to correspond to B-1a or B-1b subsets as for example described in a review by Fettke *et al.* in 2014 [20]. The detection of IgM in the supernatants of the OP9 co-culture both with and without LPS-stimulation [35] strongly supports the presence of these subsets within the AF, since natural antibodies and especially IgM are mainly produced by B-1 cells [20]. This is further backed up by the proof of CD5 expression by the majority of $CD19^+CD45R(B220)^+$ cells in a murine model of LPS induced preterm birth [35]. In 1990, Masmoudi *et al.* already stated that antibody production as response to bacterial cell wall components is mainly effected by $CD5^+$ B-1 cells [56] [8], a B cell subtype, that had already been described in other tissues of the fetal-maternal-interface [3]. After Fettke *et al.* summarized that the B-1 B cell marker CD5 is expressed especially by B-1a cells [20], it seems very likely that this subset is present within the AF.

AF B cells are able to proliferate and maturate *in vitro*. After this had partially been described by Yoshimoto et. al. in 2011 [15], it could be confirmed after the *in vitro* stimulation with OP-9 stromal cells followed by an LPS stimulation led to an increase of $CD19^+CD45R(B220)^+$ cells. These cells also expressed AA4.1/CD93, HSA/CD24 and CD43 [35], and thus again resemble Pro- or Pre- B cell stages as defined by Richard et. al. [16].

After evidencing the AF B cells' capacity for proliferation and maturation, the cells' state of activation was addressed. CD69, a surface marker that Cibrián *et al.* described as early marker of cell activation in lymphocytes [57] and which is known to be induced by LPS exposure [22] showed a significantly increased expression by $CD19^+CD45R(B220)^+$ cells upon the above given co culture and stimulation [35]. Apart from its function as activation marker Cibrián *et al.* suggested that CD69 may even play a more functional role in the orchestration of immune responses, by influencing the shifting of activated lymphocytes as well as determining cytokine-release-patterns.

As early as 1997, in his review about the production of cytokines by human B cells, V. Pistoia described that normal human B cells as well as B cells in autoimmune disease, infection and disorders of lymphoproliferation are capable of producing large quantities of cytokines. He also described that this function is strictly linked to cell activation [58].

Harris et. al. [59] also described the production of cytokines as one of the various immune functions of B cells, which, based on different cytokine profiles allows the distinction of

“effector” (Be cells) and “regulatory” (Bregs) B cell subsets [60] [30]. Cytokines themselves are known to play a crucial role in pregnancy, its maintenance and success [29] [61]. An analysis of intra-cellular production of cytokines by AF B cells revealed a low production of pro- (TNF-α, IFN- γ, IL-17A) as well as anti-inflammatory (IL-10) cytokines but no significant alteration upon OP9-co-culture and an LPS stimulus [35]. Altogether this still strongly suggests that AF cells can undergo cell activation *in vitro* and thus potentially play a role in costimulatory and or signaling cascades within the regulation of immune responses. However, based on the current experimental results, it has not yet been possible to make a clear statement about their exact immunomodulatory capacities or certain cytokine profiles. Here, functional cell assays, focusing on the cells’ functional behavior rather than its phenotype could provide valuable information. More data is needed which is made more difficult not least by the examined medium itself.

The complicated harvest of AF in mice makes the sample collection in this animal model very challenging, resulting in relatively low cell numbers for FACS analysis and ELISA. Sonographic methods for the withdrawal of AF have been described for rats [62] and in bovine models. These methods seem advantageous in terms of animal use and would make it possible to analyze multiple samples from the same animal during the same pregnancy. However, the effort still seems to outweigh the use when it comes to the usage of mice. The analysis of human AF seems to be the next suitable approach, especially when it comes to addressing the clinical relevance in humans. Amniocentesis as a well-established method could provide larger sample volumes and directly offer human data. The use of this method on a grand scale however is complicated by the compliance with high ethical requirements. Carrying out amniocentesis for purely scientific reasons would presumably remain difficult for reasons of rare but possible serious complications and therefore always be tied to prenatal diagnostics. Added to this, with non-invasive prenatal tests (NIPT) on the rise, the numbers of women undergoing amniocentesis has clearly declined in Germany over the past years [63] which makes the acquisition of samples on a grand scale even more difficult. For this reason, an expansion of harvested cells is a requirement for the performance of meaningful experiments. In the cited publication this was approached with an established stromal-cell-co-culture [15]. However, changes or adjustments to experimental settings could improve the outcome. Montecino *et al.* described a transwell culture system in which especially B-1 B cells significantly expanded and differentiated [64]. In this system, B-1 B cell precursors were seeded with the addition of thymic stromal lymphopoietin (TSLP). This stromal-cell derived factor is known to sustain the differentiation and proliferation of B cell

progenitors expressing B220 [65] [66]. Apart from that, a transwell system avoids contamination of the cells of interest and wouldn't require any subsequent purification steps prior to phenotypic or functional analyses [64]. For human samples there also are commercial "enrichment kits" available.

