IMMUNOMODULATION IN PREGNANCY AND LABOR

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Tese para obtenção do grau de Doutor em Medicina

Especialidade de Investigação Clínica (Ginecologia e Obstetrícia)

NOVA Medical School / Faculdade de Ciências Médicas

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Preface

"How does the pregnant mother arrange to nourish a fetus within herself for many months that is an antigenically foreign body?" This question was raised by Sir Peter Brian Medawar in 1953.

The relationship between mother and fetus fascinates me in the same way that it has fascinated reproductive immunologists for decades.

A large amount of research has been performed in this area in early pregnancy. Nevertheless, the phenomena that occur at the end of pregnancy, on the day of delivery and the effects of pregnancy on the immune system after birth have not yet been completely clarified.

Although an important number of discoveries in reproductive immunology have been documented in several animal models, the best model for human pregnancy remains the human female.

Therefore, our research is not designed to address all of the unanswered questions surrounding the significance of the maternal immune system during pregnancy and its influence on fetal development. Rather, our goals are to identify the gaps in knowledge and to understand the roles of specific B and T cell subsets in the peripheral blood of women during the 3rd trimester of pregnancy, on the day of delivery and postpartum. Finally, we addressed whether normal labor has an effect on maternal circulating T cell subsets, regulatory T cells, and regulatory B cells.

Research investigating reproductive immunology will advance our knowledge of immunology-related pregnancy complications and facilitate the design of novel therapies for reproductive pathologies. Overall, it might facilitate the design of new therapeutic approaches to control these complications and their impact on the health of the mother and child.

According to *Artigo 4.º* from *Diário da República*, *2.ª série*, *N.º 111*, 9th June 2015 (*Regulamento n.º* 320/2015) and with the *Artigo 19.º* from *Diário da República*, *2.ª série*, *n.º 153*, 7th August 2015 (*Regulamento n.º* 519/2015), the results presented and discussed in this thesis were published, accepted or submitted for publication in the following scientific peer-reviewed journals:

- **Lima J**, Martins C, Leandro MJ, Nunes G, Sousa MJ, Branco JC, Borrego LM. Characterization of B cells in healthy pregnant women from late pregnancy to post-partum: a prospective observational study. *BMC Pregnancy Childbirth*. 2016;16(1):139. DOI:10.1186/s12884-016-0927-7. Impact Factor: 2.180

- **Lima J**, Martins C, Leandro MJ, Nunes G, Sousa MJ, Branco JC, Borrego LM. Regulatory T cells show dynamic behavior during late pregnancy, delivery and the postpartum period. Submitted to Reproductive Sciences in July 4, 2016 (ID: RSCI-16-409).

- **Lima J**, Martins C, Nunes G, Sousa MJ, Branco JC, Borrego LM. Impact of Labor on Peripheral Blood Maternal T-Cell Subsets and on Regulatory T and B Cells [published online June 13, 2016]. *Reprod. Sci*. 2016. DOI:10.1177/1933719116653680. Impact factor: 2.429

According to the *alínea e)* of the *Artigo 19.º* from *Diário da República*, *2.ª série*, *N.º* 153, 7th August 2015 (*Regulamento n.º* 519/2015), this PhD research project was approved by *Comissão Nacional de Proteção de Dados* (*autorização n.º* 6507/2012), by the Ethics Committee of *Hospital CUF Descobertas*, and by the Ethics Committee of NOVA Medical School/*Faculdade de Ciências Médicas* (*autorização n.º 10/2013/CEFCM*).

Part of the results obtained were presented and published in the following scientific peer-reviewed journal:

- Martins C, **Lima J**, Nunes G, Borrego LM. Regulatory T and B cells in asthmatic women: variations from pregnancy to postpartum Treg and Breg: pregnancy to postpartum [published online Jun 14, 2016]. *J. Investig. Allergol. Clin. Immunol*. 2016. DOI:10.18176/jiaci.0086

Part of the results obtained were presented at following scientific meetings:

Lima J, Martins C, Borrego LM, Leandro MJ, Nunes G, Menezes MF, Trindade H, Branco JC. Are Th17 cells determinant to physiological neutrophilia during pregnancy and delivery? 23rd European Congress of Obstetrics and Gynaecology; May 7-10, 2014; Glasgow, Scotland. [Poster]

