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Adaptation mechanisms within the B cell composition for successful human and murine pregnancies

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T A B L E OF C O N T E N T S

S U M M A R Y

Introduction

A well-balanced immune maternal status is essential for favourable outcome of pregnancy. Due to their complexities, not all immune adaptations that promote tolerance during pregnancy are known. To understand the adaptation of the B cell compartment, we analysed and compared B cell lymphopoiesis in different lymphoid tissues in a number of murine models.

Furthermore, we focused on the humoral immune response during pregnancy. We analysed immunoglobulin profiles in human subjects and mice during pregnancy.

These cellular alterations are subject to the influence of chemokines, among others. Therefore, we assessed serum levels of B cell activation factor to clarify its effects during pregnancy.

Methods

For analysis of the human peripheral B cell compartment, peripheral blood samples from age-matched non-pregnant and pregnant women without pregnancy complications, immunological disease or acute/chronic inflammation were collected and sub-classified into four different groups: non-pregnant, and first, second, or third trimester of pregnancy. The experiments, based on a mouse model, were performed with 8-week-old female mice: clinically healthy non-pregnant (CBA/J ($H2^k$)), pregnant mice with normal gestation (BALB/c ($H2^d$) x CBA/J ($H2^k$)), and mice with pregnancy loss (DBA/2J ($H2^d$) × CBA/J ($H2^k$)). Subsequently, peripheral blood mononuclear cells from blood and lymphatic organs were isolated following standard protocols. The B cell analysis was performed by flow cytometry. The immunoglobulin serum levels of the human and murine subgroups were quantitated using Bio-Plex isotyping assay and analysed by a Bio-Plex reader. To quantify B cell activating factor (BAFF) in serum of pregnant and non-pregnant mice a BAFF enzyme-linked immunosorbent assay was used. The concentrations were determined by using a FLUOstar OPTIMA microplate reader. All statistical analyses were performed using the Kruskal–Wallis test with Dunn's post-test in GraphPad Prism software. P values of < 0.05 were considered statistically significant.

Results

We were able to demonstrate B cell lymphopenia in mice bone marrow downstream of pre-pro B cells, irrespective of pregnancy outcome. The mature bone marrow B cells did not show this adjustment mechanism during normal gestation.

Closer inspection of the splenic tissue revealed expansion and activation of marginal zone B cells in mice with a normal pregnancy. However, this was not observed in mice suffering from pregnancy disturbances. Natural antibodies secreted from marginal zone B cells were also present at higher concentrations in serum of pregnant mice, compared to non-pregnant animals.

We also found significantly higher levels of natural antibodies in serum of pregnant women compared to non-pregnant age-matched controls. Analysis showed significantly lower levels of BAFF in mice with normal pregnancy as compared to non-pregnant mice.

Conclusions

We are able to show mechanisms within the B cell compartment as well as the change within the natural antibodies that might be crucial for successful pregnancy in both humans and mice. Furthermore, BAFF seems to play a central role as a mediator of peripheral B cell compartment and B cell lymphopoiesis in the bone marrow for successful pregnancy.

F I G U R E S

1 Introduction

1.1 B cell development in humans and mice

In the 1890s, von Behring and Shibasaburo mentioned for the first time the presence of protective antibodies in the blood stream after exogenous antigen exposure.[1–3] This indirect proof of the antibody-producing cells was confirmed sixty-six years later by their identification in chickens by Glick and Chang.[1,4,5] Further basic research revealed that the B cells originated in the bursa fabricii lymphoid organ of young birds. This cell type was subsequently referred to as B cells.[1,4,6]

In humans, B cell development occurs in bursa-equivalent bone marrow based on self-renewing hematopoietic stem cells (HSCs).[1,7] HSCs are primarily expressed in the foetal liver and settle within the red marrow during the foetal period.[8–12] There, HSCs differentiate into common myeloid progenitors (CMP) or lymphoid progenitors (CLP), from which all blood cells arise. CLPs can differentiate into T-, B- or Natural Killer cells.[6] They pass through three development stages of B cell maturation in which the functional groups of the immunoglobulin gene segments are rearranged to encode the heavy and light chains.[8] In the first stage the D and J segment of the H-chain rearranges in CLPs, which then become early pro-B cells. This is followed by D-to-J-joining in late pro-B cells.[8] After the positive selection of pre-antigen receptor expressing cells,[13] the next development stage contains pre-B cells with characteristic κ, λ and μ locus rearrangements of the L-chain.[14,15] These pre-B cells undergo a second auto-reactivity checkpoint. Remaining pre-B cells, presenting IgM on the cell surface, are termed immature B cells as one of the final stages of maturation that occurs in bone marrow.[8] Before migrating from bone marrow into circulation, immature B cells with low-avidity IgM antibodies are positively selected to prevent auto-reactivity (Figure 1).[15,16] Thereupon, immature B cells, already flushed out of the red bone marrow, circulate into the blood vessels and settle in the spleen, lymph nodes as well as tonsils and associated lymphoid tissues where they undergo further maturation.[6,8]

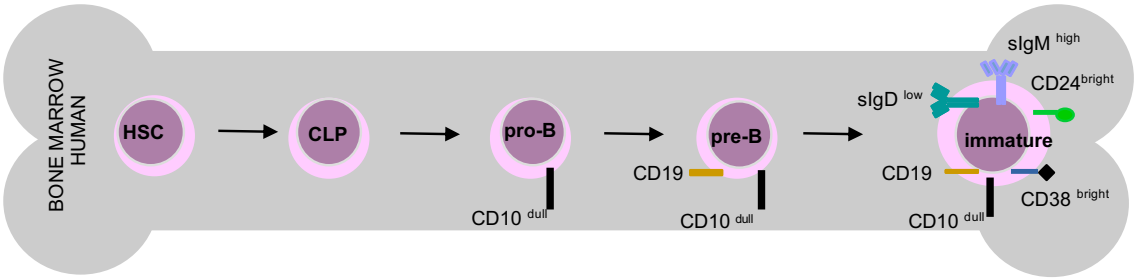

Figure 1: Model of B cell development steps in the bone marrow of humans.

From hematopoietic stem cells to pro-B cells and pre-B cells to immature B cells. The different cell maturation depends on the respective expression patterns of the surface molecules. When pro-B cells begin to express CD19, they are called pre-B cells. The additional expression of sIgM^{high}, CD24^{bright} and CD38^{bright} cells have the ability to leave the bone marrow as immature B cells. The schematic representation is based on the literature and illustrations, cited in this work.

In mice, pro- and pre-B cells also originate from HSCs.[17] In contrast to humans, the transition from pro-B cells to pre-B cells requires the loss of sialophorin (CD43).[18,19] In mice, both precursors are characterised by low expression of B220 in the absence of IgM on their cellular membrane (Figure 2).[19,20] Once IgM expression has occurred, they are also able to leave the bone marrow as immature B cells and enter the spleen for further maturation.[8,19]

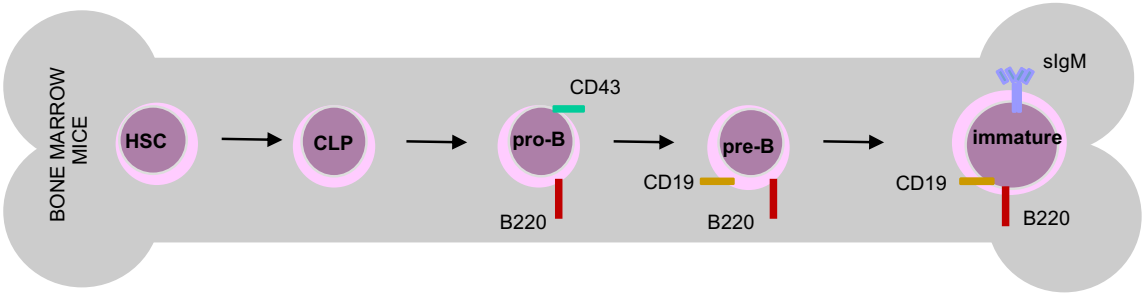

Figure 2: Model of B cell development in the bone marrow of mice.

Similarly to humans, B cell precursors in mice arise from the HSC. Pro-B cells express CD43 as early progenitor cells. If sialophorin CD43 is no longer presented on the cell membrane, these are termed pre-B cells. The additional expression of sIgM qualifies them as immature B cells. The schematic representation is based on the literature and illustrations, cited in this work.

Splenic immature B cells undergoing maturation progress through a transitional B cell stage.[21] These transitional B cells mature either in the marginal zone into marginal zone B cells (MZ) or into follicular B cells (FO) in the germinal centre (GC).[6,8] FO B cells usually differentiate to plasmablasts and short-lived plasma cells as well as the MZ B cells, whereas FO cells are also capable of providing memory (that remember the pathogens from previous antigen contact for more rapid production of antibodies against future infections) as well as long-lived plasma cells.[6,8,22] FO and long-lived plasma cells do not remain in the spleen but instead recirculate in the blood to the peripheral lymph organs and bone marrow. Within the human blood stream three main peripheral B cell populations can be identified: transitional ($CD19^{pos}CD24^{high}CD38^{high}$), memory ($CD19^{pos}CD24^{high}CD38^{neg}$) and mature ($CD19^{pos}CD24^{low}CD38^{high}$) B cells.[21] In addition, naïve ($CD19^{pos}CD27^{neg}IgD^{pos}$) and non-switched memory ($CD19^{pos}CD27^{pos}IgD^{pos}$), as well as switched ($CD19^{pos}CD27^{pos}IgD^{dull}$) B cells can be recognised by IgD in combination with CD27 expression (Figure 3).[8,23]

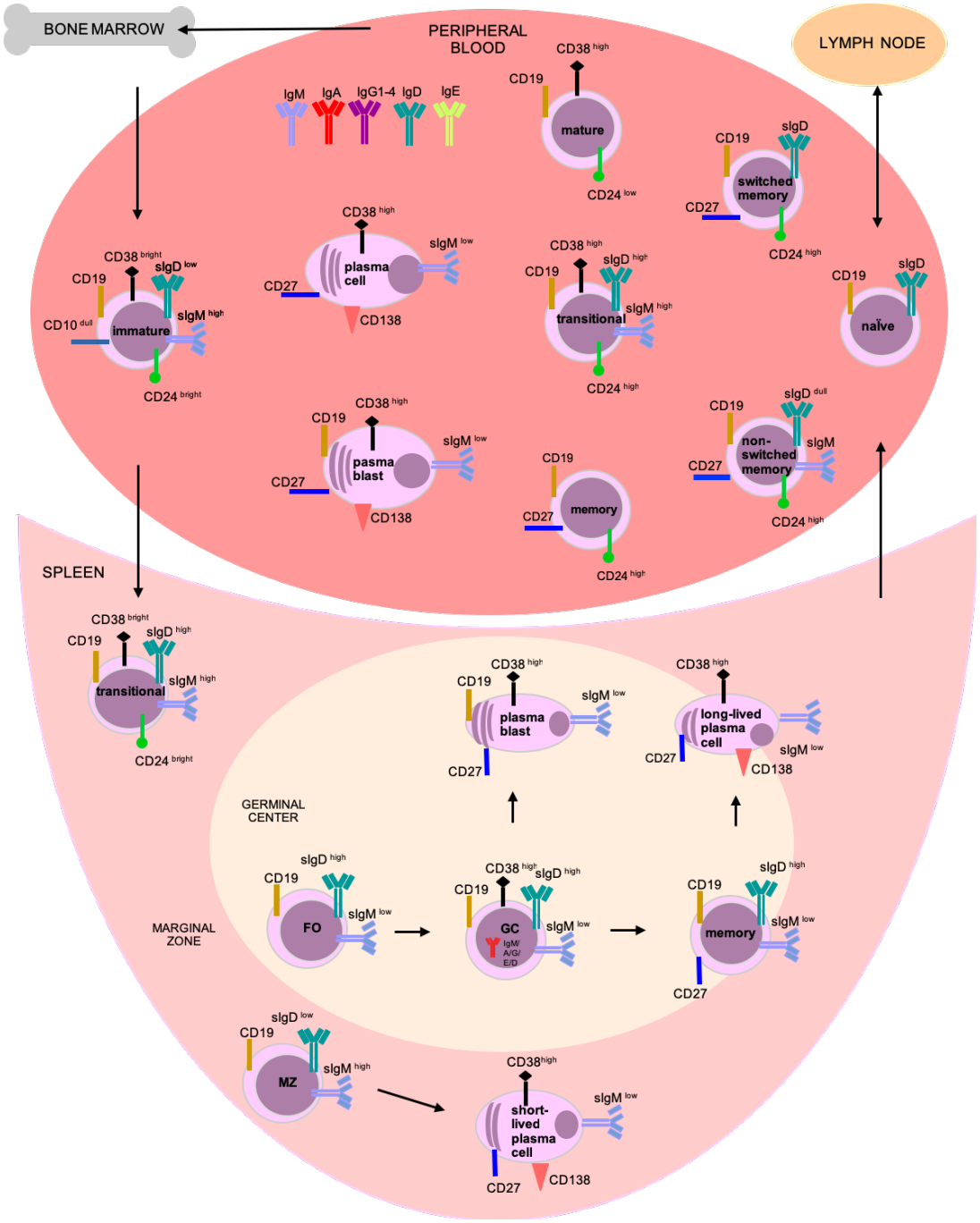

Figure 3: Schematic overview of B cell maturation in different lymphoid tissues and peripheral blood.

Immature B cells are flushed from the bone marrow into the bloodstream to mature in the different lymphoid organs. Within the spleen, immature B cells go through different stages of development in different regions. In the marginal zone they mature to MZ B cells; in the germinal centre FO B cells are formed, which in turn are connected to the peripheral bloodstream. The schematic representation is based on the literature and illustrations, cited in this work.

1.2 Modification of B cell lymphopoiesis during pregnancy in humans and mice

B cell lymphopoiesis—including B cell retention, daily output into blood stream and maturation—is regulated by various factors. Several proliferation factors (e.g. interleukin-7) and adhesion molecules (VLA-4, VCAM-1) are known to affect B cell lymphopoiesis.[24,25] To date, it is unclear to what extent B cell lymphopoiesis undergoes changes during pregnancy. The first hints came from research conducted in the late 1990s and early 2000s.[24] Those studies described changes in the lymphatic tissue as thymus involution during pregnancy. Subsequently, Kay's group reported a selective decrease in B cell lymphopoiesis in mice at day 6.5 after mating, but surprisingly not within the first 6.5 days.[24] There is also a constant production of pro-B cells during early stages of development, unlike the findings for large and small pre-B cells.[24] Similarly, red blood and granulocyte-macrophage progenitors maintain a stable number of nucleated cells in bone marrow and are unaffected by these changes.[24] Oestrogen was proposed as a regulator of murine B cell lymphopoiesis. High oestrogen levels suppress stromal cells in bone marrow through interleukin-7, whereas low interleukin-7 level stimulates lymphopoiesis.[24] In humans, this proposed mechanism was ultimately refuted.[8] Nevertheless, this observed suppression of B cell lymphopoiesis might represent an evolutionarily acquired mechanism to reduce the occurrence of autoreactive B cells, that may recognise foetal structures causing pregnancy failures.[24,26,27]

A closer look at the B cell subsets in mouse bone marrow indicates that newly formed immature B cells are the most affected bone marrow-B cell population during pregnancy. They were reported to decline by about sixty percent, whereas immature splenic cells that recently immigrated from the bone marrow declined by about forty three percent compared with baseline levels in non-pregnant mice.[24] Meanwhile, several adaptation mechanisms that could be responsible for these changes in the B cell compartment have been discussed. It is possible that the mitotic activity could be reduced and a lower proliferation rate may seem to be relevant in combination with differentiation of early progenitor cells. In addition, a more rapid transfer of matured bone marrow B cells into the blood circulation is still under discussion.[24] Furthermore, Muzzio *et al*. recently reported the reduction of pro/pre and immature B cells in the bone marrow of pregnant mice, presumably through the indirect oestradiol effect. However, the authors also demonstrated that, unlike immature B cells, mature B cells are augmented in bone marrow of pregnant mice.[27]

In summary, the findings to date show that an accelerated maturation process occurs in the bone marrow, with simultaneously reduced B cell lymphopoiesis and influx into the peripheral blood.

Consequently, a larger number of matured B cells were found in the peritoneal cavity as well as in the uterine-drained lymph nodes. The latter are in close proximity to the semi-allogeneic foetuses.[27]

1.3 Alteration of peripheral B cell subsets during pregnancy in humans and mice

In addition to bone marrow B cells, splenic B cells also undergo changes during pregnancy. These changes have different effects on the various B cell subtypes. The following two B cell subgroups, which differ in phenotype, development, localisation and function, are subject to changes during pregnancy and play a crucial role in successful pregnancy: B1 cells including their B1-a and B1-b subpopulations, as well as B2 cells.[1,27–29]

B2 cells develop continuously during postnatal life in red bone marrow and subsequently migrate to the periphery for further maturation in MZ and FO cells.[1] In more detail, naïve B cells leave the bloodstream and migrate into the lymphatic tissues.[6] There, chemokines are produced within the B cell zones to attract B cell lymphoid follicles.[6] The follicles contain a germinal centre surrounded by follicular dendritic cells.[16] In this region, antigen-presenting cells are exposed to foreign antigens to be subsequently activated.[1] Activated B cells present the epitopes to the follicular T helper cells,[30] which in turn trigger an essential B cell co-stimulation.[30] As a result, proliferation and immunoglobulin class-switching are further influenced. The so-called large centroblasts form the dark zone of the germinal centre, in which clones with high-affinity antibodies are selected.[30] In the dark zone, trapped centroblasts convert to centrocytes to express antibodies on their cell surface. After a secondary affinity selection for high-affinity surface antibodies they leave the GC.[15,16] Subsequent B cell differentiation branches off to: plasmablasts and their short-lived high-volume antibody-producing plasma cells for direct immune reaction; and to memory cells and their long-lived plasma cells for persistent protection.