Apart from enhancing the cell numbers, the phenotyping of B cells could be further improved for example by using more advanced flow cytometers, some of which are able to simultaneously detect up to 20 parameters. Also, technologies based on single cell sequencing seem very promising in terms of unbiased cell type analysis and the investigation of the cells` functional state [67],[68].

A suitable alternative to the used ELISA when dealing with low cell numbers would for example be an enzyme linked immune spot (ELISPOT). It represents an even more sensitive method for the detection of secreted cytokines or antibodies [69] and thus unfold its' strength especially in the area of low cell numbers. Technologies like immunoassays based on Luminex multi analyte profiling also produce results that are comparable to ELISA but with a multitude of detectable protein targets and a higher efficiency. These methods could therefore also represent a valuable and more precise alternative to FACS when approaching the cytokine production of AF B cells.

Although more in-depth analyses still seem necessary, the results shown in the cited publication are a timely addition to the current information on the immune cell composition of AF in healthy pregnancies as well as in preterm labor and birth. In particular, the consideration of the fetal inflammatory response and its impact on the mother's body, such as in the induction of labor [52], seems very promising for understanding the pathogenesis of inflammation induced preterm labor and birth. By continuing this research, biological markers could be identified, leading to new diagnostic and therapeutic approaches and thus helping to identify women at risk and treating those affected by this serious pregnancy complication.

5 References

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6 Publication

This cumulative dissertation consists of the following publication:

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

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REPRODUCTION

RESEARCH

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.* 2018*b*). 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.* 2018*b*). 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 &

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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.* 2018*a*).

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

AF $CD19^{+}$ 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

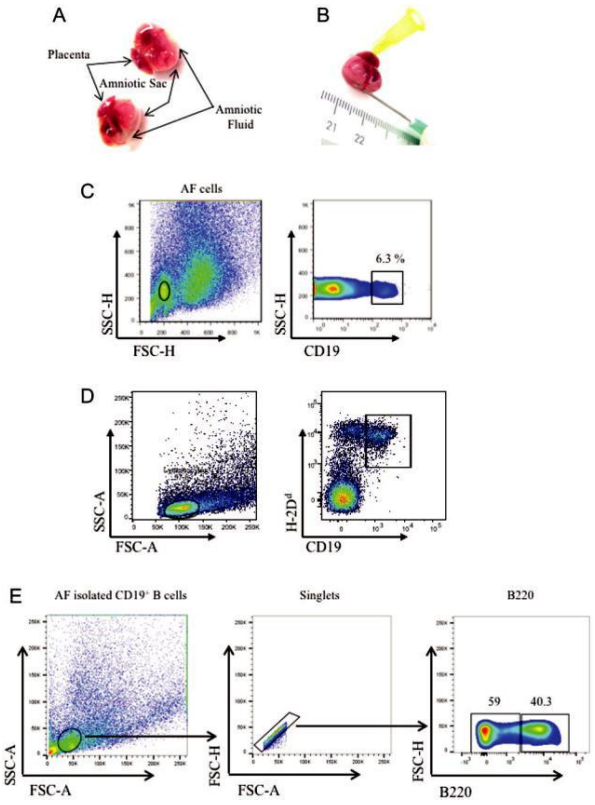

Figure 1 Isolation and characterization of AF cells. (A) Murine feto–maternal unit after removal from the maternal uterus, showing the placenta and the amniotic sac containing the AF. (B) Collection of the AF with a 0.6 × 25 mm syringe (green). (C) Gating strategy for the flow cytometric analysis of $CD19^{+}$ AF-B cells. (D) Expression analysis of paternal MHCI 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% CO_2). The medium was changed every second day. In same experiments, after 10 days in culture, cells were further stimulated with LPS (10 μg/ 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 1× + BSA 1% + 0.1 sodium azide) and incubated with Mouse BD Fc Block™ solution following manufacturer recommendations. Afterward, cells were resuspended in 100 μL 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 μL 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 (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