Martins C, **Lima J**, Leandro MJ, Nunes G, Menezes MF, Branco JC, Trindade H, Borrego LM. Impaired Foxp3 expression in late pregnancy and at delivery. 23rd European Congress of Obstetrics and Gynaecology; May 7-10, 2014; Glasgow, Scotland. [Poster]

Martins C, **Lima J**, Leandro MJ, Nunes G, Branco JC, Trindade H, Borrego LM. Peripheral blood B cells in atopy. SPI'40 - XL Annual Meeting of the Portuguese Society for Immunology; October 13-15, 2014; Lisbon, Portugal. [Poster]

Domingues T, Diamantino F, **Lima J**, Borrego LM, Martins C. Perfil Imunológico de Grávidas Atópicas: marcadores de risco para a descendência. XXII Congresso Sociedade Portuguesa de Estatística; October 7-10, 2015; Olhão, Portugal.[Oral Comunication]

Martins C, **Lima J**, Leandro MJ, Nunes G, Branco JC, Trindade H, Borrego LM. Immune profile in late pregnancy: immunological markers in atopic asthmatic women as risk factors for atopy in progeny. Pediatric Allergy and Asthma Meeting; October 15-17, 2015; Berlin, Germany. [Poster]

"You cannot create experience. You must undergo it"

Albert Camus

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Resumo

A gravidez é um desafio para o sistema imunológico, porque tem de tolerar um feto geneticamente diferente e ao mesmo tempo proteger tanto a mãe como o feto contra ameaças biológicas. Isto requer uma regulação estreita dos vários tipos de células do sistema imunitário durante as diferentes fases da gravidez. Estudos recentes revelaram que determinadas subpopulações de células B e células T têm funções reguladoras e são essenciais para a manutenção da tolerância materno-fetal durante as fases iniciais da gravidez. No entanto, não está claro se as células do sistema imunológico materno, especialmente as recém descritas células reguladoras B (Breg) e T (Treg), também podem desempenhar um papel relevante no final da gravidez normal, no parto e no pós-parto.

Com o objetivo de analisar o perfil imunológico desde o final da gravidez até ao pós-parto, foram efetuados três estudos observacionais que seguiram mulheres saudáveis, grávidas (n = 43) e não grávidas (como grupo controlo; n = 35), vigiadas na consulta externa do hospital CUF Descobertas. Em primeiro lugar, as contagens no sangue periférico das subpopulações de linfócitos B, considerando o perfil maturativo e incluindo as células Breg, foram analisadas em todas as mulheres grávidas durante o terceiro trimestre da gravidez, no dia do parto (imediatamente após o parto), e no pós-parto (pelo menos 6 semanas após o parto), e comparadas com as do grupo de mulheres não grávidas. Em segundo lugar, a evolução das subpopulações de células Treg foi avaliada no sangue periférico das mulheres grávidas nos mesmos pontos temporais do estudo anterior, e comparadas com as do grupo de controlo. Em terceiro lugar, o efeito do trabalho de parto sobre as subpopulações circulantes de células T, e sobre as subpopulações de células Treg e Breg, foi avaliado no dia do parto, comparando o subgrupo de mulheres grávidas que tiveram o parto por cesariana eletiva (sem trabalho de parto; n = 14), com as que tiveram um parto espontâneo vaginal (após o trabalho de parto; n = 18).

A quantificação e a caracterização fenotípica de células B e células T foi efetuada por citometria de fluxo, de acordo com a expressão dos marcadores de superfície celular presentes nas suas diferentes subpopulações. Especificamente, as