Those cells of the germinal centre represent the majority of spleen B cells and are subject to changes during gestation. On day 14 of pregnancy, the maximum spleen size is reached compared to the size observed in non-pregnant control mice.[27] The mature splenic $B220^{pos}$ B cell count drop included the FO B cells. From the 18th day postpartum the number of $B220^{pos}$ splenic B cells increases again concurrent with that of FO B cells.[27] At this point, the spleen parenchyma has almost returned to its original size. Within this negative correlation, between increased splenic size and decrease of total B cells within the first 14 days of pregnancy, FO and immature B cells in particular were affected.[27] Meanwhile, MZ B cells remain at a stable level. Much more crucial is the MZ/FO ratio. It shows the preponderance of MZ B cells within

the 5th to 7th days of pregnancy, which may allow short-term protection of an immunocompromised expectant mother within the first 5 to 7 days due to a delayed T cell-dependent immune response.[27,31]

Interestingly, the follicular B cell population also changes during pregnancy: Muzzio observed a marginal increase in B cell number at the end of a murine pregnancy, whereas decreases may also be observed in B cell influx and FO cell number. The physiological location of MZ B cells is the marginal zone. This highly organised zone forms a cell network with a variety of cell types, including macrophages, dendritic cells, reticular cells and sinus-lining cells.[29,31–33] Remarkably, the microvascular MZ circulatory system of humans and mice also differ in their histological structure. In both species, antigens are flushed by the bloodstream along the trabecular artery and into the central arterioles. In humans, the blood then passes from the central arteriole through smaller arterioles into an open capillary system of the red pulp and perifollicular zone[29] and recirculates back into the peripheral bloodstream.[31,32,34] The arterioles themselves are surrounded by a cuff of T lymphocytes and antigen-presenting cells, called the periarteriolar lymphoid sheaths (PALS), but less frequently in humans than in mice. In contrast to rodents, both the marginal sinus and specific cells of the mononuclear phagocytic system are absent in humans. In mice the central arterioles, which are covered by PALS, branch into follicular arterioles and drain the blood into the marginal sinus.[6,29,31,34] Both native antigens and macrophages capture antigens through the fenestrated vessels. In rodents, MZ-B cells produce 24 to 72 hours of blood-borne antigenic exposure through macrophages T cell-independent low-affinity antibodies, particularly IgM and IgA. Therefore, MZ B cells undergo rapid transformation in plasmablasts, and then settle in the red pulp.[31,35–37] Until now, this rapid activation mechanism has not been completely understood. In humans the macrophages and metallophilic macrophages are missing.[34,38] Instead, littoral cells, DCs, neutrophils and sinus lining cells are discussed as gap fillers via BAFF stimulation.[31,39,40]

B1 cells differ from B2 cells and are part of the innate-like immune response. They are formed only during the foetal and perinatal phases in liver[22], with subsequent self-renewal in the periphery.[1,41,42] While human B1 cells mainly occur in peripheral blood, murine B1 cells are found in the spleen, pleural and peritoneal cavity.[1,22,43,44] Despite being localised differently, these cells perform the same antibody-producing functions as part of a broad defence against infection. B1-a cells produce natural antibodies[1,45,46] that display lower affinity and are polyreactive.[45,47] Antibodies produced from B1-a cells are associated with birth difficulties, high pregnancy morbidity and recurrent loss of pregnancy.[1,48] They are also associated with

autoimmune diseases, including systemic lupus disease, rheumatism and antiphospholipid syndrome.[49–52] Unlike B1-a cells, B1-b cells produce lower levels of natural antibodies after antigenic staging.[1]

1.4 Marginal zone B cells and production of natural protective antibodies

Maternal humoral immunity of the acquired immune system must be adequately adjusted to prevent possible pregnancy complications. Beside the tolerance of foetal semi-allogeneic cells, the host immune processes must still provide adequate protection against infection.[53] In this reciprocal state, the natural protective antibodies (NAbs) represent the first line of defence against infection.[54,55] They are produced mainly by B1 cells and a minor portion of MZ (B2), in the absence of antigenic stimulation.[54,56–58] NAbs are characterised by their low affinity and polyreactivity.[59,60] This allows them to bind to the most diverse of their own and foreign structures, such as proteins, apoptotic cells, beta-amyloid, DNA, carbohydrate chains, lipids, bacterial molecules and virus structures.[54,59,61–64] In contrast to the classical antigen-binding pocket, the polyreactive antibody displays greater flexibility and can bind with different antigens. The reason for this is likely the germline configuration.[59,65]

Apart from protecting the foetus against various infections, the NAbs also have further essential functions in the immune system. This includes: vascular homeostasis, in which they are able to suppress allergic reactions,[66,67] protection from cancer,[68,69] and regulation of B cell response[70] and development.[54] However, the ways in which they change during pregnancy to allow a successful outcome are yet to be explored.

1.5 B cell activating factor

The B cell activating factor (BAFF) is a key protein influencing changes within the B cell subsets.[71] It stimulates B cell proliferation and differentiation and affects apoptosis. [72] BAFF is produced by B cells, trophoblasts and decidua cells.[73] It has differing binding affinity to three different receptors: BAFF receptor (BAFF-R), transmembrane activator and calcium modulator cyclophilin ligand-interactor (TACI), and B cell maturation antigen (BCMA) which is predominantly expressed on B cells.[8,72–75] Down-stream intracellular signalling pathways are unfortunately not yet fully understood. It was proposed that in bone marrow BAFF-R ligand system is expressed on immature B cells as well as on splenic MZ and FO cells. In contrast, long-lived plasma cells in the bone marrow express exclusively BCMA.[8]

During clinically unremarkable ongoing murine pregnancy, on the 7th day a slight increase in BAFF level can be detected. Seven days later a significantly lower BAFF concentration is

reached, which ultimately lasts until the 18th day before reverting to baseline levels.[27] This adjustment mechanism could be responsible for the suppression of autoreactive B cells. This supposition is supported by studies in which non-pregnant transgenic mice overexpressed BAFF. Their increased BAFF concentrations were found to strengthen B cell maturation and induce autoreactivity.[8,76]

The literature still lacks detailed investigation of the BAFF serum level in a recognised pregnant animal model suffering from immune-mediated pregnancy disturbance.

In humans, comparison of BAFF values between clinically normal pregnancies, recurrent spontaneous pregnancy loss and non-pregnant women has not been reported to date due to the lack of data from clinical studies.

The aim of this work was: Firstly, to describe cell types in the B cell compartment that are essential for successful pregnancy in humans and mice. Secondly, to characterise functional aspects of the B cells during gestation by analysing immunoglobulin production. Thirdly, to test the role of BAFF in successful pregnancy.

2 Material and Methods

2.1 Humane blood samples

The study "Characterization of the B cell compartment in peripheral blood and amniotic fluid" was approved by the Ethics Committee of the Medical Faculty, Greifswald University (BB 126/13 to FJ). All individuals gave their written consent and were fully informed of the purpose of the research before sampling.

Human anti-coagulated peripheral blood samples from non-pregnant and pregnant female volunteers, both of reproductive age and independent from their menstrual cycle, were obtained at the Department of Obstetrics and Gynecology, Greifswald University. All women lacked diagnosed immunological disease or acute or chronic inflammation at the time blood was collected. We sub-classified the blood samples into four different groups: non-pregnant (np), first trimester (1^{st}), second trimester (2^{nd}) and third trimester (3^{rd}) of pregnancy. All blood samples were processed directly after collection and peripheral blood mononuclear cells (PBMC) were isolated. Sera separation was performed by 10 minutes centrifugation at 1300 × *g* and 20 °C. Finally, sera were stored at -80 °C until analysed.

2.2 Mouse models

The inbred eight-week-old female mouse strain CBA/J ($H2^k$)[77], as well as DBA/2J ($H2^d$)[78] and BALB/c ($H2^d$)[79] males were bred and purchased from Charles River (France). The mice were kept in a 12-hour light/12-hour dark cycle at the facilities of BioTechnikum Greifswald. Water and chow were provided ad libitum. Animal experiments were carried out according to institutional guidelines as per ministerial approval (institutional review board: Landesverwaltungsamt Sachsen-Anhalt [ID: FJ2-1019 to FJ], Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei Mecklenburg-Vorpommern [7221.3-1-068/13 to F.J.]). The experiments were also carried out in compliance with the European Communities Council Directive 86/609/EEC.

For copulation, female virgin mice CBA/J were mated at 8–10 weeks of age either with BALB/c or DBA/2J males. Mated females were inspected twice daily for vaginal plugs, with the day of detection designated as day zero of pregnancy. When the vaginal plug was visible, the mating was ended by separating the animals. At day 14 of pregnancy they were scarified under ketamine/xylazine anaesthesia followed by cervical dislocation. Non-pregnant CBA/J age-matched female mice were used as controls.

Almost all of the blood volume was taken, the tibia and femur with their bone marrow as a blood-forming organ, the uterine-draining lymph nodes and retroperitoneal fluid and the spleen as a lymphatic organ.

2.2.1 DBA/2J ($H2^d$) males mated with CBA/J ($H2^k$) females

DBA is the oldest mouse strain developed by Clarence Cook Little in 1909. DBA is characterised by a homozygous allele for retinal degeneration *Pde6b*^{rd1}, which causes blindness by wean age.[78] The DBA strain is typically used for studies concerning tubule-interstitial lesions, atherosclerosis and exocrine pancreatic insufficiency syndrome, alcohol and morphine intolerance.[78] In 1920, Strong crossed a DBA male and a Bagg albino female to breed the CBA strain. CBA/J mice exhibit higher risk of cardiovascular disease, atherosclerosis and high plasma levels of triglycerides and cholesterol.[77] Meanwhile, the copulation of CBA/J females and DBA/2J males is a well-accepted mouse model of early pregnancy loss, with loss rates of the order of 20 % to 30 % reported.[80–85] Allo-immunisation between both strains causes immune-modulatory effects on the immune system[84], and immune-mediated pregnancy disturbances[86,87] through angiogenic deregulation as well as abnormal placental development and foetal growth restriction[88] are discussed.[89]

In this study, we used these biological properties to generate pregnant mice suffering pregnancy failures. We expected a median miscarriage rate of 16.7 %, (± SD) similar to reports in the literature.[85]

2.2.2 BALB/c ($H2^d$) males mated with CBA/J ($H2^k$) females

CBA/J females were mated with BALB/c males to produce a control group with clinically unremarkable pregnancy. BALB/c mice are predisposed to various forms of cancer in later life because of their systematic inbreeding.[79] However, in relation to the present study, CBA/J × BALB/c copulation promises normal pregnancy because they have the same major histocompatibility complex gene cluster H2. As described in the literature,[90] a median miscarriage rate of 0 % is expected, which was also the median miscarriage rate obtained in these studies. However, some mice displayed a few cases of embryo resorption, apparently mediated by vivarium environmental influences[81,91] such as individually insufficient adjustment period after shipping, uncomfortable bedding and notional ambient noise.[92]

2.3 Flow cytometry

Flow cytometry is a biophysical method of quantitative cell counting in which cells are identified by their surface antigens and intracellular molecule expression, also referred to as fluorescence-activated cell sorting (FACS).[93] The samples were prepared to detect extra- and intracellular molecules of B cells. Fluorochrome-tagged high-specific antibody staining was used to analyse isolated samples from human peripheral blood mononuclear cells (PBMCs), mice bone marrow (BM), spleen, peripheral lymph node (PLN) and blood cells.

Flow cytometry tubes carried extra- and/or intracellular antibody-coupled cells in suspension into the flow chamber. Inside the chamber a vacuum system was used to propel the sample as a liquid stream between the lasers and photodetectors.[94] The suspended cells passed individually through two laser beams [blue (488 nm, air-cooled, 20 mW solid-state) and red (635 nm, 17 mQ HeNe)]. The cells reflected and refracted the light depending on cell size, granularity and the presence of fluorescent molecules in the form of either antibodies or dyes.[95] Light scattering was detected by an optical sensor. An analogue-to-digital converter (ADC) then converted the sensor signals into digital form and exported them to a computer for further analysis (Figure 4A).[96]

The side scatter (SSC) detected at 90° from the incident laser beam correlates with the granularity of the cells, while the forward scatter (FSC) gives information about the volume of the cells (Figure 4B).[95] These cell characteristics were used for differentiation of cell types in

heterogeneous cell populations (PBMCs): i.e. lymphocytes, monocytes and neutrophils. Fluorochrome-linked antibodies bound to targets either on the surface or inside the cells have a fluorochrome-dependent peak of excitation and emission wavelength. The emission was detected by the sensor and allowed identification of the specific membrane and intracellular molecules. The data were analysed using FACSDiva software. Further B cell analysis was carried out using FlowJo and GraphPad.

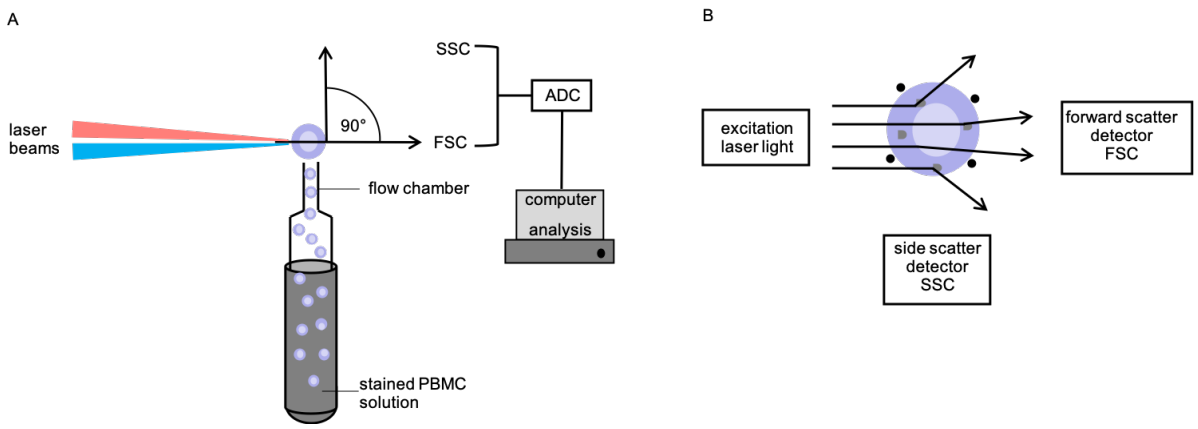

Figure 4: Schematic illustration of fluorescence-activated cell sorting.

Showing the stained cell solution, flow chamber and laser beams (A). Cell granularity as well as cell size determine forward and side light-scattering (B). The ADC digitises the analogue light signal. The schematic representation is based on the literature and illustrations, cited in this work.

2.4 Bio-Plex isotyping assay in humans and mice

Bio-Plex bead-based assays enable simultaneous quantitative determination of immunoglobulin isotypes $IgG_{1\text{-}4}$, IgA, IgM and IgE in a single reaction. The assay was carried out in a 96-well plate format with human serum. Fluorescently dyed microspheres, called beads, are coated with antibodies. These were incubated in 5 µl thawed serum samples (60 minutes, 20 °C, 850 rpm) during which the antibodies bound to the immunoglobulin of interest. Unbound antibodies were washed out then a sandwich complex of the captured antibodies and immunoglobulin of interest was created by adding biotinylated detection antibodies (30 minutes, 20 °C, 850 rpm). After washing, the addition of phycoerythrin-conjugated streptavidin enabled binding to detection antibodies. Final detection of the complex was performed by a Bio-Plex reader, based on the principles of flow cytometry. The multiplex assay suspension was passed through the detection

chamber. The red laser (635 nm) excited the bead dyes to provide bead classification, while in the meantime a green laser (534 nm) illuminated the phycoerythrin fluorescent reporters. Acquired data were analysed using GraphPad and outliers were removed. All serum samples used in this method were prepared at 1300 × *g* for 10 minutes at 20 °C and frozen at -80 °C.

To detect the outliers we used an online tool that calculates the interquartile range (IQR), defined as the difference between the 75^{th} and 25^{th} percentiles of our data set. The numbers of the data set that were called outliers, are numbers that lies below or above 1.5×IQR of the first or the third quartile.

Analogous to the analysis of human samples, murine serum levels of IgM, IgG1, IgG2a, IgG2b, IgG3, IgE and IgA were assessed using ProcartaPlex Mouse Antibody Isotyping Panel (eBioscience/Affymetrix) and subsequently analysed using Luminex test equipment.

2.5 BAFF ELISA

This enzyme-linked immunosorbent assay (ELISA) was performed using Abcams's mouse BAFF ELISA kit to measure the quantity of BAFF in serum of pregnant and non-pregnant mice. Microwell plates coated with polyclonal anti-mice BAFF antibodies were incubated with BAFF, present in the serum samples of pregnant and non-pregnant animals. To prevent non-specific binding, serum was removed after a given incubation time, followed by washing steps. BAFF was bound by a biotin-conjugated anti-BAFF antibody. Streptavidin-HRP bound these antibodies to biotin. After each incubation time, unbound antibodies were washed out. By adding a chemiluminescent substrate, Streptavidin-HRP generates a light signal based on enzyme reaction. After a stop solution was added, the absorbance was measured using a FLUOstar OPTIMA microplate reader. The concentration of BAFF was determined indirectly, based on the measured wavelength. Statistical differences between pregnant mice and non-pregnant mice were analysed using GraphPad Prism software. Outliers were removed according to the definition mentioned above.

3 Results

3.1 Role of B cells in normal pregnant mice and immune-mediated pregnancy failures

3.1.1 Alterations of precursor B cells in bone marrow

In bone marrow, significantly lower numbers of pre-pro B cells ($B220^{low}sIgM^{neg}$) are present in pregnant mice compared to non-pregnant mice (Figure 5A). Beyond this study, a strong reduction of pre-pro-B cells has been established in naturally occurring pregnancy disturbances. Therefore, all precursor B cells downstream of pre-pro B cells are accordingly reduced in the bone marrow [pre-B cells ($B220^{low}IgM^{neg}CD43^{neg}$), pro-B cells ($B220^{low}IgM^{neg}CD43^{pos}$), immature B cells ($B220^{pos}sIgM^{neg}$)] regardless of the pregnancy outcome (Figure 5B).
However, bone marrow mature B cells ($B220^{pos}sIgM^{pos}$) are significantly lower in mice with pregnancy disturbances, whereas their numbers remain stable during normal gestation (Figure 5A).

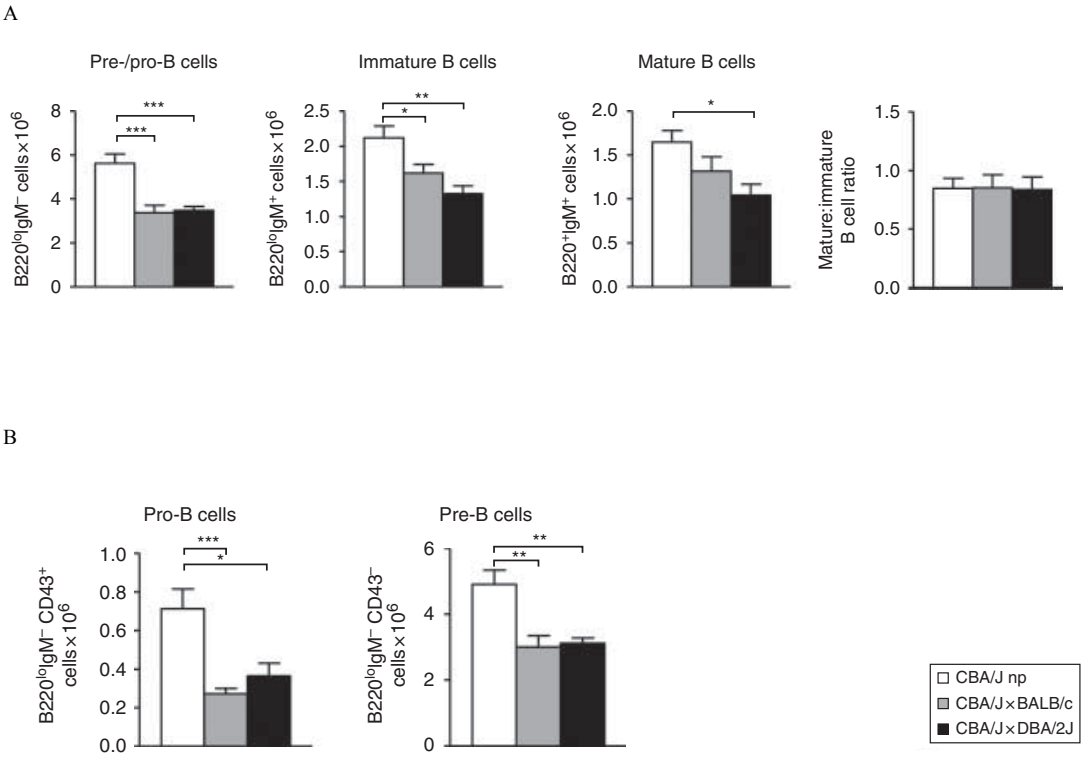

Figure 5: Mature B cells are reduced in bone marrow of mice suffering pregnancy failures.
Partial section of Figure 1, Muzzio 2016. A supplementary description and details of the gating strategies can be found in the cited publication.