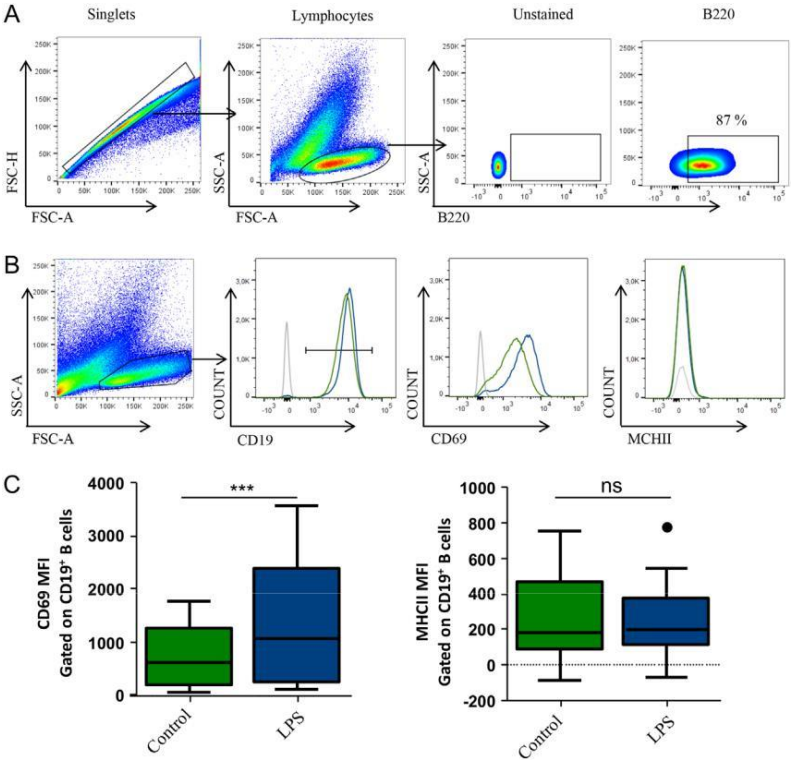

Figure 2 Phenotypic characterization of isolated AF CD19$^+$ B cells co-cultured with a bone marrow stromal cell line. (A) Magnetically isolated CD19$^+$ AF-B cells were co-cultured with a bone marrow stromal cell line (OP9) for 10 days and then stained with fluorochrome-conjugated antibodies against 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 μg/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 × SSC-A plot) and then doublets were excluded by gating on FSC-A × 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 ± 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 MHCI) in AF- $CD19^+$ cells. As shown in Fig. 1D, the vast majority of the AF-$CD19^+$ cells expressed paternal MHCI 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 ($CD19^+B220^{lo\text{-}neg}$; 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 MHCI.

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 maturate *in vitro*.