subpopulações de células B foram caracterizadas no sangue periférico pela expressão de CD19 (marcador de células B), e pela expressão de IgD e CD38 para identificar o perfil maturativo dos linfócitos B de acordo com o sistema de classificação Bm1-5: células B de transição, células B naïve, células B de memória não *switched*, células B de memória *switched* entretanto divididas em células pós centro germinativo e células de memória em repouso, e ainda plasmablastos. Os linfócitos Breg foram caracterizados através da expressão de CD24, CD27, CD38, e a produção de IL-10 foi avaliada após a estimulação com acetato de forbolmiristato (PMA), ionóforo de cálcio e lipopolissacárido (LPS) bacteriano. Relativamente às subpopulações de células T de sangue periférico, as células com expressão de CD3 (marcador de células T) que co-expressam CD4 (células T auxiliadoras) ou CD8 (células T citotóxicas) foram diferenciadas em naïve, memória central, memória efetoras, memória efetoras com diferenciação terminal utilizando os marcadores de superfície de células CD45RA e CD62L. A expressão de marcadores de ativação (HLA-DR e CD25) foi avaliada na população de linfócitos T totais e nas respetivas subpopulações de células T CD4 e CD8. Além disso, as células T *natural killer (NKT)-like* foram identificados pela co-expressão de CD3, CD16 e CD56. Finalmente, três estratégias analíticas foram utilizadas para caracterizar as células Treg ($CD4^{Dim}CD25^{Hi}$, $CD4^{+}CD25^{Hi}CD127^{-/dim}$ e $CD4^{+}CD25^{Hi}Foxp3^{+}$). Foi também efetuada a avaliação da expressão intracelular de Foxp3 na subpopulação de células Treg $CD4^{Dim}CD25^{Hi}$.

Os resultados mostraram que as contagens absolutas e percentuais dos linfócitos B foram significativamente mais baixas ($p<0,05$) nas mulheres grávidas no dia do parto, em comparação com as mulheres não grávidas. De uma forma geral, as contagens absolutas e percentagens da maioria das subpopulações de células B foram significativamente mais baixas ($p<0,05$) no terceiro trimestre da gravidez e no dia do parto. Além disso, essas contagens absolutas e percentuais das subpopulações de células B não tiveram diferenças significativas entre o período pós-parto e as mulheres não grávidas. No entanto, as exceções mais notáveis foram: as percentagens de células B naïve, que foram significativamente mais elevadas ($p<0,05$) no terceiro trimestre e no dia do parto do que no período pós-parto e o observado nas mulheres não grávidas; e as percentagens da subpopulação de células Breg $CD24^{Hi}CD38^{Hi}$, que foram significativamente mais elevadas ($p<0,05$) no

período pós-parto do que no terceiro trimestre, no dia do parto e no grupo das mulheres não grávidas.

De um modo semelhante às células Breg, as contagens absolutas de todas as subpopulações de células Treg foram significativamente maiores (p<0,05) no período pós-parto, em comparação com o terceiro trimestre da gravidez e com o dia do parto. Além disso, a expressão intracelular do fator de transcrição Foxp3 na subpopulação de células Treg $CD4^{Dim}CD25^{Hi}$ diminuiu significativamente (p<0,001) no terceiro trimestre da gravidez e no dia do parto em comparação com o observado nas mulheres não grávidas; e aumentou significativamente (p<0,001) no período pós-parto, comparativamente com o terceiro trimestre de gravidez e com o dia do parto.

Finalmente, verificou-se que as contagens absolutas e percentagens das subpopulações de células Breg e Treg circulantes não foram significativamente diferentes entre as mulheres que tiveram cesarianas eletivas (sem trabalho de parto) e aquelas que tiveram partos vaginais espontâneos (após o trabalho de parto). No entanto, as contagens absolutas dos linfócitos B e das células *NKT-like* foram significativamente menores (p<0,05) no sangue periférico das mulheres que tiveram parto vaginal.

Concluiu-se que no sangue periférico os compartimentos de células B e T sofrem alterações quantitativas do final da gravidez normal até ao período pós-parto. Particularmente, as subpopulações de células Breg e Treg aumentam no período pós-parto. Além disso, o trabalho de parto não parece ter um impacto sobre as subpopulações de células T e sobre as subpopulações de células Breg e Treg no sangue periférico materno. Portanto, estes resultados podem permitir uma melhor compreensão da imunomodulação que ocorre durante a gravidez humana e fornecem algumas evidências para o desenvolvimento de novas estratégias de diagnóstico e tratamento de patologias obstétricas. Estes resultados poderão contribuir para o estudo dos mecanismos de resposta materna à vacinação e à infeção.