3.1.2 Marginal zone B cells and mature B cells in lymphoid tissues

Bone marrow mature B cells, after entering the bloodstream, enter the peripheral lymphoid tissues, among other tissues in the spleen. The results show significantly lower numbers of total $B220^{pos}$ splenic B cells in pregnant mice, regardless of pregnancy outcome (Figure 6A). A closer look at the main B cell population revealed that the FO B cells ($B220^{pos}CD23^{high}CD21^{int}$) were significantly decreased in pregnancy compared to non-pregnant animals. MZ B cells ($B220^{pos}CD23^{low}CD21^{high}$) showed, on the one hand, increased cell number during pregnancy compared to non-pregnant mice, but not in mice with pregnancy loss (Figure 6B).

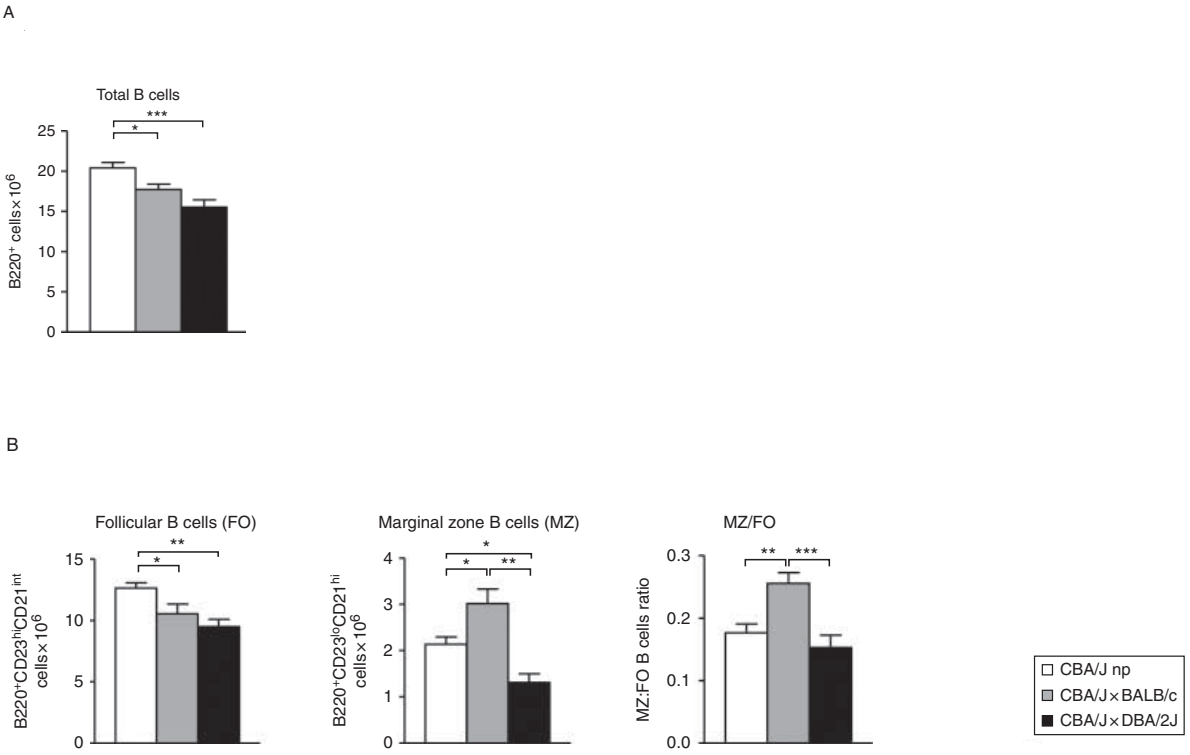

Figure 6: Reduced total B cells and expanded MZ B cells in spleen of normal pregnant mice.
Partial section of Figure 2, Muzzio 2016. A supplementary description and details of the gating strategies can be found in the cited publication.

The spleen is not the only lymphatic tissue to display alterations in its cellular composition. A significant increase in the number of mature B cells ($B220^{pos}CD21^{pos}$) was observed in uterus-drained lymph nodes during gestation regardless of pregnancy outcome (Figure 7).

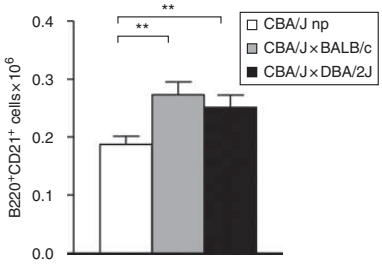

Figure 7: Mature B cell numbers in lymph nodes, draining the uterus.

Partial section of Figure 5, Muzzio 2016. A supplementary description and details of the gating strategies can be found in the cited publication.

3.1.3 Levels of immunoglobulins in serum of non-pregnant and pregnant mice

The analysis of IgM and IgA showed significantly higher serum levels of both immunoglobulins in mice with normal pregnancy compared to non-pregnant animals. It was conspicuous that the serum of pregnant mice suffering from pregnancy disturbances contained significantly less IgM compared to normal pregnant mice, but equal to non-pregnant mice. Furthermore, other immunoglobulins such as IgG1, IgG2a/b, IgG3 and IgE showed a slight but non-significant increase in serum samples obtained from normal-pregnant mice compared with non-pregnant mice and those with pathological pregnancies (Figure 8).

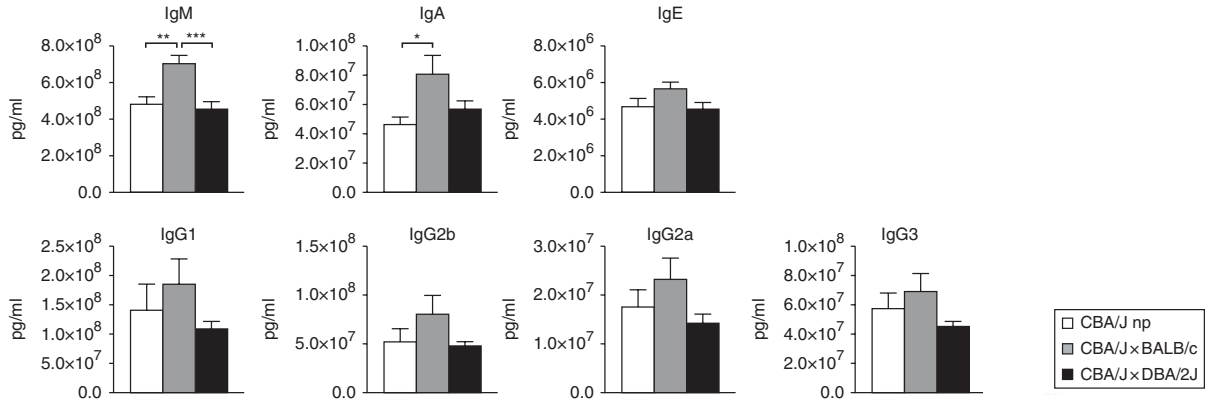

Figure 8: Dynamics of immunoglobulins during pregnancy.

Partial section of Figure 5, Muzzio 2016. A supplementary description and details of the gating strategies can be found in the cited publication.

3.1.4 Levels of BAFF in the serum of non-pregnant and pregnant mice

The analysis revealed for the first time that BAFF concentrations vary in pregnant mice undergoing pregnancy disturbance. The analysis also showed significantly lower levels of BAFF in normal pregnant mice compared to non-pregnant mice (Figure 9).

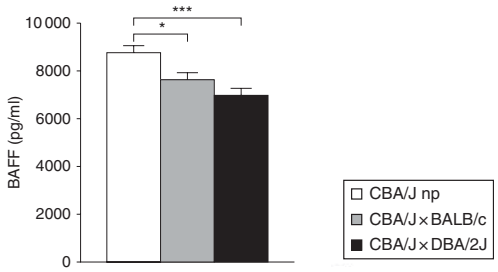

Figure 9: Pregnancy is accompanied by reduced levels of BAFF.

Partial section of Figure 5, Muzzio 2016. A supplementary description and details of the gating strategies can be found in the cited publication.

3.2 Human B cell compartment and immunoglobulin profile in peripheral blood during subclinical pregnancy

3.2.1 Changes of the main B cell populations in peripheral blood throughout the trimesters

A deeper analysis of the dynamics within the B cell compartment was still missing. In the present study a significant increase in pro-pre B cells [$CD10^{pos}IgM^{neg}(CD24^{bright}CD38^{bright})$] as the early B cell developmental stage was found in peripheral blood during the first trimester, followed by a significant decrease from the first to the second trimester. The percentages of all pro-pre B cells within the lymphocytes were analysed, and also revealed a significate rise within the first trimester followed by decrease during the following trimester.

While the early progenitor cells proliferated at the beginning of pregnancy, the more mature B cells such as immature B cells [$CD10^{pos}IgM^{low}(CD24^{bright}CD38^{bright})$] and transitional B cells [$CD24^{bright}CD38^{bright}IgD^{bright}IgM^{bright}(CD24^{bright}CD38^{bright})$] (Figure 10) from peripheral blood decreased during the second trimester.

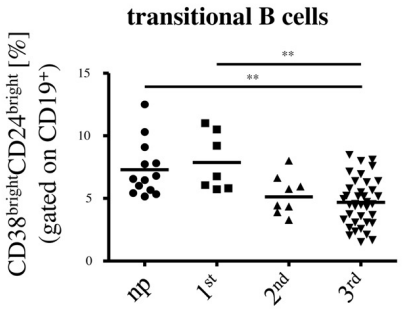

Figure 10: Dynamic of the transitional B cells of pregnant women.

Partial section of Figure 1, Ziegler & Muzzio 2018. A supplementary description and details of the gating strategies can be found in the cited publication.

3.2.2 Alteration of memory and naïve B cells during pregnancy

Immature and transitional B cells are reduced during mid-pregnancy. Therefore, the dynamics of the main B cell populations in peripheral blood—consisting of naïve ($CD19^{pos}CD27^{neg}IgD^{pos}$), non-switched ($CD19^{pos}CD27^{pos}IgD^{pos}$) and switched ($CD19^{pos}CD27^{pos}IgD^{neg}$) memory B cells—must be examined during the three trimesters of pregnancy for further basic research. The population of naïve B cells [$CD19^{pos}CD27^{neg}IgD^{pos}(CD19^{pos})$] actually increased in the first weeks of pregnancy and until the third trimester but without alteration of memory B cells.

3.2.3 IgG- and IgA-producing plasmablasts

Ultimately, the most advanced B cell stage in the differentiation represents the antibody-producing plasmablasts ($CD138^{pos}CD27^{pos}$). The percentages of plasmablasts were slightly but not significantly increased throughout all three trimesters. However, IgA- and IgG-expressing plasmablasts [[$CD138^{pos}IgA^{pos}(CD138^{pos})$], [$CD138^{pos}IgG^{pos}(CD138^{pos})$]] showed significant percentage increases. In IgM- and IgD-expressing plasmablasts, no significant change was observed (Figure 11).

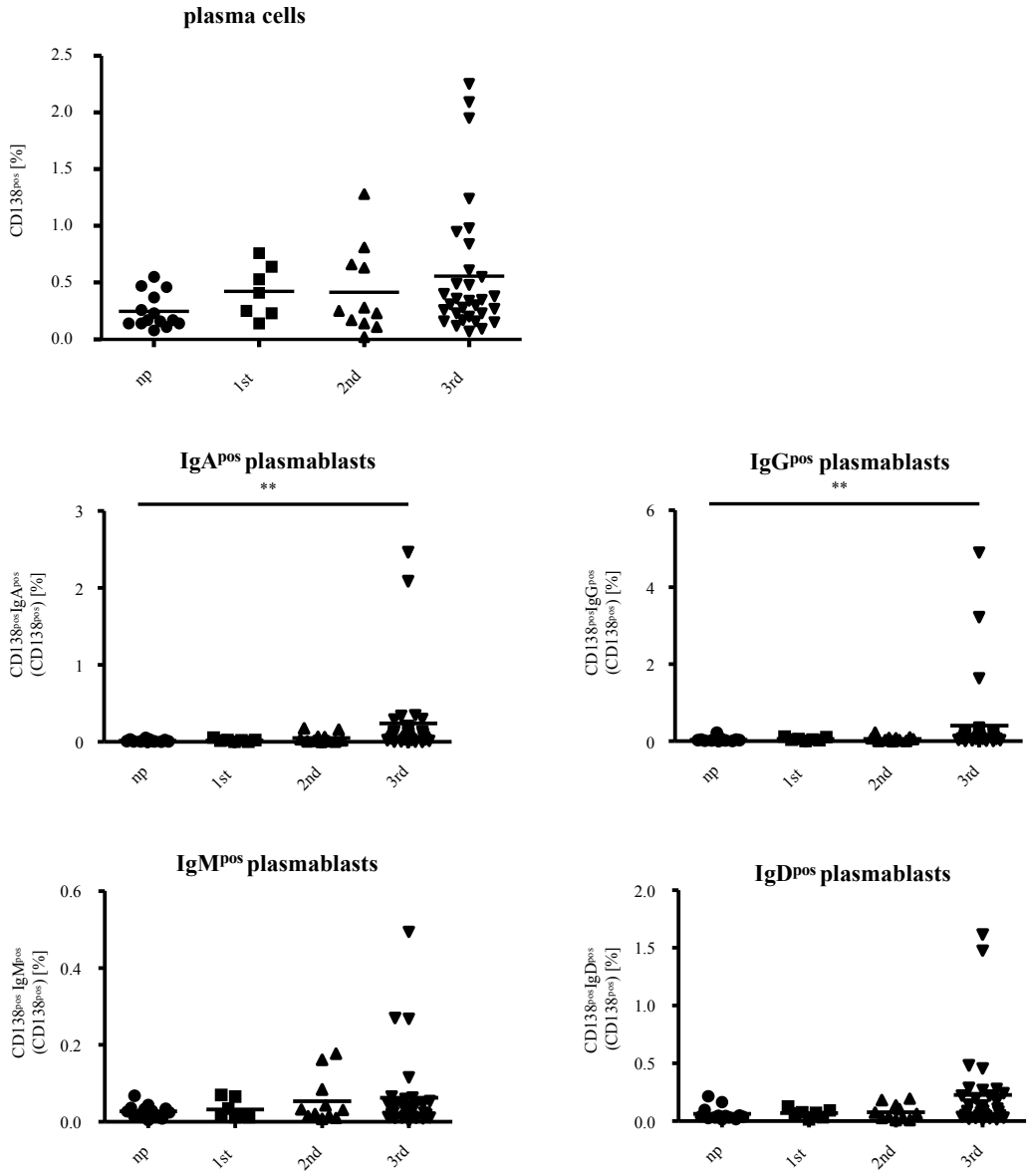

Figure 11: Plasma cells in peripheral blood of pregnant and non-pregnant women.

Partial section of Figure 1, Ziegler & Muzzio 2018. A supplementary description and details of the gating strategies can be found in the cited publication.

3.2.4 Immunoglobulin profile production of natural antibodies during human pregnancy

Analogous to rodents, human IgM as well as IgA serum levels were significantly increased during the first trimester among pregnant women compared to non-pregnant controls. Subsequently, during the second trimester, the IgM and IgA serum readings among pregnant women declined to the levels observed in non-pregnant control subjects. Within the IgG subclasses, IgG_1 serum level was significantly increased during all trimesters compared with the serum levels of non-pregnant women. In comparison, IgG_3 serum levels were moderately increased during the first trimester, peaked during the second trimester, dropping again toward the third trimester. IgG_2 and IgG_4 showed constant serum level during all trimesters. In conclusion, in both human patients and a mouse model, pregnancy alters the immunoglobulin profile and boosts production of natural antibodies (Figure 12).

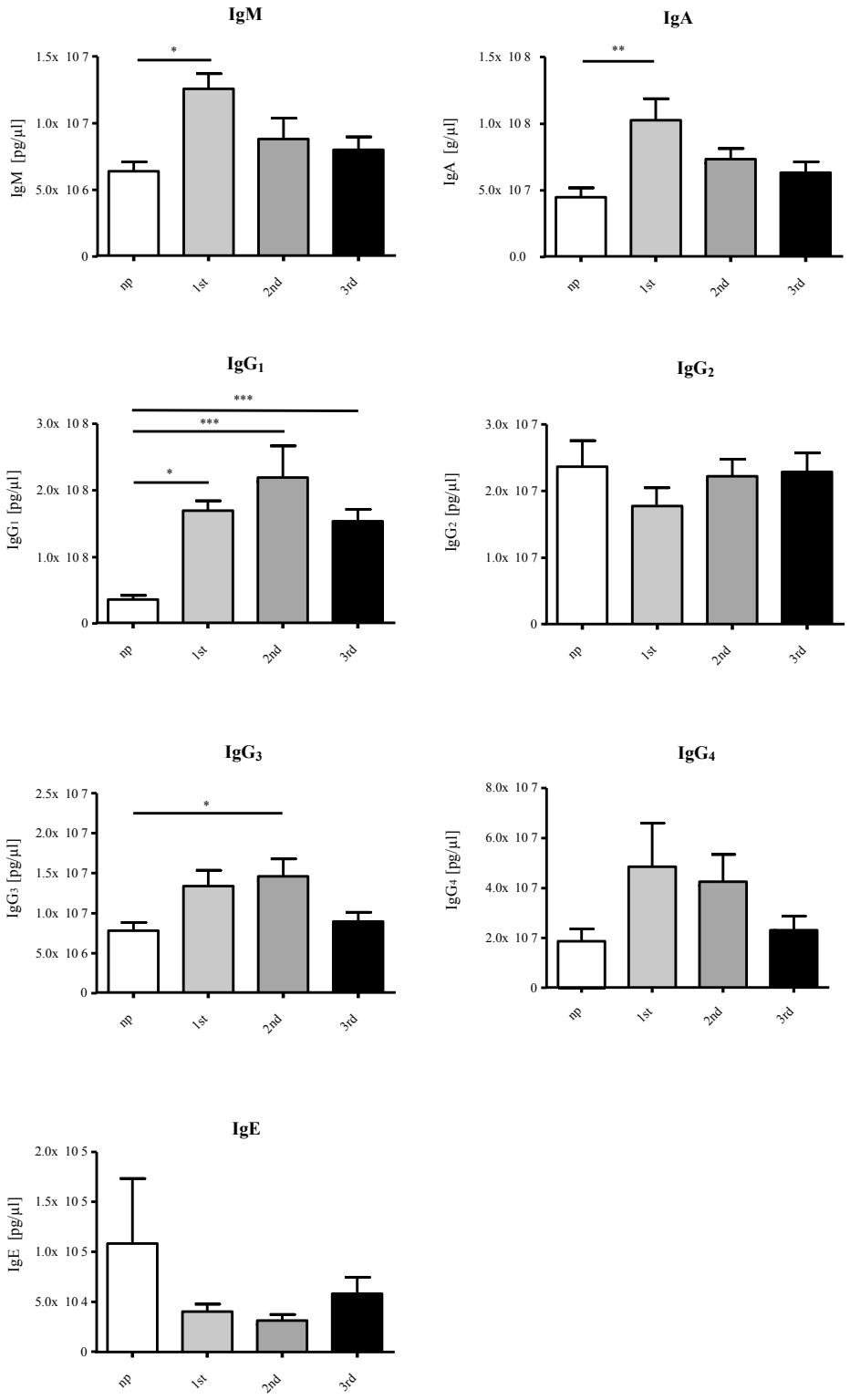

Figure 12: Levels of immunoglobulin in serum of pregnant and non-pregnant women.