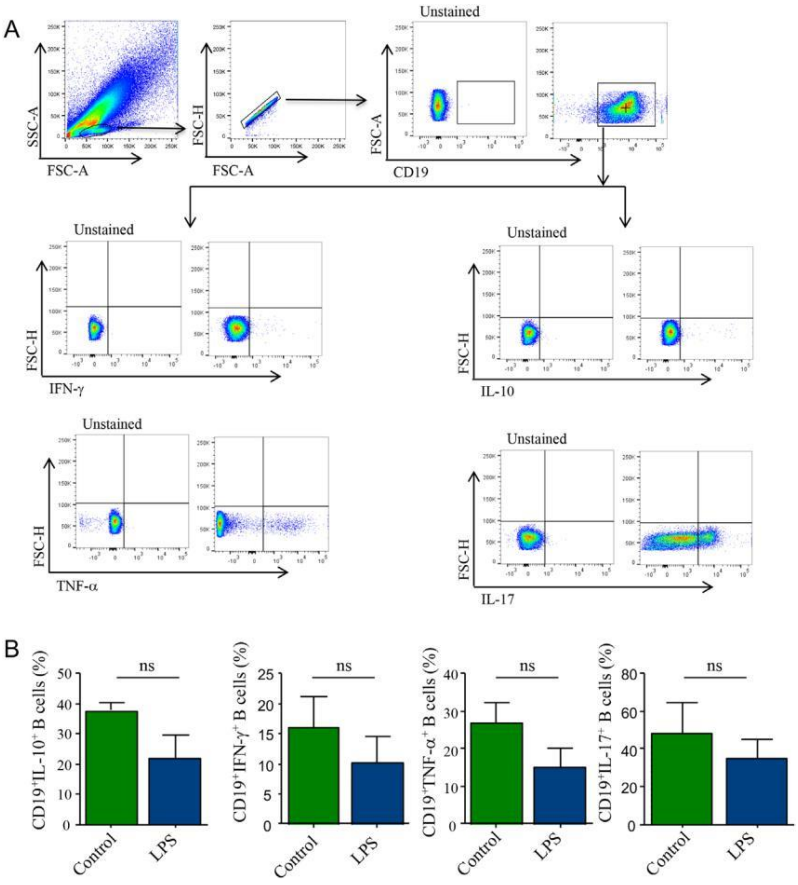

Figure 3 Amniotic fluid B cells produce pro as well as anti-inflammatory cytokines. (A) Representative pseudocolor plots showing gating strategy used to analyze intracellular cytokine production by AF-B cells. Pure isolated $CD19^+$ AF-B cells were co-cultured with OP9 stromal cells for 10 days and then 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.

Lipopolysaccharide (LPS) induces the activation of amniotic fluid B cells in vitro

Knowing that AF-B cells are capable to mature *in vitro*, we next analyzed whether these cells can also be activated *in vitro*. To do so, AF CD19$^+$ isolated B cells were co-cultured for 10 days with OP9 bone marrow stromal cells and then further stimulated with LPS for 48 h. As shown in Fig. 2C, we observed a significant increase in the expression of B cell activation marker CD69 in CD19$^+$ AF-B cells upon LPS stimulation (Fig. 2B and C). However, no differences were observed in the expression of MHCII in AF-CD19$^+$ B cells (Fig. 2C). We additionally analyzed intracellular production of pro-inflammatory TNF-α, IFNγ and IL-17A as well as anti-inflammatory IL-10 cytokines in AF-B cells upon LPS stimulation. No differences were observed related to cytokines production by AF-B cells upon LPS stimulation (Fig. 3). In summary, these results demonstrate that albeit AF-B cells can be activated with LPS *in vitro,* LPS does not significantly affect the production of IL-10, IFN-γ, TNF-α and IL-17A by AF-B cells in the conditions we tested.

Amniotic fluid B cells spontaneously produce IgM in vitro

One of the main features of the B cells compartment is the capacity to give raise to fully differentiated cells (plasma cells) capable to produce antibodies. To investigate whether AF-B cells can differentiate into antibody producing cells, we co-cultured pure isolated AF-CD19$^+$ B cells with OP9 bone marrow stromal cells for 10 days with or without the addition of LPS for the last 48 h. Afterward, levels of IgM were measured in the supernatants. Notably, unstimulated AF-B cells produced detectable quantities of IgM (Fig. 5). Lipopolysaccharide stimulation did not alter the production of IgM by AF-B cells as compared to non-stimulated cells (Fig. 4). In summary, these results illustrate the capacity of the AF-B cells to spontaneously differentiate into IgM producing cells, strongly suggesting that AF-B cells belong to the B1 B cell subset.

Maternal LPS challenge induces an increase in the numbers of amniotic fluid B cells in a mouse model of preterm birth

In an attempt to evaluate how AF-B cells behave in the context of preterm birth we took advantage of a well-established model of LPS-induced PTB. Briefly, pregnant females were systemically challenged (i.p.) with 10 μg of LPS on day 15 of pregnancy, a dose that induces 100% of preterm births (Kaga *et al.* 1996, Huang *et al.* 2017, unpublished data from our laboratory) and were killed 5 h later. As control, pregnant females were injected with sterile PBS (vehicle). We observed that LPS induced a significant increase in the percentages as well as total numbers of CD19$^+$ AF-B cells compared to PBS-treated mice (Fig. 5). Moreover, percentages of B220 expressing CD19$^+$ AF-B cells were significantly increased upon LPS challenge as compared to PBS-treated control mice (Fig. 5). Interestingly, the vast majority of AF-B cells in both groups expressed the B1 B cell marker, CD5.

Overall, the results presented here indicate that the presence of bacterial components in maternal circulation during pregnancy affects the numbers and phenotype of AF-B cells.

Discussion

In this work, we demonstrated that murine amniotic fluid contains B cells in different stages of development which express paternal MHCI molecule, suggesting an embryonic origin. Furthermore, we were able to show that AF-B cells can continue their development *in vitro*, become mature and produce pro- as well as anti-inflammatory cytokines and more importantly spontaneously release IgM.