Abstract

The immune system is challenged during pregnancy because it must tolerate a genetically foreign fetus and protect both the mother and the fetus against biological threats. This achievement requires a tight regulation of the various immune system cell types during the different stages of pregnancy. Recent studies have reported that subsets of B cells and T cells have regulatory functions that are essential for the maintenance of tolerance during early pregnancy by suppressing the maternal alloreactivity against the fetus. However, it is unclear whether the maternal immune system cells, especially the recently described regulatory B cell (Breg) and T cell (Treg) subsets, can also play a relevant role in normal late pregnancy, labor and in postpartum period.

To analyze the immunological profile from late pregnancy to postpartum, three observational studies have been performed encompassing the follow-up of healthy pregnant women (n = 43) and non-pregnant women (as a control group; n = 35) who were attending a hospital-based outpatient clinic (Hospital CUF Descobertas). First, the levels of peripheral blood B cell subsets, considering the different maturational stages and including Breg, were analyzed in all pregnant women during the 3rd trimester of pregnancy, on the day of delivery (immediately after labor), and in the postpartum period (at least 6 weeks after delivery), and compared with those of the non-pregnant women. Second, the variation in Treg subsets was assessed in peripheral blood of pregnant women at the same time points used in the previous study and compared with the control group. Third, the effect of labor on the peripheral blood T cell subsets and on the Treg and Breg subsets was assessed on the day of delivery, comparing the subgroup of pregnant women who gave birth by elective cesarean (no labor; n = 14) with those who had a spontaneous vaginal delivery (after labor; n = 18).

Quantification and phenotypic characterization of B cells and T cells according to the expression of specific cell surface markers has been performed by flow cytometry. Specifically, circulating B cell subsets were characterized by the expression of CD19 (B cell marker) and by the expression of IgD and CD38 to

identify the different B cell maturational stages according to the Bm1-5 classification system: transitional B cells, naïve B cells, unswitched memory B cells and switched memory B cells, which were subsequently divided into post-germinal memory B cells and resting memory B cells; and plasmablasts. Breg subsets were characterized by the expression of CD24, CD27, CD38, and the production of IL-10 after phorbol 12-myristate 13-acetate (PMA), calcium ionophore and lipopolysaccharide (LPS) stimulation. Regarding the circulating T cell subsets, cells expressing CD3 (T cell marker) and co-expressing either CD4 (helper T cells) or CD8 (cytotoxic T cells) were discriminated into naïve, central memory, effector memory, and terminally differentiated effector memory cells based on the cell surface markers CD45RA and CD62L. The expression of activation markers (HLA-DR and CD25) was assessed in total T lymphocytes and in CD4 and CD8 T cells subsets. In addition, natural killer T (NKT)-like cells were identified by the co-expression of CD3 with CD16 and CD56. Finally, three analytical strategies were used to characterize Treg cells ($CD4^{Dim}CD25^{Hi}$, $CD4^{+}CD25^{Hi}CD127^{-/dim}$ and $CD4^{+}CD25^{Hi}Foxp3^{+}$ T cells). Additionally, the expression levels of the transcription factor Foxp3 in the $CD4^{Dim}CD25^{Hi}$ Treg cells were analyzed.

The results showed that the absolute counts and percentages of B cells were significantly lower ($p<0.05$) in pregnant women on the day of delivery compared with non-pregnant women. Overall, the absolute counts and percentages of the majority of the B cell subsets were significantly lower ($p<0.05$) in the 3rd trimester of pregnancy and on the day of delivery. Moreover, these counts and percentages did not differ significantly between the postpartum period and non-pregnant women. However, the most notable exceptions were the percentages of naïve B cells, which were significantly higher ($p<0.05$) in the 3rd trimester and on the day of delivery compared with the postpartum period and in non-pregnant women, and the percentages of the $CD24^{Hi}CD38^{Hi}$ Breg subset, which were significantly higher ($p<0.05$) in the postpartum period compared with the 3rd trimester, the day of delivery, and in non-pregnant women.

Similarly to Breg cells, the absolute counts of all Treg subsets were significantly higher ($p<0.05$) in the postpartum period compared with the 3rd trimester and the day of delivery. In addition, intracellular expression of Foxp3 in the $CD4^{Dim}CD25^{Hi}$ Treg

subset significantly decreased (p<0.001) in the 3rd trimester of pregnancy and on the day of delivery compared with non-pregnant women; they also significantly increased (p<0.001) during the postpartum period compared with the 3rd trimester and on the day of delivery.