Partial section of Figure 1, Ziegler & Muzzio 2018. A supplementary description and details of the gating strategies can be found in the cited publication.

4 Discussion

4.1 Alterations of B cell precursors during mouse pregnancy

For successful pregnancy, the maternal immune system has to tolerate the semi-allogenic foetus while simultaneously defending the mother and foetus from infection.[97] This antagonistic characteristic of suppression and activation is finely balanced and appears to be subject to adjustments within the B cell compartment. To further investigate the modifications that are required for successful pregnancy, we used a mouse model of clinically unremarkable pregnancy and one of immune-mediated pregnancy loss.[88–90]

Previous studies showed suppression of B cell lymphopoiesis during normal pregnancy, which was considered to be a physiological adaption within the B cell compartment to diminish auto-reactive B cell formation.[24,27] The suppression of all B cell precursors downstream of pre-pro B cells was already demonstrated in bone marrow during gestation. However, the mature B cells in the bone marrow are not subject to these changes and therefore maintain stable numbers. This suggests that fewer B precursor cells are produced or become apoptotic, whereas mature B cells are retained in the bone marrow. Alternatively, it would also be possible that the precursor B cells are increasingly stimulated in their maturation to mature B cells. Consequently, the mature B cells of the immune system are held back in bone marrow. This should be one of those adaptive mechanisms that reduce the occurrence of auto-reactive B cells that recognise foetal structures and thereby prevent foetal rejection.[24,26] Compared to mice with uneventful pregnancy, the present findings show that mice with immune-mediated pregnancy failures[80–83] have significantly reduced counts of mature B cells in bone marrow. B cell stages that follow progenitor cells show unchanged behaviour in comparison to mice with normal pregnancy. Therefore, it can be assumed that the mature B cells will increasingly be flushed out into the blood circulation, or the course of B cell lymphopoieses from immature to mature will be interrupted without new progenitor cells ripening. To support this hypothesis, we analysed the B cell compartment of the lymphatic organs.

4.2 Alterations of the murine B cell compartment in lymphatic tissues

Muzzio and colleagues reported an increasing total numbers of B cells of uterus-draining lymph nodes during normal pregnancy.[27] We demonstrated that peritoneal cavity and uterine-drained lymph nodes showed a larger number of mature B cells during gestation regardless of the pregnancy outcome. On the one hand, more of the mature B cells could be flushed out of the bone marrow and, at the same time, more B cells mature in the context of a rightward shift. On the other hand, it would be conceivable that the mature B cells already present in the periphery are increasingly localised into the lymph nodes in close proximity to the semi-allogeneic foetuses.

Not only the B cell populations of the peritoneal fluid and uterus-draining lymph nodes are affected, but also the spleen as the largest lymphatic organ alters its cellular composition. Pregnant mice showed significantly lower total numbers of $B220^{pos}$ splenic B cells compared with non-pregnant animals, regardless of pregnancy outcome. It is already known that the B cells, egressing from bone marrow into the blood stream and then migrating into the spleen, further differentiate into MZ and FO B cells.[1] Muzzio *et al.* previously described splenic B cell lymphopenia and the composition of the MZ and FO cells during normal pregnancy. They detected a reduction of FO B cells, while the numbers of MZ B cells are increased during normal gestation.[27] They hypothesised that the population of splenic MZ B cells expands considerably as a compensatory mechanism to balance the lower influx of B cells, thus maximising the capacity of the maternal immune system to control pathogens.[27] Our analyses confirm this increase of MZ B cells. In contrast, this alteration was not detected in mice with pregnancy loss. Thus, there may be a correlation between pregnancy pathologies and MZ B cell numbers. We attribute this form of splenic B cell alteration to their different cell functions.[28,98] The FO B cells undergo somatic hypermutations after previous antigen contact regardless of whether they are foreign antigenic, self-antigenic or foetal antigens. After 5 days they encode, express and release high-affinity antibodies and thereby boost the maternal immune system to control infections during pregnancy.[31] Until this fifth day is reached, the mother's immune system is in a phase in which it does not seem sufficiently robust to ward off pathogens.[21] Therefore, it is likely that the MZ B cells increase in order to produce more of their low-affinity and polyreactive natural antibodies, until finally the described T cell-dependent immune response can be adequately performed to prevent pregnancy risk.[21] Crucially, it does not require prior antigenic contact to express natural antibodies such as IgM.[27,46,99] This provides more rapid immunological response against invading pathogens. To test this hypothesis, we further

analysed the immunoglobulins from MZ B cells in peripheral blood serum. In mice with normal pregnancy, serum IgM levels are significantly higher than in non-pregnant animals. This indicates that the expansion of MZ B cells and their upregulated antibody production might be necessary for the immunity of the mother and foetus. In addition to MZ B cells, B1 cells also produce a considerable proportion of natural antibodies.[62,63]

However, because the MZ cell numbers increase significantly, we attribute the increase in IgM to the MZ B cells. Considering that mice with pregnancy disturbances showed reduced MZ B cells in the spleen, it would be conceivable that the antibody expression is upregulated as a compensatory response in order to shield the mother and foetus from infection. Alternatively, immunoglobulins already present in the cell could be increasingly secreted into peripheral blood. However, our analysis showed that was not the case. Instead, a reduced serum concentration of their produced natural antibodies was detected. Thus, pregnancy well-being is confirmed by the expansion and activation of the MZ B cell compartment. Whether the miscarriages are due to a weakened immune system or foetal structure recognition is not clear. Therefore, IgM is discussed as potentially playing a role in preventing autoimmune disease.[1] Previous studies have shown that IgM binds to sets of epitopes of apoptotic cells and induces phagocytic clearance to prevent innate immune reactions.[1,62] It is also reported that in the periphery of the spleen IgM promotes phagocytosis of apoptotic material, thereby activating the Il-10-producing B cells to avoid inflammatory reactions.[62] In another study, in the same mouse model of pregnancy disturbances used in the present study, mice were injected with natural antibodies that had been isolated from blood serum of several mice with normal pregnancy. The administration of natural antibodies showed a demonstrably positive influence on pregnancy outcomes in mice.[90]

4.3 Alterations within human peripheral blood B cell compartment during pregnancy

Such a study of the B cell compartment from bone marrow, spleen, lymph nodes and peritoneal cavity cells from non-pregnant women and those with normal pregnancy presents risks to the subjects and the unborn child, and is therefore not possible for ethical reasons. Only the B cell compartment of peripheral blood could be analysed.

The population of naïve B cells without previous antigen contact increased in the first weeks of pregnancy and until the third trimester but without alteration of memory B cells. This result is complemented by the work of Lima *et al*., who demonstrated that naïve B cells from peripheral blood showed significantly higher cell counts in the third trimester as well as postpartum compared to non-pregnant women. In contrast to this finding, the total number of B cells in

peripheral blood remained unchanged.[100] The increase of naïve B cells could be explained by the increasing concentration of progesterone during pregnancy. Progesterone can act as an inhibitor of B cell maturation.[101] Consequently, the differentiation of naïve B cells in memory B cells or in plasmablasts would be slowed down. Likewise, Lana *et al*. explained that the increase in naïve B cells may lead to a cell shift into lymphoid tissues.[101] For example, chemokines could attract immune cells as memory cells to the uterus.[100] Consequently, higher values of naïve B cells would be measurable in peripheral blood.

4.4 Crucial dynamics of immunoglobulins during inconspicuous pregnancy

So far, the kinetics of different immunoglobulin subtypes throughout human gestation is not precisely defined. In order to determine the extent to which natural antibodies could also contribute to well-being during human pregnancy, we first analysed serum from women of reproductive age during the three trimesters of pregnancy, compared to non-pregnant women of the same age independent from their menstrual cycle. We excluded women with active and/or chronic diseases as well as positive history of miscarriage. As expected, some serum levels of natural antibodies were significantly increased during gestation. IgM showed the most pronounced increase during the first trimester, whereas IgG increased in the last trimester. At this point, it would be interesting to determine how the immunoglobulins would behave during pregnancy among subjects with recurrent miscarriage. We have started to investigate this issue; however, we were unable to use the results due to the small number of study participants. In this context, the literature reports that in the past, women with recurrent pregnancy loss were treated with high-dose intravenous immunoglobulins (IVIG) from healthy donors to prevent spontaneous loss. This reprocessed blood product contained natural antibodies, and the approach was shown to result in higher live birth rates.[102] Currently, IVIG is preferred for use off-label in women younger than 35 years with morphologically ideal embryos or euploid ova in order to improve implantation, since treatment at older ages was less successful, possibly due to chromosomal abnormalities.[103] However, immunoglobulin-mediated cytokine stimulation and suppression through natural antibodies are discussed. Moreover, other disciplines also report that higher levels of IgM are detectable in the blood of patients with low disease activity than in patients with higher activity and organ damage.[62] Taking all of those findings together, it appears that low serum concentration of natural antibodies could—among other factors—be responsible for potential autoimmune and foetal rejection.[104]

The theory that lower IgM serum levels may lead to an increase of pathogenic IgG natural antibodies[105] with autoimmunity, that may result in autoimmune diseases or abortions cannot be confirmed in our study. Our data showed no elevation of IgG levels in mice that experienced spontaneous abortion. In addition, higher IgG_1 and IgG_3 levels were measured during the second and third trimester among women who had an inconspicuous pregnancy course. This seemingly physiologically delayed increase in $IgG_{1/3}$ might be explained by an immunoglobulin class switch from IgM to IgG stimulated through cytokines.[106] Nevertheless, we do not exclude the possibility that an overall higher level of immunoglobulins in a secondary hypergammaglobulinemia may lead to pregnancy complications. A primary hypogammaglobulinemia is very unlikely because women with acute and chronic disease were excluded as well as women with a known tumour disease that could relate to hypergammaglobulinemia.

Based on significantly increased immunoglobulin levels in serum of pregnant women, either the plasma cells increase or their production increases. We observed the latter and not the expansion of antibody-producing cells.

4.5 BAFF as mediator between suppression and activation of maternal immune system

The regulatory mechanisms responsible for modulation within the B cell compartment during pregnancy are manifold and are still not fully understood. It appears that not only are immunoglobulins associated with autoimmune diseases[64] and spontaneous pregnancy loss: overproduction of tumour necrosis factor BAFF may also be associated with autoimmune diseases and may also play a role in a successful pregnancy without complications.[8,73,107] Therefore, we focused on BAFF, which is regarded as a major factor influencing changes within the B cell subsets.[8] It was previously reported that mice display lower BAFF concentrations during normal pregnancy.[27] That observation was supported by the findings of the present study. Of note, cells that express the BAFF-R ligand system, such as the increased splenic MZ B cells during gestation, are associated with an increased risk of autoimmunity due to their stimulating effect on antibody production.[108] It would be conceivable that these low levels of BAFF represent a protective mechanism to suppress autoreactive B cells.[27] The ways in which BAFF concentrations vary in pregnant mice undergoing pregnancy disturbance were unknown prior to the present study. We were able to reproduce these low levels during gestation and expanded these by studying the BAFF levels of pregnant mice with foetal rejection. The latter showed even more significant declines in BAFF levels than did pregnant mice without gestation

complications. This insight supports the work of Bienertova-Vasku *et al*., who described lower BAFF levels in blood serum from preeclamptic patients.[109] A different group reported lower BAFF concentration in the bloodstream of women with spontaneous abortion than in those with inconspicuous early pregnancy.[73] Given all the results and possible interpretations, the even lower BAFF levels now detected in mice with miscarriages compared to normal pregnant mice do not necessarily question BAFF as an immunosuppressant. Such an effect may be possible, but the BAFF influence on immune crosstalk between mother and infant seems to be much more far-reaching. Presumably, the immuno-histochemical expression of BAFF on the cell tissue part of the placenta is crucial. In decidual tissues, the level of BAFF expression among pregnant women is demonstrably higher than in decidual tissues of women with spontaneous pregnancy loss.[73,109] In more detail, BAFF is believed to be expressed and secreted by cytotrophoblast and syncytiotrophoblast cells. As part of the TNF family, BAFF may act as a cytokine and mediate NF-kB signalling .[8,72,73,109] This leads to the suggestion that lower BAFF level causes immoderate apoptosis from cells involved in maternal–foetal interface.[73] It is also notable that another observation showed that BAFF expressed in trophoblasts, decidua cells and endothelial cells of the blood vessels of pregnant women, but not in the endothelial cells of women who suffered two or more consecutive miscarriages.[73] Consequently, BAFF is also crucial in angiogenesis at the maternal–foetal interface for foetal growth and reproductive success.[73] Symptoms such as foetal grow restriction, angiogenic deregulation and abnormal placental development are characteristic for our mouse model.[88] Nevertheless, the effect of BAFF may not be considered as isolated, since BAFF acts in combination with an enormous number of molecules, which in turn triggers complex and not yet fully understood cascades that also have different effects on pregnancy depending on the stimulus.[73] The last point discussed in this context is that it would also be plausible to speculate that BAFF also represents a type of mediator between the peripheral B cell compartment and B cell lymphopoiesis in the bone marrow. It is known that immature B cells in bone marrow express BAFF and a higher level of BAFF receptors as in precursors.[110] Some researchers are also convinced that BAFF-R is not represented on murine pro-B and pre-B cells.[111,112] It would thus be conceivable that during pregnancy the lower BAFF levels in peripheral blood due to BAFF recession/recursion into the bone marrow could affect the maturation of bone marrow. In cell cultures of immature B cells of bone marrow it was shown that high BAFF concentrations favour the survival of these cells and suppress apoptosis.[110] Thus, physiologically low BAFF concentrations, such as those present in mice during pregnancy, could lead to the physiological suppression of B cells in bone

marrow. Pathologically low levels of BAFF, as in mice with foetal rejection, could result in an excessive apoptosis of the bone marrow B cells. However, these speculations should not be considered in isolation from other cellular processes. BAFF is also able to bind BCMA and TACI expressed on the cells, cascades of which can in turn influence maturation. In precursors, BCMA and TACI vary in their level of expression and they are also subject to adjustment mechanisms. In addition, there are also non-lymphatic cells present in bone marrow that also produce and perceive BAFF.[113] They or the B cells themselves could react reflexively at pathologically low levels of BAFF. Further studies are required in order to determine the BAFF concentrations in the bone marrow of both mouse models, especially compared to those produced by non-lymphatic cells.

In summarising the results presented here, it can be stated that the expansion of the MZ B cell compartment and their upregulated antibody production are important for immunity at the maternal–foetal interface. Furthermore, BAFF seems to act as a mediator of peripheral B cell compartment and B cell lymphopoiesis in the bone marrow for successful pregnancy and B cell homeostasis and angiogenesis.[73]

It should also be emphasised that future studies may also face limited availability of human samples. Samples from live donors, pregnant or non-pregnant, will not be made available to use without an urgent medical indication of bone marrow, lymph node or liver aspiration. Even then, there is usually a clinical picture that could basically not be assigned to a patient group. However, a control group with a meaningful number of people will most likely not be achieved. Nevertheless, it is a topic on which basic research should continue, as families suffer greatly from the loss of the unborn. Approximately 5 % of all couples experience more than three consecutive miscarriages[114], although there is certainly a high number of unreported cases as it often remains a taboo topic due to shame, grief and guilt.[115] Moreover, many miscarriages are not perceived as such but as an irregularity in the menstrual cycle and only a third of all conceptions result in a live birth.[115]

4.6 Study design, limitations of models and statistics

In addition to the cellular and humoral changes, it is necessary to critically discuss the group of study participants, the mouse models and the methodology and statistical evaluations.

Starting with the evolutionarily older model,[116] the number of mice used has to be discussed. From an ethical point of view, we opted for the smallest possible number of mice, but sufficient

to establish significance. It is also worth noting that we provided leftover cells as well as unused organs to other doctoral students for their studies as part of the approved project "Charakterisierung des B-Zell Kompartiments im peripheren Blut und in der Amnionflüssigkeit während der Schwangerschaft". Consequently, we were able to undertake effective research while also keeping in focus the lives of murine subjects.

The use of mouse models as a preclinical model of human pregnancy, listed above, should also be viewed critically. CBA/J × DBA/2J mice experience early pregnancy loss, as described in the literature, through angiogenic deregulation as well as abnormal placental development and foetal growth restriction.[88,89] This seems to be based on different immunogenetically determined processes[85,89] but also involves as yet unexplained issues[85,89] apart from exogenous factors.[92] The interpretation of the results is therefore clearly limited, since the changes are multifactorial. We tried to control the exogenic factors in which the mice, all purchased from the same source and of the same age, lived in the same stable with the same food, water and light conditions. The mice were allowed to acclimate to the conditions for 14 days before copulation. Thus, we aimed to avoid miscarriages that might be caused by stress resulting from transportation with relocation to a new barn or loud ambient noise.[81,91,92] To avoid a miscarriage due to high age, mice were paired at 8–10 weeks.

