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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 (1st), late second trimester (2nd) and late third trimester (3rd) 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	1st trimester of pregnancy (n = 11) Mean ± SD	2nd trimester of pregnancy (n = 15) Mean ± SD	3rd 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

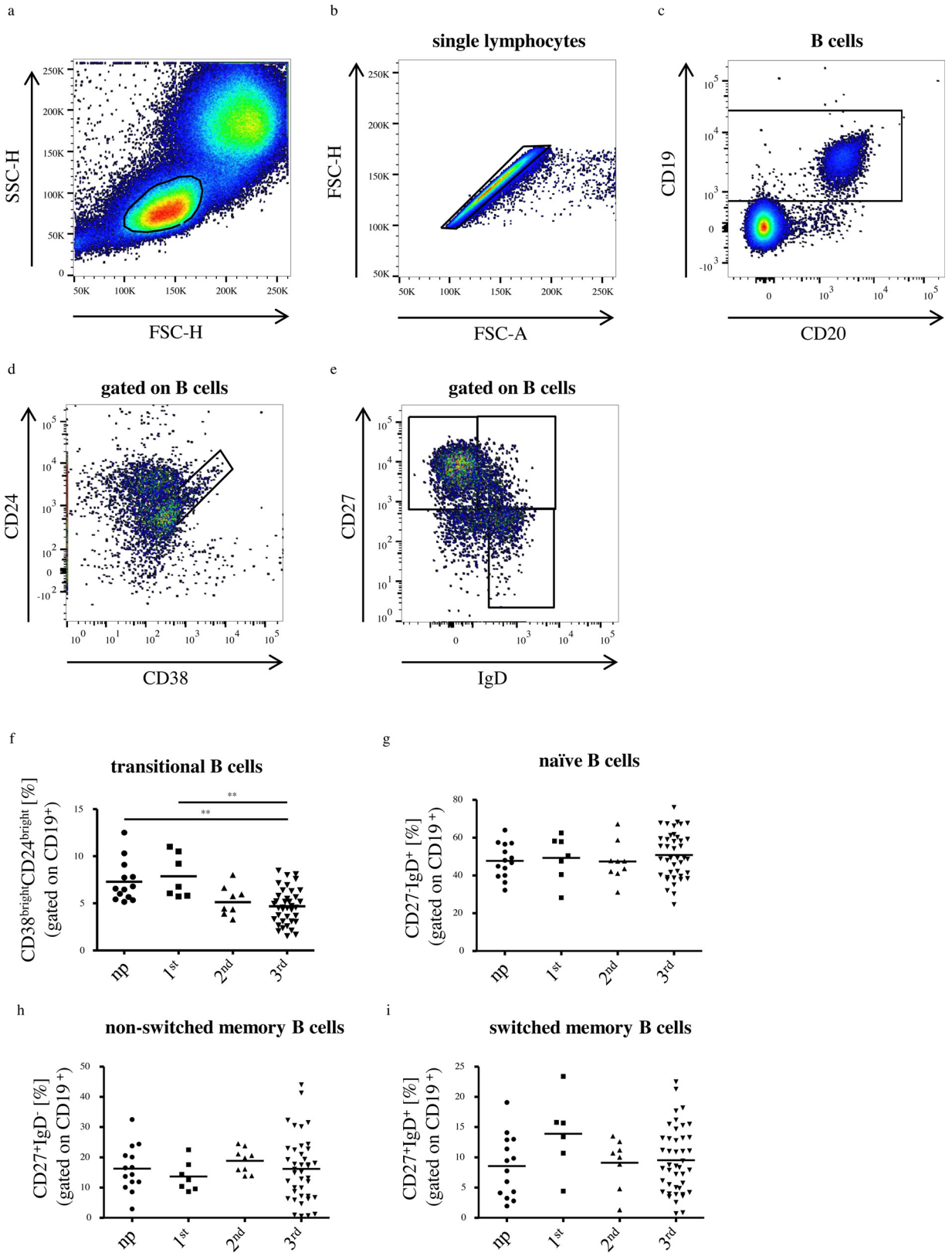

Fig. 1. Analysis of naïve, switched and non-switched memory B cells in peripheral blood of pregnant and non-pregnant women. Representative dotplots showing gating strategy used to analyze naïve as well as memory B cells (a–e). $CD19^+$ gated B cells (c) were analyzed for the expression of CD27 and IgD (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 (1st) second (2nd) and third (3rd) 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.