Finally, the absolute counts and percentages of circulating Breg and Treg subsets were not significantly different between women who had elective cesareans (no labor) and those who had spontaneous vaginal deliveries (after labor). Nevertheless, the absolute counts of B cells and of NKT-like cells were significantly lower (p<0.05) in the peripheral blood of women who had vaginal deliveries.

These findings indicated that the peripheral blood B cell and T cell compartments undergo quantitative changes from normal late pregnancy to the postpartum period. In particular, the results presented in this thesis suggest that circulating Breg and Treg subsets increase during the postpartum period, despite their well-known involvement in early pregnancy. In addition, labor does not seem to have a major impact on maternal peripheral blood T cell subsets and on Breg and Treg subsets. Therefore, these findings may improve our understanding of immunomodulation during human pregnancy and provide some evidence for the development of new strategies to diagnose and treat pregnancy-associated disturbances. These investigations may also contribute to the study of the mechanisms of maternal responses to vaccination and infection.

Table of Contents

List of Figures

List of Tables

List of Abbreviations, Acronyms and Symbols

-	Negative antigen expression
+	Positive antigen expression
BAFF	B cell-activating factor
BCR	B cell receptor
Bm	Mature B cell
BM	Bone marrow
Breg	Regulatory B cell
CD	Cluster of differentiation
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
DC	Dendritic cell
Dim	Low or dim antigen expression intensity
ER	Estrogen receptor
FasL	Fas ligand
Foxp3	Forkhead box p3
GC	Germinal center
G-CSF	Granulocyte-colony stimulation factor
GM-CSF	Granulocyte-macrophage colony-stimulation factor
hCG	Human chorionic gonadotropin
Hi	High or bright antigen expression intensity
HLA	Human leukocyte antigen
HLA-DR	Human leukocyte antigen – antigen D related complex
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin

Low	Low antigen expression intensity
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MALT	Mucosa-associated lymphoid tissue
MC	Mast cell
MHC	Major histocompatibility complex
mPR	Membrane progesterone receptor
Mφ	Macrophage
NK	Natural killer cell
NKT-like	Natural killer-like T cell
PC	Plasma cell
PIBF	Progesterone-induced blocking factor
PMA	phorbol 12-myristate 13-acetate
sIg	Surface immunoglobulin
TCR	T cell receptor
TGF	Transforming growth factor
Th	T helper cell
TNF	Tumor necrosis factor
Tr1	Type 1 regulatory T cell Foxp3$^-$
Treg	Regulatory T cell
u	Uterine
VIP	Vasoactive intestinal peptide

Thesis Outline

Chapter I provides a general introduction regarding current knowledge of the immunological aspects of pregnancy.

Chapter II presents the main objectives of this thesis.

The subsequent chapters present three studies on which this PhD thesis is based. Each chapter consists of the content of a manuscript that has been published or submitted for publication, subdivided into main sections of abstract, introduction, materials and methods, results, discussion and conclusion.

Chapter III addresses the variation of B cell subsets and regulatory B cells in the peripheral blood of healthy pregnant women during the 3rd trimester, the day of delivery, and the postpartum period:

- **Lima J**, Martins C, Leandro MJ, Nunes G, Sousa MJ, Branco JC, Borrego LM. Characterization of B cells in healthy pregnant women from late pregnancy to post-partum: a prospective observational study. *BMC Pregnancy Childbirth*. 2016;16(1):139.

Chapter IV focuses on the variation in regulatory T cells in the peripheral blood of healthy pregnant women through the 3rd trimester, the day of delivery, and the postpartum period:

- **Lima J**, Martins C, Leandro MJ, Nunes G, Sousa MJ, Branco JC, Borrego LM. Regulatory T cells show dynamic behavior during late pregnancy, delivery and the postpartum period. Submitted to Reproductive Sciences in July 4, 2016.

Chapter V discusses the effect of labor on the T cell subsets and on the regulatory T and B cells in the peripheral blood of healthy women by comparing the differences between spontaneous vaginal deliveries and elective cesareans:

- **Lima J**, Martins C, Nunes G, Sousa MJ, Branco JC, Borrego LM. Impact of Labor on Peripheral Blood Maternal T-Cell Subsets and on Regulatory T and B Cells [published online June 13, 2016]. *Reprod. Sci*. 2016.