Apart from our mouse models, there is generally no identical preclinical model to humans. However, the question arises of whether one can use this mouse model to observe isolated single immunological processes and transfer the findings to humans. This is especially pertinent, since mice have other surface molecules, antibody classes, cytokine expression and immune cells than humans.[116] It would be too simple to use a mouse model with evolutionary differences of 65 million years[116] to explore and then transfer a highly complex process—such as pregnancy course and miscarriage based on B cell compartment—to humans. It is unlikely that these processes occur in the same way in different species.[116] With this awareness, some researchers are now using highly immunodeficient and HSC-transplanted mice—so-called humanised mice—with human haematolymphoid system.[117] In order to make our work even more meaningful, this should also be taken into account in the future. Following a transplant, these mice can go through human haematopoiesis and can express human cytokines.[117] Nevertheless, there are some unresolved issues in the model. They can be very similar to humans, but cannot perfectly imitate them.[117] However, this does not mean that the mouse model is inadequate for in vivo trials, since it can nevertheless provide important clues that may be crucial for human research. One should, however, keep such differences in mind. Especially in the past, several

clinical studies on humans were conducted on the basis of previously very promising mouse experiments, which ultimately led to an exacerbation of the clinic and had to be stopped contrary to all expectations.[116] Exactly for that reason, basic human immunological research should be emphasised in the future. From the knowledge of the human system, the development of a specific drug might be possible as pharmacological therapies become increasingly closely targeted.[116]

Before starting our study with humans, several considerations were made to avoid errors. A primary definition of the patient groups was done to minimise possible variables that could influence the B cell compartment and that are outside the focus of the study. The study only included female participants who had no diagnosed immunological disease or acute or chronic inflammation. However, this inclusion process was based on anamnesis rather than on laboratory tests and therefore such factors cannot be ruled out with complete certainty. For the control group, women in the climacteric were excluded as well as girls outside the reproductive phase, in order to enable a control group of women who corresponded to the pregnant subjects. Within this control group, it was not possible to pay attention to the participants' menstrual cycle, due to its individual course. Differing oestrogen and progesterone concentrations can alter the B cell compartment and thus have an incalculable influence on our results.[101] Within the group of pregnant women there are also variables that were not addressed. Although the main diagnoses were noted for outpatient/inpatient consultation by means of an anonymised data sheet, the keywords [nicotine abuse], [alcohol consumption], [endocrine abnormalities] and [obesity] were not systematically queried in the groups of pregnant or non-pregnant women. Maternal alcohol and nicotine consumption, higher levels of glycosylated haemoglobin and obesity have to be mentioned because of their known positive associations with pregnancy loss.[118,119] This information is listed in the participants' patient records, but was not approved for use in this study. For this, a renewed application would have to be submitted to the ethics committee in addition to newly informing and obtaining consent from the study participants. Ultimately, we were not able to define the group of participants too narrowly, otherwise the number of eligible patients would have been too small to reliably calculate statistical significance from the findings. Nevertheless, the number of participants plays a crucial role in the value of a study. In order to ensure a high number of serum samples, the clinical personnel from ambulatory and stationary gynaecology obligingly provided us with blood samples from all women who were willing to participate in the study. Some candidates could not be included

due to the exclusion criteria listed above, e.g. acute inflammation. This was to be expected, as rarely a woman visits a gynaecological department without pregnancy problems. In order to increase the number of participants, especially in the first trimester, several private practices were approached, as they primarily oversee pregnancies as long as no complications arise. Unfortunately, collaboration with these clinics was not forthcoming due to the significant amount of time required to obtain informed consent from patients and collect blood samples, as well as uncertainty on the part of potential participants. In addition to ensuring sufficient time and financial resources, cooperation with different disciplines should be established in future studies, in order to increase the number of samples available from different patient groups, thereby making such studies less reliant on animal models. Furthermore, trauma surgeons (for example) could expand the spectrum of samples by obtaining bone marrow intraoperatively from pregnant and non-pregnant polytrauma patients with surgically supplied fractures, without presenting additional risk to the mother or child. The same is true for visceral surgeons, who need to perform surgical procedures on pregnant and non-pregnant women in order to sustain their lives. Polytrauma patients show often an acute spleen and/or liver lesion. Both injuries are partly surgically referred and the corresponding cell material for research could be obtained during the intervention.[120] Polytrauma patients could be particularly well suited, since they are mainly young people of reproductive age and are systematically registered at the hospital by the shock room.[121]

In case of minor injuries, it should be possible to conduct an informative discussion about a possible tissue donation and the subsequent declaration of consent, especially since not every fracture or organ lesion has to be operated on as an emergency. However, the trauma could result in the release of cytokines, which in turn change the immune system decisively and make it difficult to interpret the results.[122]

Another possibility, which would require prior ethical approval, is post-interventional investigation in the event of an emergency splenectomy or a partial liver resection. In such cases, remaining biomaterial as part of a regular healing treatment is usually routinely stored for routine histopathological examination, and would otherwise be discarded. Apart from this, a systematic and complete medical history should be carried out, as well as laboratory chemical tests to rule out endocrine dysregulation and inflammation among prospective study subjects.

In addition to the models, methods of sample processing and measurement have to be discussed in terms of detecting and avoiding potential errors. In this work, the experiments were carried

out according to a standardisation protocol with the same reagents and devices in a single laboratory. Consequently, the experimental protocol and equipment were always the same and therefore should not be a source of variation or inconsistency. In retrospect, it would be preferable to perform the FACS measurement using viability dyes to discriminate dead cells, to prevent unspecific binding of the antibodies with dead cells, which can lead to false-positive signals.

When performing statistical analysis, unique values outside the interquartile range (IQR) were generally excluded. The IQR was defined as the difference between the first (Q1) and third (Q3) quartiles. Values more or less than ±1.5 times the IQR were excluded from the statistical analysis. These outliers could occur not only through incorrect classification of patients but also by unexplained scattered values. Nevertheless, it is also possible that false-high or false-low values are present within the expected range of dispersion, which cannot be identified by this mathematical calculation and which therefore influence the analysis.

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6 Publications

This cumulative dissertation consists of the following publications:

6.1 Marginal zone B cells emerge as a critical component of pregnancy well-being

Muzzio DO, Ziegler KB, Ehrhardt J, Zygmunt M, Jensen F.
Reproduction, 2015, S. REP-15-0274.

REPRODUCTION
RESEARCH

Marginal zone B cells emerge as a critical component of pregnancy well-being

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Abstract

The success of eutherian mammal evolution was certainly supported by the ability of the already existing immune system to adapt to the presence of the semi-allogeneic fetus without losing the capability to defend the mother against infections. This required the acquisition of highly regulated and coordinated immunological mechanisms. Failures in the development of these strategies not only lead to the interruption of pregnancy but also compromise maternal health. Alongside changes on the cytokine profile – expansion of tolerogenic dendritic and regulatory T cells – a profound adaptation of the B cell compartment during pregnancy was recently described. Among others, the suppression of B cell lymphopoiesis and B cell lymphopenia were proposed to be protective mechanisms tending to reduce the occurrence of autoreactive B cells that might recognize fetal structures and put pregnancy on risk. On the other hand, expansion of the pre-activated marginal zone (MZ) B cell phenotype was described as a compensatory strategy launched to overcome B cell lymphopenia thus ensuring a proper defense. In this work, using an animal model of pregnancy disturbances, we demonstrated that the suppression of B cell lymphopoiesis as well as splenic B cell lymphopenia occur independently of pregnancy outcome. However, only animals undergoing normal pregnancies, but not those suffering from pregnancy disturbances, could induce an expansion and activation of the MZ B cells. Hence, our results clearly show that MZ B cells, probably due to the production of natural protective antibodies, participate in the fine balance of immune activation required for pregnancy well-being.

Reproduction (2016) **151** 29–37

Introduction

The evolution of the eutherian mammal has surely represented a challenge to the rules of the already existing immune system, as it faced the dilemma to simultaneously tolerate the presence of a semi-allogeneic fetus without losing the capacity to defend the mother against pathogens. This dichotomy seems to have been resolved by the acquisition of highly regulated immune strategies ensuring a fine balance between immune suppression, allowing the presence of the fetus, and immune activation, guaranteeing a proper defense.

Alongside well-known and deeply investigated immune adaptations involving dendritic cells (Segerer *et al.* 2012), T cells (Ruocco *et al.* 2014), NK cells (Parham 2004) and macrophages (Faas *et al.* 2014), the B cell compartment was recently demonstrated to undergo profound modifications and adaptations during pregnancy.

B lymphocytes are fundamental components of the adaptive arm of the immune system. B cells are continuously originated from pluripotent hematopoietic stem cells in the bone marrow (BM). The pro- and pre-B cells represent the earliest B cell stages and are characterized by low expression levels of B220 and the absence of IgM expression on their cellular membrane (Hardy & Hayakawa 1991, Hardy 2003). The transition from pro- to pre-B cell stage requires the loss of expression of the sialoglycoprotein CD43. Once pre-B cells start expressing IgM on their cellular membrane, they become immature B cells, leave the BM and migrate to the spleen where they continue their development (Hardy 2003). In this tissue, immature B cells give rise to either follicular (FO) or marginal zone (MZ) B cells, the most prominent mature B cell subsets in the body (Allman & Pillai 2008). These two B cell populations have major differences in terms of phenotype, localization and functionality. FO B cells, localized in the follicular niche of the spleen and lymph nodes, are

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defined as B220^{+}CD23^{hi}CD21^{int} (Allman & Pillai 2008, Chu *et al*. 2008). Once encountering an antigen, FO B cells move into the germinal center and, upon additional signals given by T cells, differentiate into high affinity IgG-producing plasma cells and memory B cells (Allman & Pillai 2008, Chu *et al*. 2008). This process lasts ~5 days, a time that can be crucial especially for fast replicating pathogens. However, this gap is covered by the B220^{+}CD23^{lo}CD21^{hi} MZ B cells that, due to their pre-activated phenotype and strategic localization in the marginal sinus area of the spleen, rapidly respond against pathogens, giving rise to short-life plasma cells producing low affinity antibodies (Cerutti *et al*. 2013). MZ B cells are also involved in the production of IgM and IgA natural antibodies that control the first wave of an infection.

In previous studies, we and others have demonstrated that pregnancy induces a strong suppression of B cell lymphopoiesis and proposed that this might represent an evolutionarily acquired mechanism tending to reduce the occurrence of autoreactive B cells that may recognize fetal structures causing pregnancy failures (Medina *et al*. 1993, Ait-Azzouzene *et al*. 1998, Muzzio *et al*. 2014). Despite B cell lymphopenia, we also showed that pregnant mice display an expansion of the pre-activated MZ B cells, suggesting an attempt to compensate for the reduced number of B cells during gravidity thus ensuring a proper defense (Muzzio *et al*. 2014).

In this study, using a well-established animal model of immune-mediated pregnancy failures, we aimed to deeply investigate the participation of B cells in pregnancy outcome.

Materials and methods

Animals

Eight-week-old female CBA/j (H2^{k}) as well as DBA/2J (H2^{d}) and BALB/c (H2^{d}) males were purchased from Charles River (Sulzfeld, Babavia, Germany). All mice were maintained in the facilities of the BioTechnikum Greifswald under a 12h light:12h darkness cycle with free access to water and chow. Animal experiments were carried out according to institutional guidelines after ministerial approval (institutional review board: Landesverwaltungsamt Sachsen-Anhalt (ID: FJ2-1019 to F J) and 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.

For our study, we used a well-established model of immune-mediated pregnancy disturbances (Clark *et al*. 1986). Shortly after CBA/J females were mated to DBA/2J males, a median of 20–30% of the embryos resorbed (aborted). In addition, DBA/2J pregnant CBA females also displayed angiogenic deregulation, abnormal placental development and fetal growth restriction, all features of pre-eclampsia (Kanasaki & Kalluri 2009, Ahmed *et al*. 2010). BALB/c-mated CBA/J females represented the normal pregnancy combination with a median of 0% abortions and none of the symptoms described above for the DBA/2J-mated CBA/J females. Age-matched virgin CBA/J females were mated with BALB/c or DBA/2J males. Females were inspected daily for vaginal plugs, and the presence of a vaginal plug was designated as day 0 of pregnancy. Pregnant females were sacrificed at day 14 of pregnancy.

Cell preparation and flow cytometry

For flow cytometry analysis, single cell suspensions were obtained from the isolated samples following previously described procedures (Muzzio *et al*. 2014). Briefly, cells were isolated from BM of the femur and tibia, spleen, blood and para-aortic lymph nodes that drain the uterus. Plasma was separated from heparinized whole blood after 10 min centrifugation at 1300***g*** and stored at −80 °C for further analysis. Organs were crushed in a 100 µm cell strainer to obtain a single cell suspension and red blood cells were lysed for 5 min. After washing, cell suspensions were counted using a Neubauer chamber, and 1×10^{6} cells were stained 30 min at 4 °C with specific labeled antibodies (Table 1) or isotype controls. It is important to note that, although CD45R (B220) is mainly expressed by the B cell lineage from early pro-B to mature B cells and it has been classically used as a B cell marker, according to eBioscience, it might also be expressed in some activated T cells, lymphokine activated killer cells, NK cell progenitors in the BM and T cells of the lpr/lpr mutant mouse. Therefore, in this study, apart from the analysis of total numbers of B cells in the spleen, this antibody has always been used in combination with other B cells markers. Data were acquired on FACSCalibur or FACS Canto (BD Biosciences, Hidelberg, Baden-Württemberg, Germany) and analyzed by using FlowJo software (Tree Star, Inc., Ashland, OR, USA). B cell populations were defined using gating strategies and the percentage was later referred to the cell count obtained by the

Table 1 Antibodies used in FACS analysis of the B cell populations in CBA/J mice.

Molecule	Antibody clone	Label	Alternate nomeclature
CD23	B3B4	PE	FcεRII
CD21	7G6	FITC	
CD19	1d3	PE-Cy7	
CD43	R2/60	FITC	
CD45R	RA3-6B2	PE-Cy7	B220
IgM	II/41	APC	
IgD	11-26c	PE	
CD93	AA4.1	FITC	C1qRp

FACS, fluorescence-activated cell sorting.

Neubauer chamber to obtain the absolute numbers of cells (Table 2).

Analysis of immunoglobulin in serum

Concentrations of IgM, IgG1, IgG2a, IgG2b, IgG3, IgE and IgA in plasma were quantified using the ProcartaPlex Mouse Antibody Isotyping Panel (eBioscience/Affymetrix, Frankfurt am Main, Hessen, Germany) and subsequently analyzed on Luminex test equipment (Bio-Plex® 200 Systems (Bio-Rad, Munich, Bavaria, Germany)). Before starting the assay, frozen plasma samples were thawed and centrifuged at 4 °C for 10 min at 1000***g***. A 1:10 000 dilution of the samples was made and the multiplex assay was performed on a 96-well filter plate as described in the manufacture's instructions. The standard curve range was as follows: IgG1 (0.69–500 ng/ml), IgG2a (5.49–4000 ng/ml), IgG2b (4.12–3000 ng/ml), IgG3 (10.25–7500), IgA (4.12–3000 ng/ml), IgE (0.69–500 ng/ml) and IgM (4.12–3000 ng/ml).

BAFF ELISA

Levels of the B-cell activating factor (BAFF) of the TNF family in serum were assayed using a commercially available ELISA kit from R&D Systems (Minneapolis, MN, USA), following the supplier's recommendations.

Statistical analysis

Data were analyzed with PRISM software (v. 5.01; GraphPad, La Jolla, CA, USA). To evaluate the differences of means among the groups, ANOVA followed by Tukey's multiple comparison test or Kruskal–Wallis test were used. Significant differences are indicated with asterisks (*$P=0.05$, **$P=0.01$, ***$P=0.001$).

Results

Normal pregnant as well as pregnant mice suffering from pregnancy disturbances display strong suppression of B cell lymphopoiesis

Suppression of B lymphopoiesis in the BM during pregnancy has been previously demonstrated and was postulated to represent a physiological mechanism tending to reduce the occurrence of autoreactive B cells, thus preventing pregnancy failures (Medina *et al.* 1993, Muzzio *et al.* 2014). Here, using an animal model of immune-mediated pregnancy disturbances, we observed that both pregnant mice undergoing normal pregnancies and those naturally suffering from pregnancy disturbances display significantly lower numbers of $B220^{lo}sIgM^{-}$ pre- and pro-B cells as well as $B220^{+}sIgM^{-}$ immature B cells in the BM as compared to non-pregnant control mice (Fig. 1A). Further analysis showed that a number of $B220^{lo}IgM^{-}CD43^{-}$ pre-B cells as well as $B220^{lo}IgM^{-}CD43^{+}$ pro-B cells were significantly decreased in the BM of normal pregnant mice and pregnant mice naturally suffering from pregnancy disturbances compared to non-pregnant animals (Fig. 1B). However, when the number of $B220^{+}sIgM^{+}$ mature B cells was evaluated, a significant reduction was observed in the BM of pregnant mice suffering from pregnancy disturbances compared to non-pregnant control mice (Fig. 1A). Pregnant mice undergoing normal pregnancies showed a slight, but not significant, reduction in the number of $B220^{+}sIgM^{+}$ mature B cells compared to non-pregnant mice (Fig. 1A). In summary, B cell lymphopoiesis is reduced in the BM of pregnant mice regardless of pregnancy outcome.

Table 2 B cell subsets analyzed with their corresponding markers.