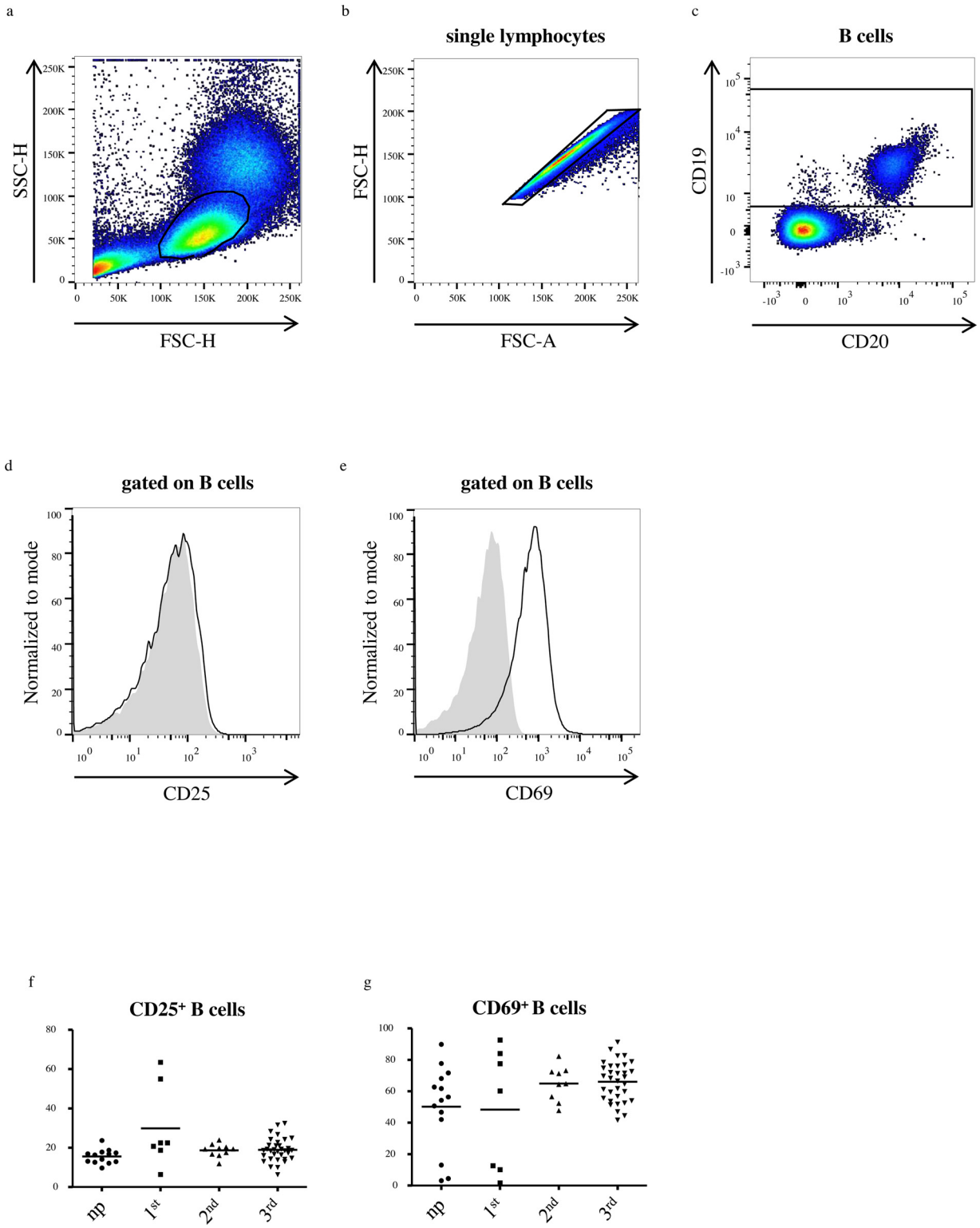

Fig. 2. Expression analysis of CD25 and CD69 on peripheral B cells during pregnancy. Representative dot plots (a–c) and histograms (d and e) displaying gating strategy used to analyze expression of CD25 and CD69 (empty histogram) against FMO control (filled histogram) on B cells during pregnancy. Graphs show percentages of CD25 (f) and CD69 (g) expressing B cells in peripheral blood of pregnant women on the first (1st) second (2nd) and third (3rd) 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