Based on phenotype, localization and function, B cells can be divided into B1 and B2 subpopulations (Hardy & Hayakawa 2001). Unlike B1 B cells, which are originated almost exclusively during the embryonic life, B2 B cells are continuously produced throughout

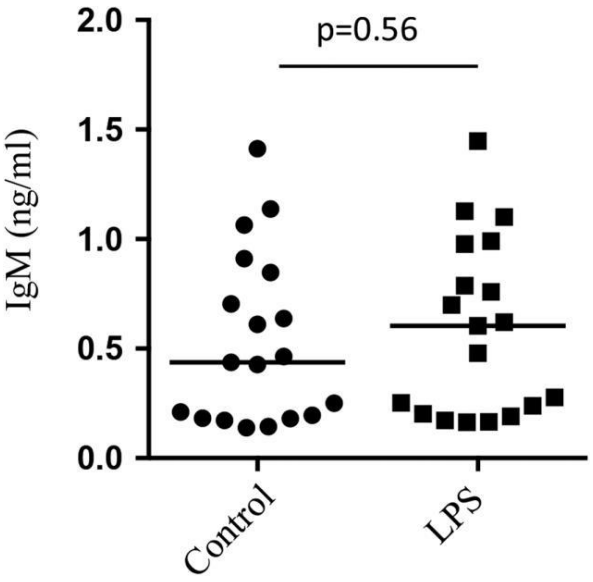

Figure 4 Amniotic fluid B cells spontaneously produce IgM. Pure magnetically isolated CD19$^+$ AF-B cells were co-cultured with OP9 stromal cells for 10 days and then further stimulated with LPS (10 μg/ mL) for 48 h. Levels of IgM were quantified in supernatants by ELISA. Data are shown as single dot, whereas means are indicated. No statistically significant differences were observed among the groups as analyzed by Mann–Whitney test.

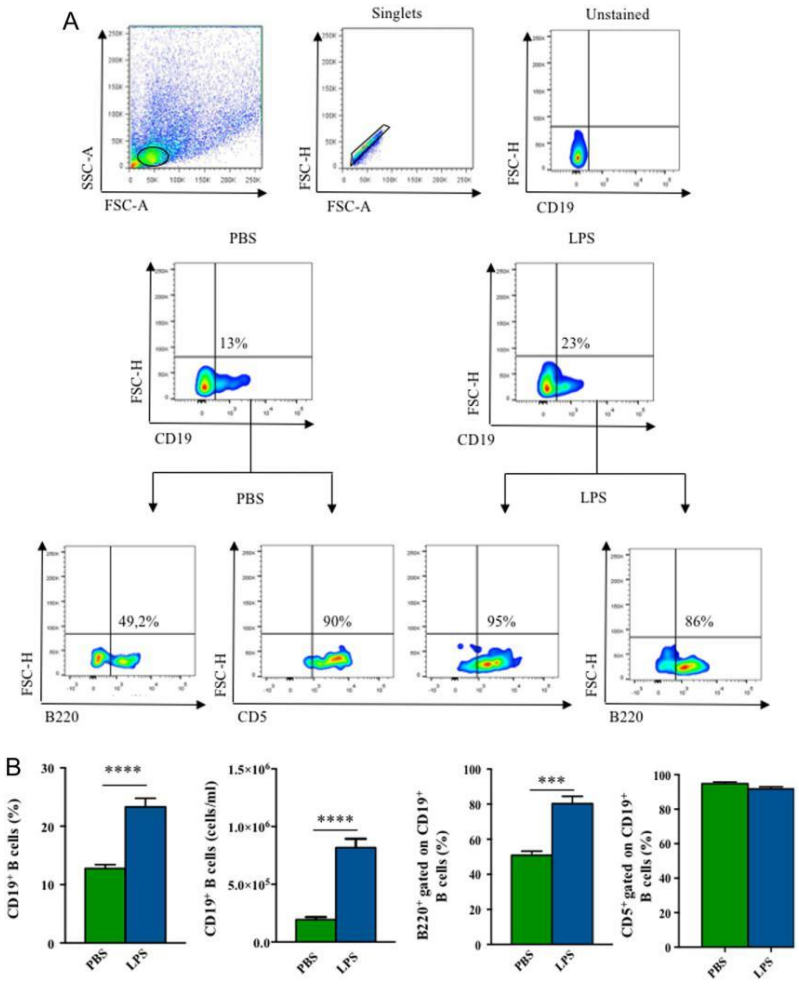

Figure 5 B cells are augmented in the amniotic fluid during acute phase of LPS-induced PTB. Pregnant mice were injected (i.p.) with LPS (10 μg/mice) on day 16 of pregnancy and killed 5 h later. (A) Representative pseudocolor plots showing gating strategy used to analyze different B cell subpopulations 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.* 2018*b*). 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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