Phenotype	B cell subset	Organ
$B220^{int}sIgM^{-}$	Pre/pro	Bone marrow
$B220^{int}sIgM^{-}CD43^{-}$	Pre	Bone marrow
$B220^{int}sIgM^{-}CD43^{+}$	Pro	Bone marrow
$B220^{int}sIgM^{+}$	Immature	Bone marrow
$B220^{+}sIgM^{+}$	Mature	Bone marrow
$B220^{+}CD23^{lo}CD21^{hi}$	MZ	Spleen
$B220^{+}CD23^{hi}CD21/35^{int}$	FO	Spleen
$B220^{+}CD21^{+}$	Mature	Para-aortic lymph node

MZ B cells are expanded in the spleen of normal pregnant mice but not in those suffering from pregnancy disturbances

In a previous study, we demonstrated that MZ B cell numbers were expanded in the spleen during pregnancy and proposed that this could be a compensatory mechanism launched to balance the lower influx of B cells observed in this tissue, thus maximizing the capacity of the maternal immune system to control pathogens (Muzzio *et al.* 2014). To test this hypothesis, we began analyzing the number of total B cells in the spleen of normal pregnant as well as pregnant mice naturally suffering from pregnancy disturbances and observed that, regardless of pregnancy outcome, pregnant mice showed a significantly lower number of total $B220^{+}$B cells in the spleen compared to non-pregnant control animals (Fig. 2A). These results prompted us to further investigate whether the distribution of main populations of mature B cells, namely, follicular and MZ B cells, in the spleen could also be altered during normal and pathological pregnancies. Indeed, we observed that the number of $B220^{+}CD23^{hi}CD21^{int}$ FO B cells was significantly decreased ($12.63\pm0.44\times10^{6}$ cells to $10.56\pm0.78\times10^{6}$ cells) in normal pregnant mice compared to non-pregnant controls (Fig. 2B). Diminution of FO B cell numbers was even more pronounced in pregnant mice suffering from pregnancy disturbances ($12.63\pm0.44\times10^{6}$ cells to $9.49\pm0.61\times10^{6}$ cells; Fig. 2B). Interestingly, unlike FO B cells, the number of $B220^{+}CD23^{lo}CD21^{hi}$ MZ B cells was

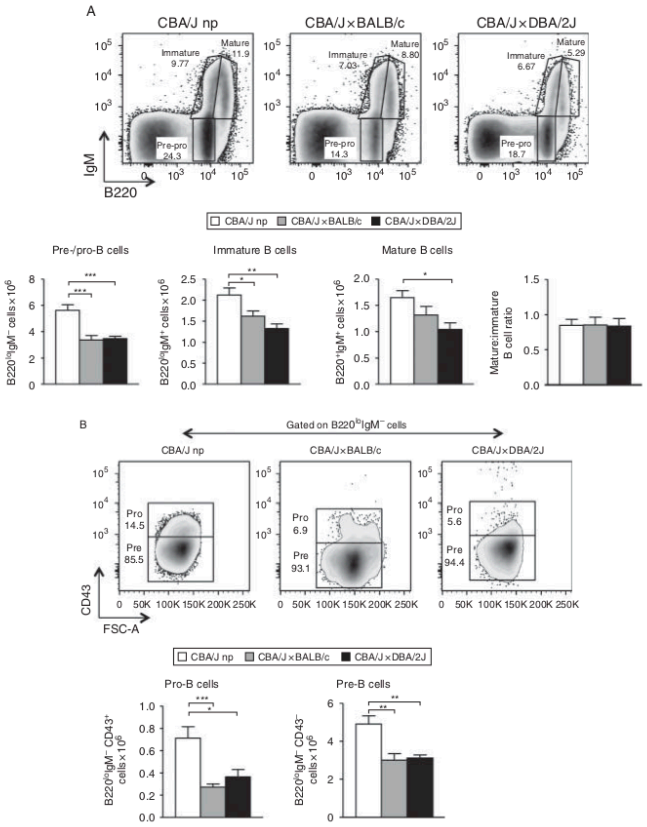

Figure 1 B cell lymphopoiesis is strongly reduced in normal pregnant as well as in pregnant mice suffering pregnancy failures. (A) Representative density plots displaying percentage and gating strategy used to analyze the population of pre- and pro- ($B220^{lo}sIgM^{-}$), immature ($B220^{lo}sIgM^{+}$) and mature ($B220^{+}sIgM^{+}$) B cells in the bone marrow (BM) of non-pregnant (np) CBA/J mice, BALB/c-mated CBA/J females (normal pregnant combination) and DBA/2J-mated CBA/J females (pregnant mice suffering pregnancy disturbances). Bar graphs show quantification of total numbers of pre- and pro-, immature and mature B cells as well as the ratio of mature vs immature B cells in the BM of non-pregnant CBA/J ($n=13$), BALB/c-mated normal pregnant mice ($n=12$) and DBA/2J-mated CBA/J females (mice suffering pregnancy disturbances) ($n=10$). (B) Density plots showing percentage and gating strategy applied to differentiate pro-B cells ($B220^{lo}IgM^{-}CD43^{+}$) from pre-B cells ($B220^{lo}IgM^{-}CD43^{-}$) in the BM of non-pregnant CBA/J mice, BALB/c-mated CBA/J and DBA/2J-mated CBA/J females. Bar graphs show quantification of total numbers of pre- and pro-B cells in the BM of non-pregnant CBA/J ($n=13$), BALB/c-mated ($n=12$) and DBA/2J-mated CBA/J ($n=10$) females. Data are expressed as mean ±S.E.M. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ as analyzed by the one-way ANOVA, followed by a Tukey multiple comparison test.

significantly higher in normal pregnant mice compared to non-pregnant animals and pregnant mice suffering from pregnancy disturbances (3.02 ± 0.312–2.14 ± 0.16 and $1.31\pm0.18\times10^{6}$ cells respectively; Fig. 2B). We have additionally performed the ratio between MZ and FO B cells and observed a clear preponderance of MZ over FO B cells in the spleen of normal pregnant mice compared to non-pregnant and pregnant mice suffering from pregnancy disturbances (Fig. 2B). In summary, normal pregnant mice, but not pregnant females, suffering from pregnancy disturbances exhibited an expansion of the MZ B cells compartment despite B cell lymphopenia observed in the spleen.

Enhanced levels of MZ-produced immunoglobulin in serum of normal pregnant mice

Having observed that a number of MZ B cells were increased in the spleen of normal pregnant mice even though total B cell influx was reduced in this tissue, we next focused on the functionality of these cells in terms of antibody production. As shown in Fig. 3, serum levels of IgM, the most prominent MZ B cell-produced immunoglobulin (Guinamard *et al.* 2000, Martin & Kearney 2002), were significantly higher in the serum of normal pregnant mice compared to non-pregnant control animals as well as pregnant mice suffering from pregnancy disturbances. Similarly, serum levels of IgA, another immunoglobulin classically produced by MZ B cells (Martin & Kearney 2002, Cerutti *et al.* 2013), were also significantly higher in the serum of normal pregnant mice compared to non-pregnant control mice. When compared to pregnant mice having pathological pregnancies, the levels of IgA in the serum of normal pregnant mice were slightly, but not significantly, higher (Fig. 3). We have additionally analyzed the levels of IgG1, IgG2a, IgG3 and IgE and observed that all of these immunoglobulin were barely, not significantly, augmented in the serum of normal pregnant mice as compared to non-pregnant and pregnant mice suffering from

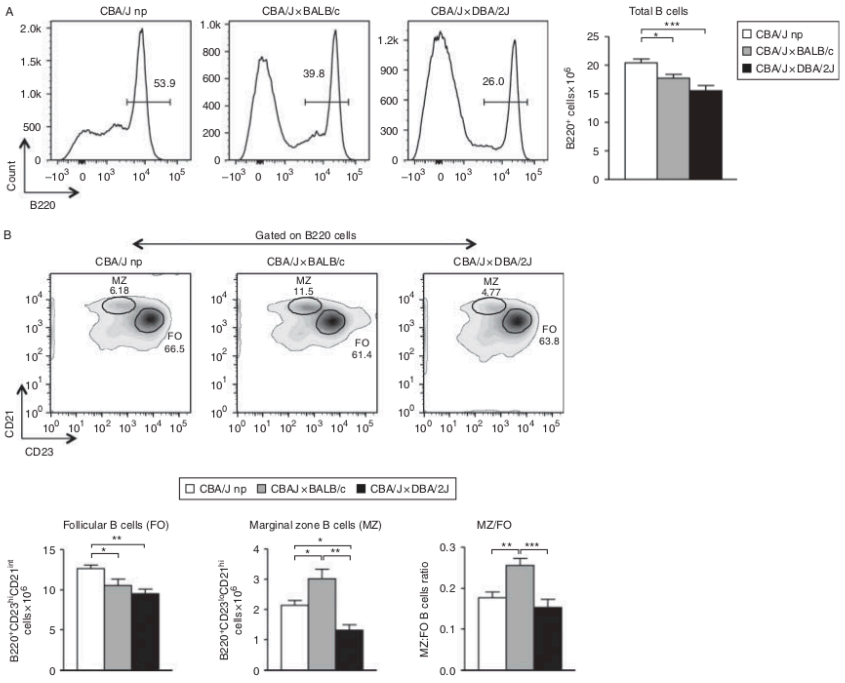

Figure 2 Marginal zone B cell numbers are increased in the spleen of normal pregnant mice. (A) Representative histograms showing the expression of B220 in splenocytes from non-pregnant (np) CBA/J as well as BALB/c-mated CBA/J females (normal pregnant combination) and DBA/2J-mated CBA/J females (pregnant mice suffering pregnancy disturbances). Numbers on histogram indicate the percentage of $B220^+$ cells. Bar graph shows total numbers of B220 B cells in the spleen of non-pregnant CBA/J ($n=13$), BALB/c-mated CBA/J ($n=12$) and DBA/2J-mated CBA/J ($n=10$) females. (B) Representative density plots displaying percentage and gating strategy used to analyze $B220^+CD23^{hi}CD21^{int}$ follicular (FO) B cells (low right circle) and $B220^+CD23^{lo}CD21^{hi}$ marginal zone (MZ) B cells (upper left circle). Bar graphs show total number of FO and MZ B cells as well as the ratio between MZ and FO B cells in the spleen of non-pregnant CBA/J ($n=13$), BALB/c-mated CBA/J ($n=12$) and DBA/2J-mated CBA/J ($n=10$) females. Data are expressed as mean ± S.E.M. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ as analyzed by the one-way ANOVA, followed by a Tukey multiple comparison test.

pregnancy disturbances. In summary, normal pregnancy seems to be associated with an enhanced production of immunoglobulin by MZ B cells.

Reduced levels of BAFF in normal pregnant as well as pregnant mice suffering from abortions

Taking into account the fact that we have previously described a reduction in the levels of BAFF during normal pregnancies in mice (Muzzio *et al*. 2014), we next wondered whether this is also true for pregnant animals suffering from immune-mediated pregnancy disturbances. As expected, we observed a significant reduction in the levels of BAFF in the serum of normal pregnant mice compared to non-pregnant control animals (from 8745 ± 312.1 to 7622 ± 284.8 pg/ml; Fig. 4). Interestingly, the reduction in the levels of BAFF was even more pronounced in the case of pregnant mice undergoing pregnancy disturbances compared to non-pregnant control animals (from 8745 ± 312.1 to 6959 ± 283.4 pg/ml; Fig. 4). There were no significant differences in the levels of BAFF between normal pregnant mice and those suffering from pregnancy failures (Fig. 4).

Increased number of mature B cells in the lymph nodes draining the uterus of normal pregnant as well as pregnant mice suffering from abortions

It has been previously demonstrated that the proportion (Newport & Carter 1983) as well as the total number of B cells (Muzzio *et al*. 2014) in the para-aortic lymph nodes (PLN) draining the uterus is increased during pregnancy. In this study we first confirmed this by showing that

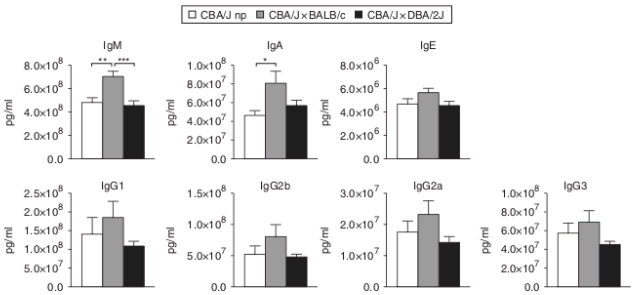

Figure 3 Levels of immunoglobulin in serum of non-pregnant and pregnant mice. Concentrations of IgM, IgA, IgG1, IgG2b, IgG3, IgG2a and IgE were evaluated in the serum of non-pregnant (np) CBA/J females ($n=13$) as well as BALB/c-mated (normal pregnant mice) ($n=12$) and DBA/2J-mated CBA/J females (suffering pregnancy disturbances) ($n=10$). Data are expressed as mean±S.E.M. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ as analyzed by the one-way ANOVA, followed by a Tukey multiple comparison test.

normal pregnant mice displayed a significantly higher number of $B220^{+}CD21^{+}$ mature B cells in PLN compared to non-pregnant mice (Fig. 5) and then extended these results to show that pregnant animals suffering from abortions also displayed a significantly higher number of $B220^{+}CD21^{+}$ mature B cells in PLN compared to non-pregnant mice (Fig. 5). In summary, during pregnancy, the number of mature B cells is increased in the uterine-draining lymph nodes regardless of pregnancy outcome.

Discussion

We demonstrated in this study that normal pregnant mice but not those suffering from immune-mediated pregnancy failure can compensate for the strong reduction of B cell lymphopoiesis as well as splenic B cell lymphopenia occurring during gravidity by inducing mechanisms tending to favor the presence of the pre-activated MZ B cells and the production of natural protective antibodies. Successful pregnancy in mammals relies on the capacity of the maternal immune system to simultaneously tolerate the semi-allogeneic fetus and protect the mother against potential infections (Arck & Hecher 2013). This requires strong and highly regulated adaptations of maternal immunity. Suppression of B cell lymphopoiesis has been postulated to be one of these mechanisms, representing an evolutionarily acquired strategy tending to reduce the occurrence of autoreactive B cells that might recognize fetal structures and put pregnancy at risk (Medina *et al.* 1993, Ait-Azzouzene *et al.* 1998, Muzzio *et al.* 2014). However, the fact that a lower number of B cells are being produced during this critical period of time can also compromise the capacity of the maternal immune system to fight pathogens. Hence, it becomes evident that additional adaptations are demanded. Indeed, using a mouse model of immune-mediated pregnancy failure, we demonstrated in this study that the suppression of B cell lymphopoiesis seems not to be directly associated with pregnancy outcome as both normal pregnant and pregnant mice suffering from immune-mediated pregnancy disturbances showed a significant reduction of B cell lymphopoiesis in their BM. These results prompted us to further investigate whether, independently of a direct effect on pregnancy well-being, the reduction of B cell lymphopoiesis during gravidity might affect B cell differentiation in the periphery. To analyze this, we then focused on the spleen, which is the tissue in which immature B cells arriving from the BM become either FO or MZ B cells, the most prominent mature B cell in the body (Monroe & Dorshkind 2007, Allman & Pillai 2008). These two B cell subtypes have different but complementary functions (Monroe & Dorshkind 2007, Allman & Pillai 2008). In a process that lasts ~5 days after encountering an antigen, FO B cells produce T cell-dependent high affinity antibodies, mainly IgG subtypes (Cerutti *et al.* 2013). In contrast, due to their pre-activated phenotype, shortly after encountering an antigen, MZ B cells produce, in a T cell independent fashion, low affinity antibodies that are crucial for controlling the first wave of infection (Martin *et al.* 2001, Colino *et al.* 2002). In the context of pregnancy,

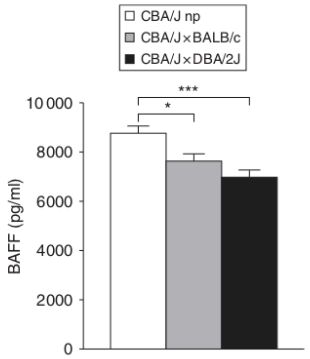

Figure 4 Levels of B cell-activating factor (BAFF) in the serum of non-pregnant and pregnant mice. The concentration of BAFF was evaluated in serum of non-pregnant CBA/J females ($n=13$) as well as BALB/c-mated CBA/J normal pregnant mice ($n=12$) and DBA/2J-mated CBA/J pregnant mice suffering from pregnancy disturbances ($n=10$). Data are expressed as mean±S.E.M. $^{*}P<0.05$, $^{***}P<0.001$ as analyzed by the one-way ANOVA, followed by a Tukey multiple comparison test.

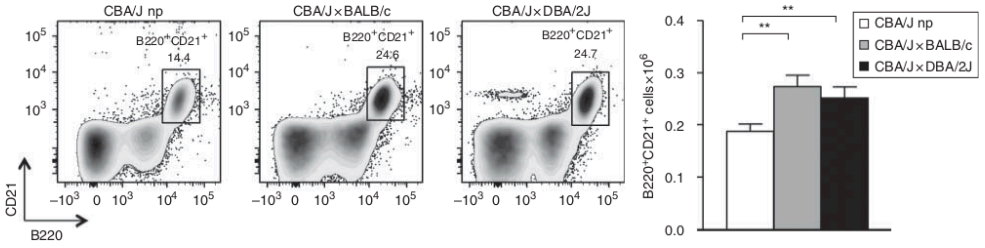

Figure 5 Mature B cell numbers are expanded in the lymph nodes draining the uterus during pregnancy. Representative density plots displaying the percentage as well as gating strategy used to analyze $B220^+CD21^+$ mature B cells in the para-aortic lymph nodes of non-pregnant (np) CBA/J females ($n=13$) as well as BALB/c-mated CBA/J normal pregnant mice ($n=12$) and DBA/2J-mated CBA/J pregnant mice suffering from pregnancy disturbances ($n=10$). Data are expressed as mean ± S.E.M. $^{**}P<0.01$ as analyzed by the one-way ANOVA, followed by a Tukey multiple comparison test.

we have recently demonstrated that, although the total number of B cells as well as FO B cells is reduced, the number of pre-activated MZ B cells is increased in the spleen of pregnant mice (Muzzio *et al.* 2014). Based on these results we proposed that the expansion of the pre-activated MZ B cell compartment may represent an acquired mechanism launched to compensate the lower influx of B cells into the spleen, thus maximizing the capacity of the maternal immune system to control infections (Muzzio *et al.* 2014). Indeed, we confirm here that normal pregnant mice, but not those suffering from immune-mediated pregnancy failures, were able to induce an expansion of MZ B cells even in the presence of a splenic B cell lymphopenia. Interestingly, it has been previously proposed that the high incidence of pregnancy failure naturally occurring in the DBA/2J-mated CBA/J females is correlated with their higher susceptibility to environmental antigens (Hamilton & Hamilton 1987) and can be prevented or reduced by transferring natural antibodies from normal pregnant BALB/c-mated CBA/J females (Chaouat *et al.* 1985, Hamilton & Hamilton 1987). Natural antibodies are crucial components of the first-line defense against invading pathogens and also have a homeostatic role in regulating the clearance of necrotic and apoptotic cells, thus preventing inflammatory reactions (Schwartz-Albiez *et al.* 2009). They are produced by MZ and B-1a B cells in the absence of antigen stimulation (Kaveri *et al.* 2012, Silverman *et al.* 2013). The majority of natural antibodies consist of IgM with a smaller proportion of IgA subtype (Schwartz-Albiez *et al.* 2009). Notably, in addition to an expansion of MZ B cells, we observed significantly higher levels of IgM and, in a minor extent, IgA, in the serum of normal pregnant mice compared to non-pregnant or mice suffering pregnancy disturbances. Hence, it becomes evident that pregnancy well-being relies on a proper expansion and activation of the MZ B cell compartment.

We next concentrated on investigating factors involved in the differentiation of immature B cells into MZ B cells or FO B cells. The TNF family ligand BAFF/Blys is an essential growth factor for B cell development and maintenance (Schiemann *et al.* 2001, Schneider *et al.* 2001, Pillai & Cariappa 2009). We have previously shown that pregnant mice display lower levels of BAFF as compared to non-pregnant animals (Muzzio *et al.* 2014). Here we first confirmed and then extended these results by demonstrating that mice suffering from immune-mediated pregnancy failures showed a more pronounced reduction in the levels of BAFF when compared to non-pregnant animals. However, we did not observe significant differences in the levels of BAFF between normal pregnant mice and those suffering pregnancy disturbances. Hence, it becomes evident that other factors rather than BAFF control the dynamic of MZ B cells during gravidity. Pregnancy well-being has been classically associated with a shift toward a Th2 profile, which favors the development of humoral over cellular immunity (Saito *et al.* 2010). In this regard, our results concerning elevated levels of IgM and IgA – immunoglobulin more likely produced by MZ B cells – in serum of normal pregnant mice seem to at least partially support this idea. Noteworthy, levels of IgG, a FO B cell-produced immunoglobulin, did not show significant differences among the groups. T follicular helper (Tfh) cells are known to be crucial in regulating antibody production by controlling isotype class switching and plasma cell differentiation, a process involving FO B cells (Crotty 2011). Furthermore, Tfh cells are also able to induce MZ B cell apoptosis (Tortola *et al.* 2010). Taking this into account, our results might suggest a lack of Tfh activity during pregnancy, favoring a survival of MZ B cells and a shift toward T cell independent antibody response.