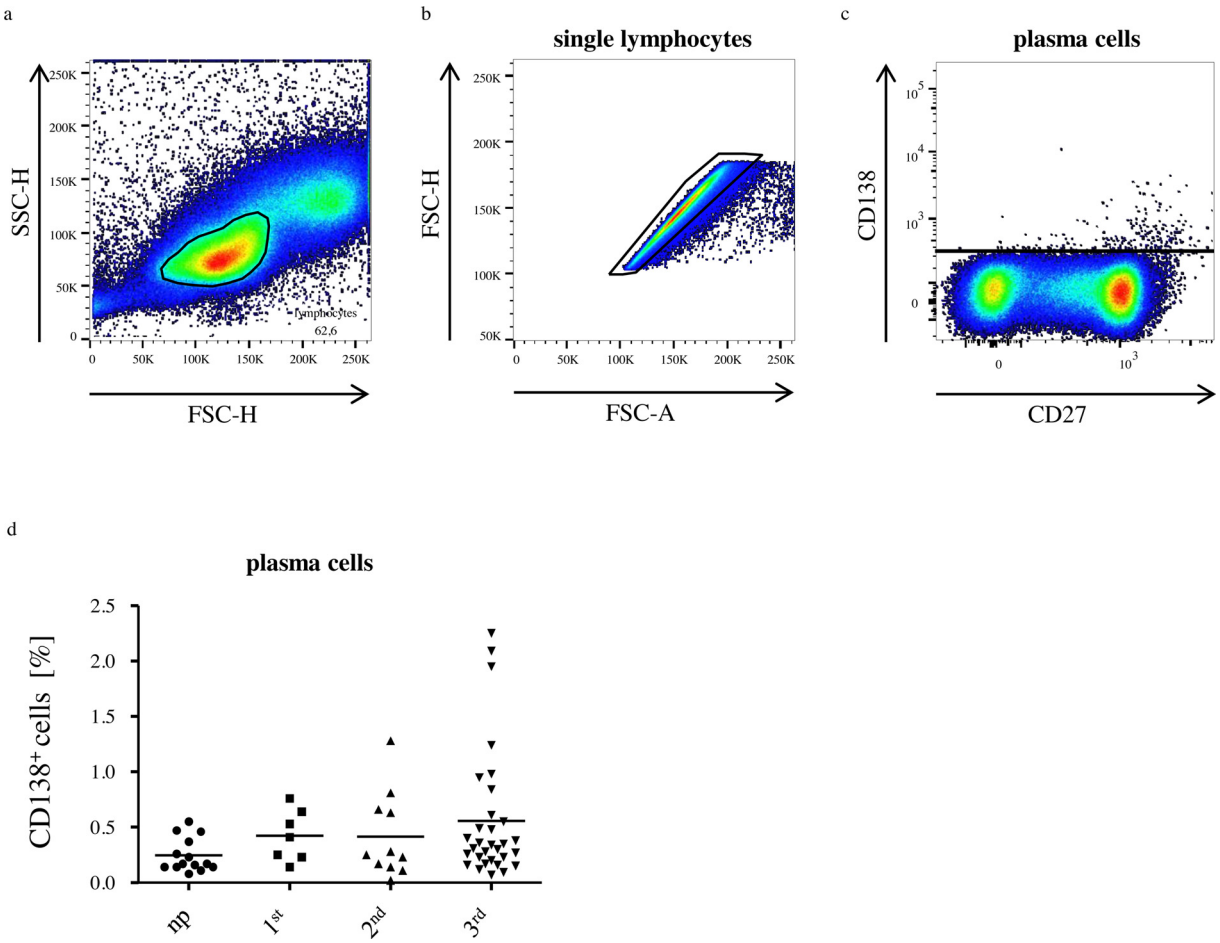

Fig. 3. Percentages of plasma cells in peripheral blood of pregnant and non-pregnant women. Representative dot plots displaying gating strategy used to analyze plasma cells during pregnancy (a–c). Graphs show percentages of CD138$^+$ plasma cells in peripheral blood of pregnant women on the first (1st) second (2nd) and third (3rd) 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 (1st, 2nd and 3rd 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

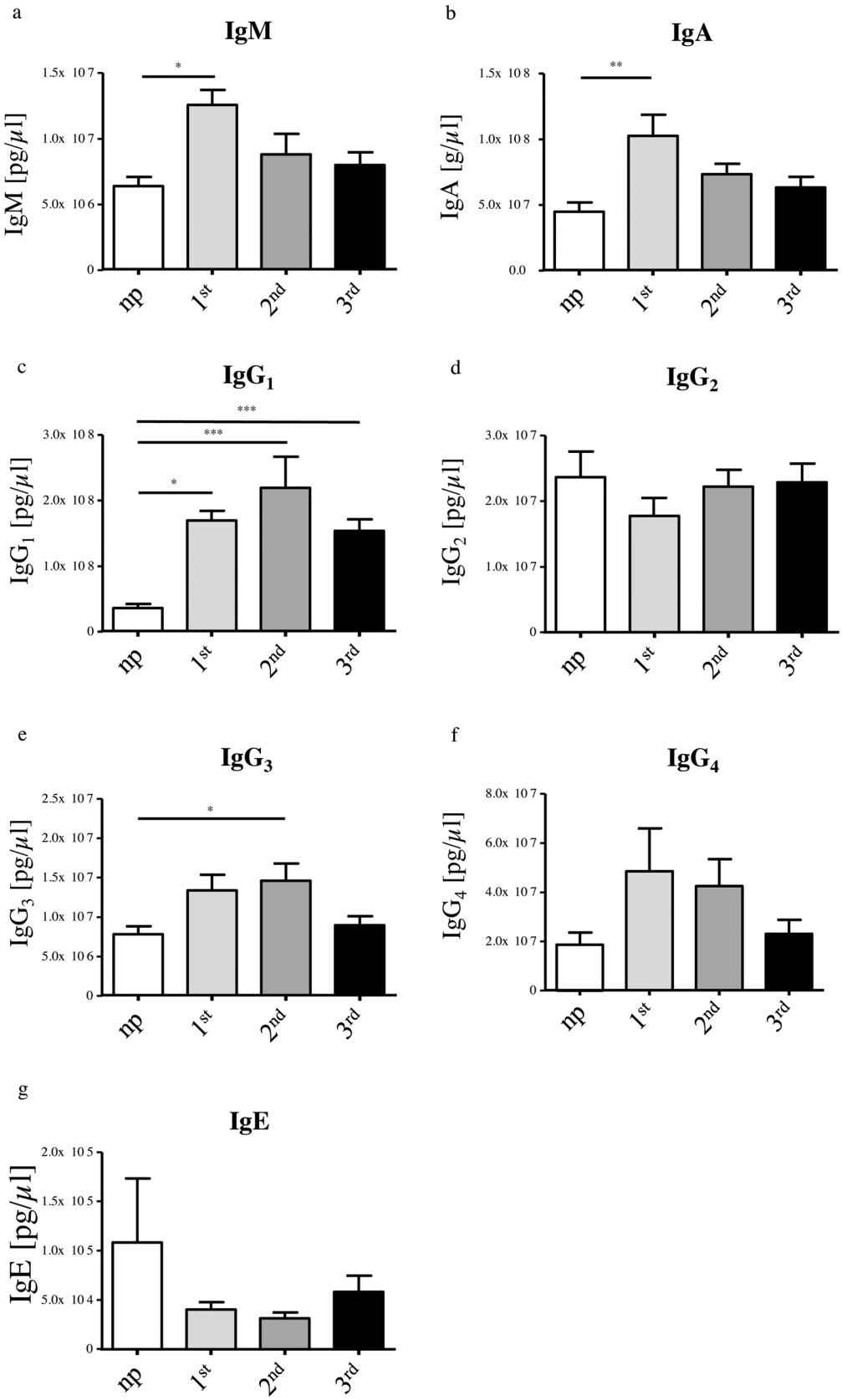

Fig. 4. Levels of immunoglobulin in serum of pregnant and non-pregnant women. Bar graphs (a–g) show the concentration of IgM, IgA, IgG_1, IgG_2, IgG_3, IgG_4 and IgE in serum of pregnant women on the first (1st) second (2nd) and third (3rd) trimester as well as in non-pregnant (np) women (n = 9, 13, 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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