In summary, we have demonstrated in this study that the suppression of B cell lymphopoiesis occurring during gravidity in mice does not seem to directly affect pregnancy outcome. However, it has a strong impact on the influx of B cells into the spleen, as a splenic B cell lymphopenia was observed in pregnant mice. Notably,

normal pregnant animals, but not those suffering from immune-related pregnancy failures, could compensate B cell lymphopenia by inducing an expansion of the pre-activated MZ B cells and the production of natural antibodies, thus maximizing the capacity of immune defense and avoiding undesired inflammatory reactions. Whether the increased number of MZ B cells, observed during normal pregnancy, is due to a preferential differentiation process of this B cell lineage or simply an expansion of pre-existing MZ B cells in the spleen due to an increased proliferation or decreased apoptosis rate should be addressed in the future.

Our results clearly highlight the fundamental role of the MZ B cells in the intricate balance between immune suppression and activation launched during pregnancy to simultaneously tolerate the presence of the semi-allogeneic fetus and ensure a proper defense of the mother against pathogens. Further studies are required in order to identify factors controlling this phenomenon.

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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6.2 Human pregnancy is accompanied by modifications in B cell development and immunoglobulin profile

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Human pregnancy is accompanied by modifications in B cell development and immunoglobulin profile

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ABSTRACT

Though human pregnancy success has been classically linked with a shift into a Th2 immunoglobulin producing cell response, a clear picture concerning B cell development and immunoglobulin profile during human pregnancy is missing. We analyzed in this work the dynamic of different B cell populations in peripheral blood of pregnant women on the first, second and third trimester of pregnancy. As control, age-matched non-pregnant fertile women were included. Additionally, we quantified the levels of immunoglobulin (IgG1, IgG2, IgG3, IgG4, IgM, IgA and IgE) in the serum of pregnant and non-pregnant women. We observed a significant decrease in the percentages of transitional B cells in peripheral blood of pregnant women as compared to non-pregnant control women. Besides, percentages of naïve as well as switched and non-switched memory B cells in peripheral blood of pregnant women were similar to those in non-pregnant control women. Interestingly, although we did not observe differences in the activation status of B cells as well as in the percentages of plasma cells between pregnant and non-pregnant women, we observed significantly higher levels of IgM, IgA, IgG_3, more likely natural antibodies, as well IgG_4 in serum of pregnant women compared to non-pregnant age matched control women.

1. Introduction

It is very well known that the maternal cellular as well as humoral arms of the acquired immune system have to undergo an adequate adaption to prevent pregnancy complications: on the one hand to tolerant fetal semi-allogeneic cells expressing foreign surface molecules, on the other hand to ensure a quick and efficient local protection against infections. B cells, through their antibody production and regulatory capacities, are mayor players in the maintenance of immune homeostasis (LeBien and Tedder, 2008). In the adult, B cells are generated in the bone marrow and then migrate to the periphery at the immature or transitional stages. At this point, they are still short-lived and functionally immature (Chung et al., 2001, 2003). B cell lymphopoiesis, including B cell retention, daily output and maturation, is commanded by growth factors and adhesion molecules as well as not complete known mechanisms. Previously, our group and others have demonstrated that in mice, pregnancy induces a strong suppression on B cell lymphopoiesis that affects the distribution of main B cell populations in the periphery (Muzzio et al., 2014, 2016; Medina et al., 1993). We have further demonstrated that this suppression significantly affects the numbers as well as the distribution of main B cell populations in the periphery (Muzzio et al., 2014, 2016). Transitional B cells are transported by the bloodstream to the spleen where they develop into long-lived mature B cells and subsequently recirculate into different tissues (Carsetti et al., 2004). Transitional B cells mature either into marginal zone B cells (MZ) or to follicular B cells (FO). While FO B cells differentiate to plasmablasts and short-lived plasma cells as well as the MO B cells, FO B cells are capable for providing memory as well as long-lived plasma cells. FO and also long- lived plasma cells do not remain in spleen rather recirculate in blood and peripheral lymph organs and bone marrow. Hence, human peripheral B cells can be taken as hallmark of the state of B-cell production and function (Caraux et al.,

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2010). In this regards, different B cell markers as well as gating strategies have been used in order to identify B cell subpopulations in peripheral blood. Carsetti et al. have shown that using IgD in combination with CD27 permits the discrimination between naïve and switched/non- switched memory B cells. Besides, the same group showed that human peripheral transitional B cells express high levels of CD38 and CD24 (Carsetti et al., 2004; Maecker et al., 2012). Furthermore, immunoglobulin producing cells or plasma cells represents the final stage on B cell development (Calame et al., 2003). In human peripheral blood, plasma cells are identified by the expression of CD27 and CD138 (Fernandes and Snider, 2010).

Recently, we have shown that several B cell subsets as well as their immunoglobulin signature are highly modified in murine pregnancies (Muzzio et al., 2014, 2016). The main aim of this work was to perform a detailed characterization of the B cell compartment in peripheral blood during pregnancy including immunoglobulin profile in serum.

2. Material and methods

2.1. Human subjects

All experiments including samples from human subjects were reviewed and approved by the Ethics Committee of the Medical Faculty, Greifswald University (BB 126/13 to FJ). All individuals were properly informed concerning the purpose of our research and gave their written consent before sampling. The characteristics of the recruited participants are summarized in Table 1.

Human blood samples from voluntary non-pregnant and pregnant women were obtained by the Department of Obstetrics and Gynecology Greifswald University. Pregnant women included in this study lacked of diagnosed immunological disease or acute or chronic inflammation by the time blood was collected. The pool of non-pregnant women represents women in reproductive age independent from their menstrual cycle.

We sub-classified the blood samples in four different groups: non-pregnant (np), late first trimester (1^{st}), late second trimester (2^{nd}) and late third trimester (3^{rd}) of pregnancy. All blood samples were processed directly after collection for the peripheral blood mononuclear cell (PBMC) isolation and serum separation. Sera separation was performed by 10 min centrifugation at 1300 × *g* and 20 °C. Sera were stored at − 80 °C until analyzed.

2.2. Reagents

Following reagents were used in this work: Lymphoprep from STEMCELL Technologies (Oslo, Norway), PBS from Biochrom GmbH (Berlin, Germany), Cytofix/Cytoperm (BD, Plymouth, UK), Perm/Wash (BD, Plymouth, IK), BIO Plex Pro Human Isotyping Assay (Bio-Rad Laboratories GmbH, Munich, Germany) and Lysis Buffer (Qiagen, GmbH, Hilden, Germany).

2.3. Antibodies

The following antibodies were used: CD27 PE-Cy7 (M-T271), CD38 APC (HIT2), CD138 PE (M115), IgM PerCP-Cy5.5 (G20-127), CD24 PE (ML5), IgD FITC (IA6-2), CD20 APC-H7 (2H7), CD19 PerCP-Cy5.5 (HIB19), CD25 APC (M-A251), CD69 PE-Cy7 (FN50).

For a better understanding of the different B cell populations analyzed in this work please see Table 2.

Table 2
B-cell subsets and their corresponding phenotype.

Phenotype	Name	Reference
$CD24^{bright}CD38^{bright}IgD^{+}IgM^{+}$	Transitionals	Carsetti et al. (2004)
$CD24^{bright}CD38^{bright}CD10^{+}IgM^{+}$	Immature	Carsetti et al. (2004)
$CD19^{+}CD27^{+}IgD^{-}$	Memory IgD^{-}	Weller et al. (2008)
$CD19^{+}CD27^{+}IgD^{+}$	Memory IgD^{+}	Weller et al. (2008)
$CD19^{+}CD27^{-}IgD^{+}$	Naive	Weller et al. (2008)
$CD138^{+}CD27^{+}$	Plasmablasts	Cepok et al. (2005)

2.4. Cell preparation and flow cytometry

Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood of pregnant and non-pregnant age matched control women by density gradient centrifugation using Lymphoprep medium (STEMCELL Technologies, Cologne, Germany). PBMCs (1×10^6 cells) were stained for 30 min at 4 °C with specific anti-human antibodies (Table 2). Gates were based on published bibliography and set by comparison with Fluoresence Minus One (FMO) controls. Stained cells were preserved at 4 °C and analyzed by flow cytometry. Data were acquired by using FACSCantoTM flow cytometer (BD Bioscience, Heidelberg, Germany). Data analysis was done by FlowJo software V10 (TreeStar Inc., Ashland, Oregon).

2.5. Analysis of immunoglobulin in serum

Quantitative serum determination of immunoglobulin isotypes IgG1, IgG2, IgG3, IgG4, IgA, IgM and IgE was performed by using a Bio-Plex ProTM Human Isotyping Assay (Bio-Rad, Munich, Germany) and subsequently analyzed on Bio-Plex ManagerTM software, Version 5.0 (Bio-Rad, Munich, Germany).

2.6. Statistical analysis

Statistical analysis was performed using PRISM software (ver. 5.01; GraphPad, La Jolla, USA). To estimate the significance of differences between the groups, Kruskal-Wallis test with Dunn's post test was used with a significant level alpha = 0.05 (95% confidence intervals). Significant differences are indicated with asterisks (*P < 0.05; **P < 0.01; ***P < 0.001).

3. Results

3.1. Percentages of transitional B cells are diminished while naïve and memory B cells are not altered in peripheral blood during pregnancy

We began this work by analyzing main B cells populations in peripheral blood of pregnant and non-pregnant women. As shown in Fig. 1, we observed that percentages of $CD19^{+}CD24^{bright}CD38^{brigth}$ transitional B cells were significantly diminished toward third trimester in peripheral blood (PB) of pregnant women as compared to non-pregnant control women and pregnant women on the first trimester (Fig. 1).

Table 1
Characteristics of the studied population of women in reproductive age without immunological disease or inflammation.

	Non-pregnant (n = 13) Mean ± SD	1^{st} trimester of pregnancy (n = 11) Mean ± SD	2^{nd} trimester of pregnancy (n = 15) Mean ± SD	3^{rd} trimester of pregnancy (n = 64) Mean ± SD
Age (years)	24.54 ± 3.072	30.55 ± 6.157	28 ± 6.612	30.08 ± 4.745
Week of gestation		9.250 ± 2.062	21.09 ± 4.805	35.56 ± 3.457

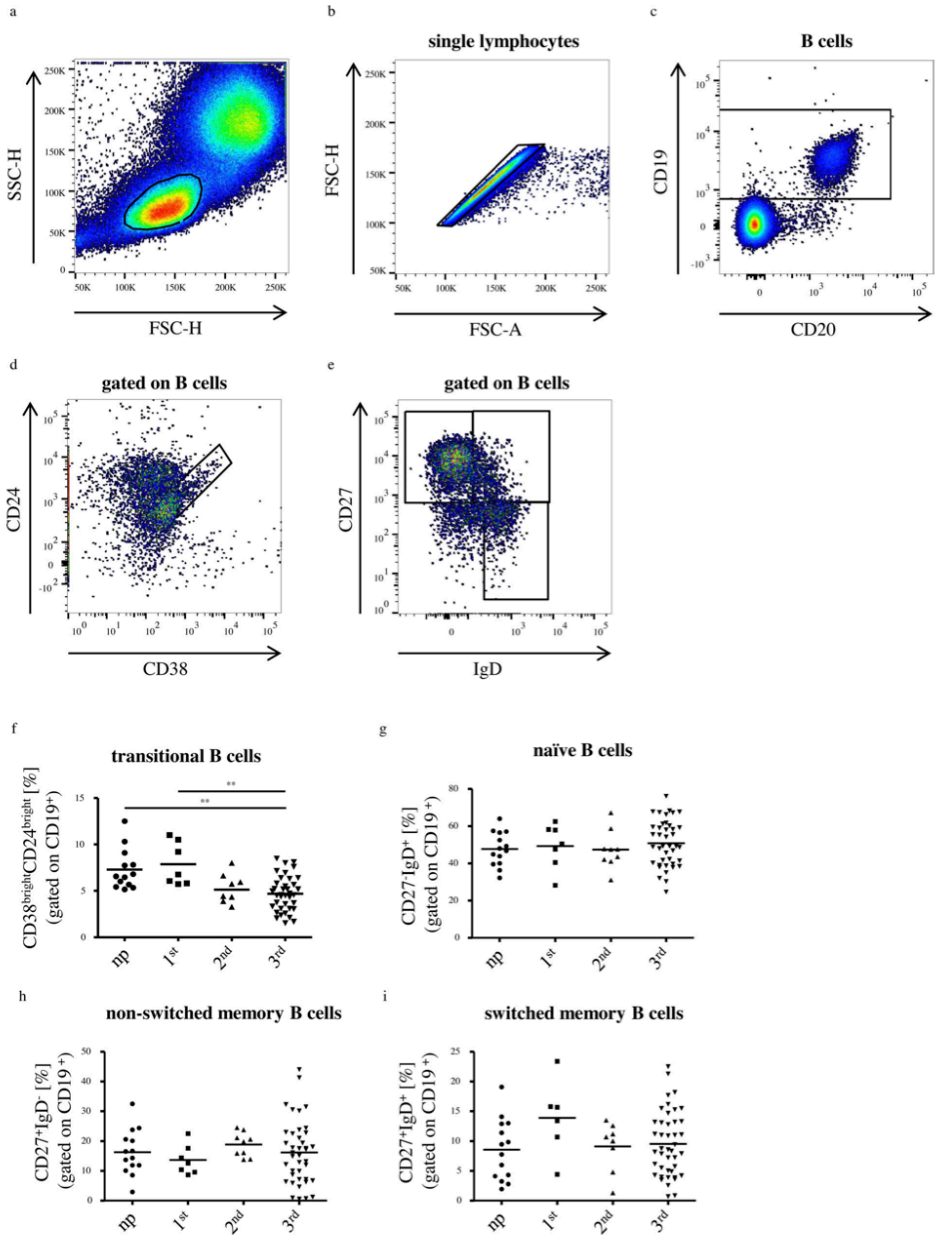

Fig. 1. Analysis of naïve, switched and non-switched memory B cells in peripheral blood of pregnant and non-pregnant women. Representative dotplots showing gating strategy used to analyze naïve as well as memory B cells (a–e). $CD19^+$ gated B cells (c) were analyzed for the expression of CD27 and IgD (e). Naïve B cells were defined as $CD19^+IgD^+CD27^-$, non-switched memory B cells as $CD19^+IgD^-CD27^+$ and switched-memory B cells as $CD19^+IgD^+CD27^+$. Graphs show percentages of transitional (f), naïve (g), non-switched memory (h) and switched (i) B cells in peripheral blood of pregnant women on the first (1^{st}) second (2^{nd}) and third (3^{rd}) trimester as well as in non-pregnant (np) women (n = 7, 7, 40 and 14 respectively). Statistical differences were analyzed by Kruskal-Wallis test with Dunn's post-test.

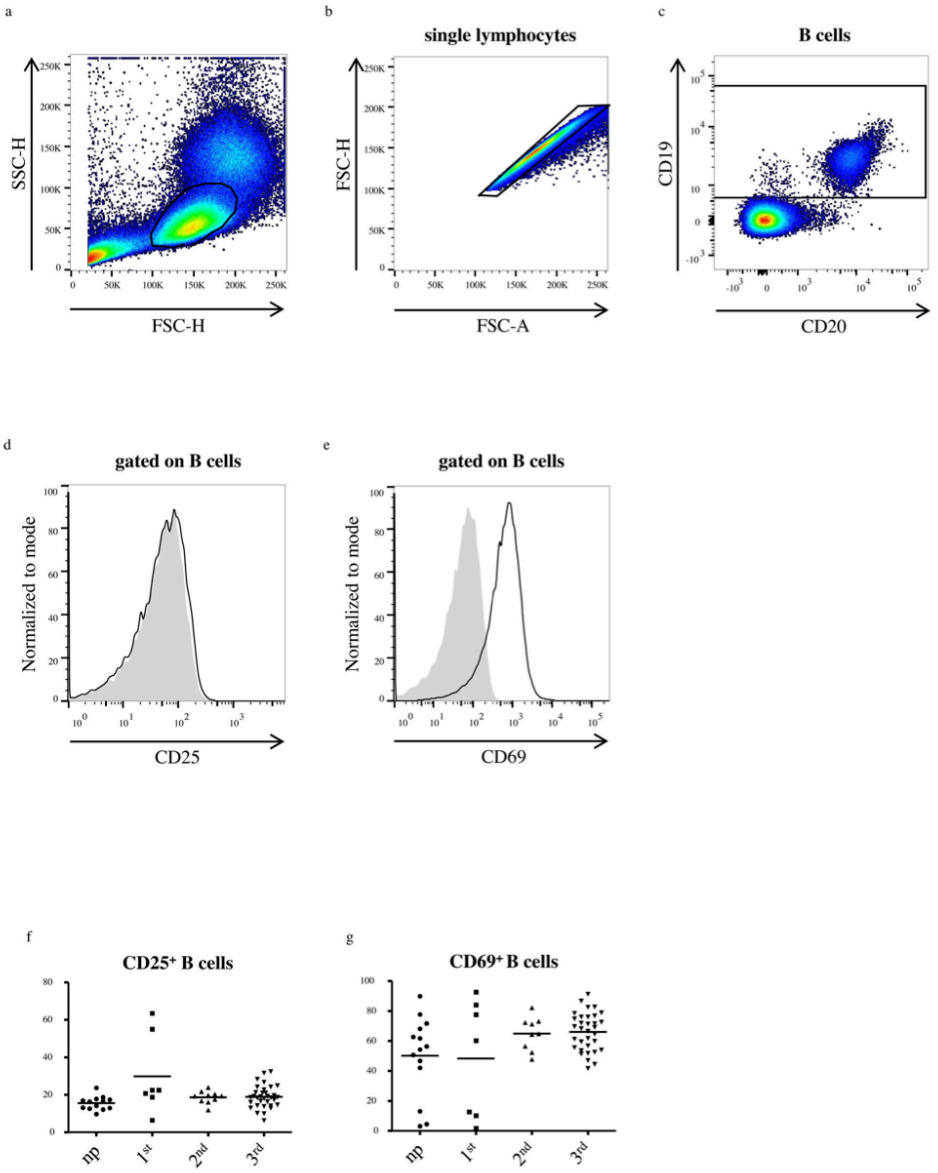

Fig. 2. Expression analysis of CD25 and CD69 on peripheral B cells during pregnancy. Representative dot plots (a–c) and histograms (d and e) displaying gating strategy used to analyze expression of CD25 and CD69 (empty histogram) against FMO control (filled histogram) on B cells during pregnancy. Graphs show percentages of CD25 (f) and CD69 (g) expressing B cells in peripheral blood of pregnant women on the first (1^{st}) second (2^{nd}) and third (3^{rd}) trimester as well as in non-pregnant (np) women (n = 7, 11, 39 and 14 respectively). Statistical differences were analyzed by Kruskal-Wallis test with Dunn's post-test.

Unlike transitional B cells, percentages of $CD19^+CD27^-IgD^+$ naïve B cells as well as $CD19^+CD27^+IgD^-$ non-switched and $CD19^+CD27^+IgD^+$ switched memory B cells were not significantly modified throughout pregnancy (Fig. 1).

3.2. B cells activation status seems not to be affected by pregnancy

Next, we investigated whether pregnancy induces modifications in B cell activation. To do so, we analyzed the expression levels of B cell activation markers: CD69 and CD25 in $CD19^+$ gated B cells in

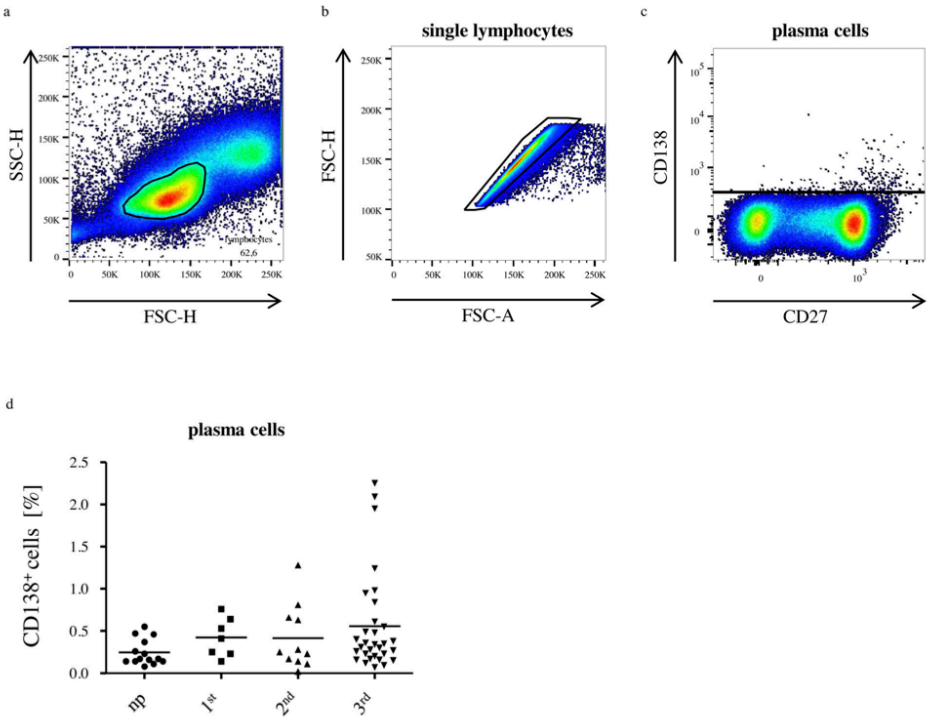

Fig. 3. Percentages of plasma cells in peripheral blood of pregnant and non-pregnant women. Representative dot plots displaying gating strategy used to analyze plasma cells during pregnancy (a–c). Graphs show percentages of $CD138^+$ plasma cells in peripheral blood of pregnant women on the first (1^{st}) second (2^{nd}) and third (3^{rd}) trimester as well as in non-pregnant (np) women (d) (n = 7, 11, 31 and 14 respectively). Statistical differences were analyzed by Kruskal-Wallis test with Dunn's post-test.

peripheral blood of pregnant and non-pregnant women. As shown in Fig. 2, neither expression levels of CD25 nor CD69 on $CD19^+$ B cells were significantly modified throughout pregnancy as compared to non-pregnant control women (Fig. 2).

3.3. Plasma cells levels are not modified in peripheral blood of pregnant women

The final stage in B cell development is the differentiation into antibody-producing cells or plasma cells. We next evaluated the percentages of $CD138^+$ plasma cells in peripheral blood of pregnant and non-pregnant women. As shown in Fig. 3, besides a slight not significant increase in the percentages of $CD138^+$- plasma cells during pregnancy (1^{st}, 2^{nd} and 3^{rd} trimester), percentages of plasma cells were not significantly altered as compared to non-pregnant control women (Fig. 3).

3.4. Pregnancy induces alterations in immunoglobulin profile

It is broadly accepted that pregnancy wellbeing is associated with a shift from Th1 ("cell mediated") into Th2 ("humoral") immune profile (Raghupathy, 1997). Yet, the kinetic of different immunoglobulin subtypes throughout pregnancy was not properly defined. We observed here that the levels of IgM and IgA were significantly increased in the serum of pregnant women on the first trimester and then drops (second and third trimester) to the levels observed in non-pregnant control women (Fig. 4). The analysis of the different IgG subclasses depicted a significant increase of IgG_1 in the serum of pregnant women already on the first trimester and maintained on the second and third trimester as compared to non-pregnant control women (Fig. 4). Compared to non-pregnant women, levels of IgG_3 showed a modest, not significant increase in serum of pregnant women on the first trimester that reached significance at the second trimester, dropping again toward the third trimester (Fig. 4). Levels of IgG_2 and IgG_4 did not show significant changes through pregnancy (Fig. 4). Similarly, levels of IgE in serum were not significantly modified during pregnancy (Fig. 4).

4. Discussion

Human pregnancy wellbeing has been classically associated with a shift into a Th2-humoral-mediated immunity (Wegmann et al., 1993; Lin et al., 1993), which protects the semi-allogeneic fetus from being rejected by Th1-cell mediated immunity (Raghupathy, 1997; Krishnan et al., 1996). Though, a clear picture about how antibodies producing cells behave through gestation has not been provided so far. In this study, we performed a detailed characterization of the B cell compartment during human pregnancy. We showed here that percentages of transitional B cells are significantly decreased in peripheral blood of pregnant women. B-lymphocytes are continuously produced by precursors located in the bone marrow (Chen et al., 2008) and then migrate to the periphery as immature or transitional cells, to continue their maturation in the spleen (Chen et al., 2008). Interestingly, in previous works from our laboratory we demonstrated that in the mice, pregnancy induces strong suppression of B cell lymphopoiesis in the

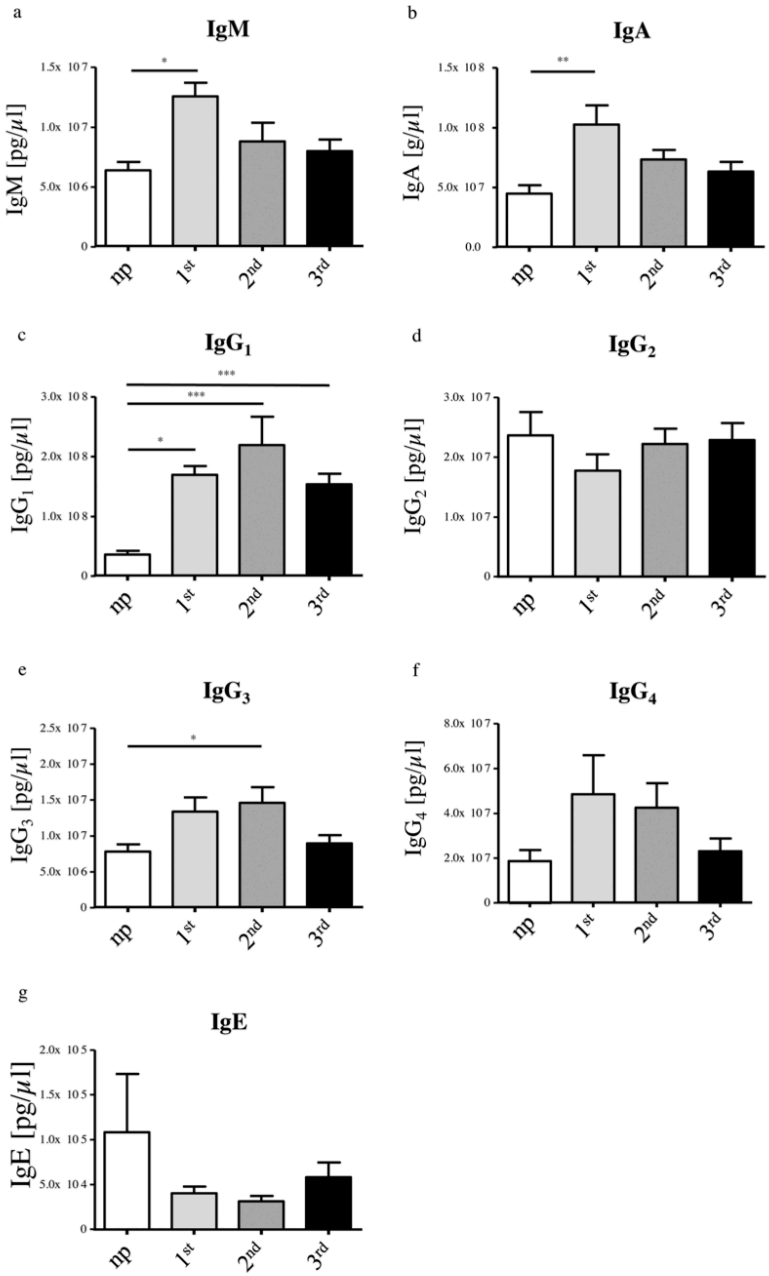

Fig. 4. Levels of immunoglobulin in serum of pregnant and non-pregnant women. Bar graphs (a–g) show the concentration of IgM, IgA, IgG_1, IgG_2, IgG_3, IgG_4 and IgE in serum of pregnant women on the first (1^{st}) second (2^{nd}) and third (3^{rd}) trimester as well as in non-pregnant (np) women (n = 9, 13, 49 and 31 respectively). Data are expressed as mean ± SEM. * ≤ 0.05, ** ≤ 0.01 and *** ≤ 0.001 as analyzed by Kruskal-Wallis test with Dunn's post-test.

bone marrow (Muzzio et al., 2014, 2016). In addition to a decrease on transitional B cell numbers, we showed here that percentages of main peripheral B cell populations; namely naïve as well as switched and non-switched memory B cells remain unaltered in normal pregnant women when compared to non-pregnant control subjects. Notably, it has been recently described a case of a woman suffering recurrent pregnancy loss and RA showing increased numbers of non-switched memory B cells (Ota et al., 2014). Similarly, Carbone and co-authors showed that non-pregnant women with a history of recurrent miscarriages display higher numbers of non-switched memory B cells in peripheral blood as compared to non-pregnant women who had children but no history of miscarriages and to women with no previous pregnancies (Carbone et al., 2016). All together these data suggest a possible relationship between levels of non-switched memory B cells and pregnancy wellbeing. Future studies are needed in order to confirm or reject this suggestion.

After antigen-independent development, immature B cells leave the bone marrow and gather in the longer-lived mature, naive IgD^+CD27^- B cell pool (Warnatz et al., 2002). When these cells are properly stimulated by antigen in the presence of T cell co-stimulation, they will engage in a germinal center (GC) reaction and develop into antibody producing plasma cells, which produce antigen specific immunoglobulin (Berkowska et al., 2011). In addition to antigen dependent antibodies, a great proportion of circulating immunoglobulin is represented by natural antibodies, which are produced by some B cells in the absence of antigen stimulation (Zhou et al., 2007). Natural antibodies belong to the IgM, IgA and IgG_3 isotypes and play a crucial role in many immune processes (Lobo, 2016). They can direct pathogen neutralization, induce classical complement activation, boost antibody–dependent cell mediated cytotoxicity by NK cells, maximize the clearance of apoptotic cells avoiding or reducing inflammation and prevent autoimmunity by promoting the clearance of DAMPs, such as dsDNA (Panda and Ding, 2015). Hence, in addition to their protective role against pathogens, natural antibodies are crucial in maintaining immune homeostasis (Panda and Ding, 2015). Interestingly, we showed here that levels of IgM, IgA, IgG_1 and IgG_3 are significantly augmented in serum during pregnancy. As we specifically excluded from our study pregnant women with symptoms of acute or chronic inflammation as well as diagnosed immunological disease and pregnancy loss, it can be argued that these immunoglobulin are natural antibodies and further speculate a possible role of natural antibodies in pregnancy well-being. Reinforcing this idea, we have previously showed that in mice, natural antibodies are augmented in serum during pregnancy (Muzzio et al., 2014). Furthermore, using a murine model of immune-mediated pregnancy failure, we could later confirm the role of natural antibodies in pregnancy by showing that their levels are increased in serum of normal pregnant mouse but not in those suffering from pregnancy failures (Muzzio et al., 2016). Moreover, using the same animal model, it has been shown that transfer of natural antibodies isolated from normal pregnant mice into pregnant mice naturally suffering pregnancy failures is enough to significantly improve pregnancy outcome (Chaouat et al., 1985). Remarkably, intravenous immunoglobulin administration (IVIG), which basically consists in a preparation of natural antibodies, has long been used as a treatment for recurrent miscarriage as well as peri-implantation embryo failure in patients undergoing *in vitro* fertilization and embryo transfer (IVF) (Clark et al., 2006). Moreover, IVIG is widely used to treat autoimmune diseases and inflammatory disorders (Kaufman et al., 2015).

Albeit we found higher levels of immunoglobulin in serum of pregnant women, no differences were observed concerning levels of antibody producing cells, suggesting that indeed antibody production per cell is increased during pregnancy rather than expansion of plasma cells.

Hence, the data presented here highlight the importance of more likely natural antibodies during pregnancy as a potential immunological mechanisms launched in order to control undesired inflammation that might put pregnancy on risk. This opens new avenues to explore both, levels of natural antibodies in serum as predictors of pregnancy outcome as well as the use of natural antibodies to treat pregnancy failures.

Authors' role

K.B.Z. and D.M. performed experiments and analyzed data. F.M., I.B., K.M. M.S.V and J.E. performed experiments. M.Z. contributed with study design and data analysis. F.J. conceived and designed the study, supervised the experiments and wrote the paper.

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Conflict of interest

The authors have no conflicts of interest to declare.

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bone marrow (Muzzio et al., 2014, 2016). In addition to a decrease on transitional B cell numbers, we showed here that percentages of main peripheral B cell populations; namely naïve as well as switched and non-switched memory B cells remain unaltered in normal pregnant women when compared to non-pregnant control subjects. Notably, it has been recently described a case of a woman suffering recurrent pregnancy loss and RA showing increased numbers of non-switched memory B cells (Ota et al., 2014). Similarly, Carbone and co-authors showed that non-pregnant women with a history of recurrent miscarriages display higher numbers of non-switched memory B cells in peripheral blood as compared to non-pregnant women who had children but no history of miscarriages and to women with no previous pregnancies (Carbone et al., 2016). All together these data suggest a possible relationship between levels of non-switched memory B cells and pregnancy wellbeing. Future studies are needed in order to confirm or reject this suggestion.

After antigen-independent development, immature B cells leave the bone marrow and gather in the longer-lived mature, naive IgD^+CD27^- B cell pool (Warnatz et al., 2002). When these cells are properly stimulated by antigen in the presence of T cell co-stimulation, they will engage in a germinal center (GC) reaction and develop into antibody producing plasma cells, which produce antigen specific immunoglobulin (Berkowska et al., 2011). In addition to antigen dependent antibodies, a great proportion of circulating immunoglobulin is represented by natural antibodies, which are produced by some B cells in the absence of antigen stimulation (Zhou et al., 2007). Natural antibodies belong to the IgM, IgA and IgG_3 isotypes and play a crucial role in many immune processes (Lobo, 2016). They can direct pathogen neutralization, induce classical complement activation, boost antibody–dependent cell mediated cytotoxicity by NK cells, maximize the clearance of apoptotic cells avoiding or reducing inflammation and prevent autoimmunity by promoting the clearance of DAMPs, such as dsDNA (Panda and Ding, 2015). Hence, in addition to their protective role against pathogens, natural antibodies are crucial in maintaining immune homeostasis (Panda and Ding, 2015). Interestingly, we showed here that levels of IgM, IgA, IgG_1 and IgG_3 are significantly augmented in serum during pregnancy. As we specifically excluded from our study pregnant women with symptoms of acute or chronic inflammation as well as diagnosed immunological disease and pregnancy loss, it can be argued that these immunoglobulin are natural antibodies and further speculate a possible role of natural antibodies in pregnancy well-being. Reinforcing this idea, we have previously showed that in mice, natural antibodies are augmented in serum during pregnancy (Muzzio et al., 2014). Furthermore, using a murine model of immune-mediated pregnancy failure, we could later confirm the role of natural antibodies in pregnancy by showing that their levels are increased in serum of normal pregnant mouse but not in those suffering from pregnancy failures (Muzzio et al., 2016). Moreover, using the same animal model, it has been shown that transfer of natural antibodies isolated from normal pregnant mice into pregnant mice naturally suffering pregnancy failures is enough to significantly improve pregnancy outcome (Chaouat et al., 1985). Remarkably, intravenous immunoglobulin administration (IVIG), which basically consists in a preparation of natural antibodies, has long been used as a treatment for recurrent miscarriage as well as peri-implantation embryo failure in patients undergoing *in vitro* fertilization and embryo transfer (IVF) (Clark et al., 2006). Moreover, IVIG is widely used to treat autoimmune diseases and inflammatory disorders (Kaufman et al., 2015).

Albeit we found higher levels of immunoglobulin in serum of pregnant women, no differences were observed concerning levels of antibody producing cells, suggesting that indeed antibody production per cell is increased during pregnancy rather than expansion of plasma cells.

Hence, the data presented here highlight the importance of more likely natural antibodies during pregnancy as a potential immunological mechanisms launched in order to control undesired inflammation that might put pregnancy on risk. This opens new avenues to explore both, levels of natural antibodies in serum as predictors of pregnancy outcome as well as the use of natural antibodies to treat pregnancy failures.

Authors' role

K.B.Z. and D.M. performed experiments and analyzed data. F.M., I.B., K.M. M.S.V and J.E. performed experiments. M.Z. contributed with study design and data analysis. F.J. conceived and designed the study, supervised the experiments and wrote the paper.

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Conflict of interest

The authors have no conflicts of interest to declare.

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7 Eidesstattliche Erklärung

Hiermit erkläre ich, dass ich die vorliegende Dissertation selbständig verfasst und keine anderen als die angegebenen Hilfsmittel benutzt habe.

Die Dissertation ist bisher keiner anderen Fakultät, keiner anderen wissenschaftlichen Einrichtung vorgelegt worden.

Ich erkläre, dass ich bisher kein Promotionsverfahren erfolglos beendet habe und dass eine Aberkennung eines bereits erworbenen Doktorgrades nicht vorliegt.

Greifswald

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