Chapter VI presents the final remarks and future perspectives of this work.

CHAPTER I

General Introduction

1 The Immune System

The immune system is a host defense system comprising a network of molecules, cells, tissues and organs that protect the body against viruses, bacteria and other toxins. It can be divided into innate (a non-specific reaction to foreign antigens) and adaptive (to specific antigens) compartments (Figure 1).[1]

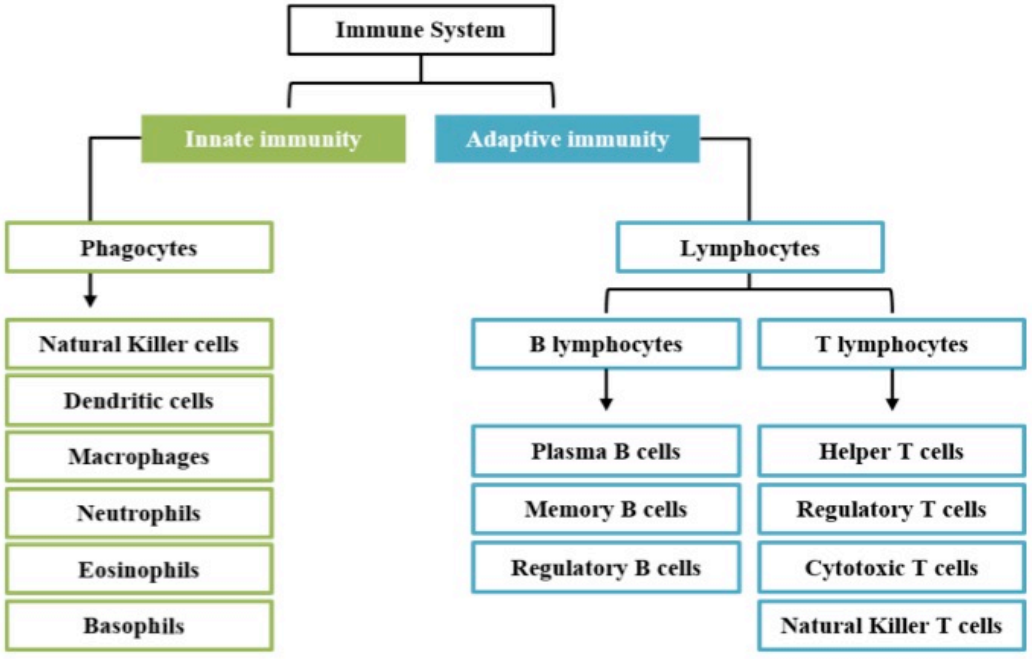

Figure 1. Components of the immune system, innate and adaptive compartments, and various immune cells involved in defense. Adapted from Nouroz et al., 2016.[1]

1.1 Innate Immune System

The innate immune system is a first line of defense because it acts against invaders that cross physical barriers such as the skin and mucosa. The innate immune response consists of various phagocytes such as macrophages, neutrophils, natural killer (NK) cells, dendritic cells (DC), basophils and eosinophils.[1,2] NK cells are involved in the destruction of tumor and virus-infected cells, while DCs, macrophages, basophils, eosinophils and neutrophils facilitate the inflammatory response, which is immediate but has a short duration. The innate immune response

requires no special preparation to act against pathogens and consists of chemical, mechanical, microbiological and cellular defense systems.[3]

1.2 Adaptive Immune System

The adaptive immune response is activated by the innate immune response through specific signals. There are two types of adaptive immune responses: humoral immunity, which is mediated by antibodies produced by B cells, and cell-mediated immunity, which is mediated by T cells.

1.2.1 B cells

B cells are lymphocytes that function in the humoral immunity component of the adaptive immune system by secreting antibodies with a vast range of antigen specificity.[4-6] B cells also secrete cytokines and can act as antigen-presenting cells to activate T cells.[7] B cells are also involved in the development of autoimmune diseases, partly through their ability to produce pathogenic autoantibodies.[7]

In the current model of human peripheral B cell development, B cells leave the bone marrow and enter the circulation as immature transitional B cells (still unable to respond to antigen), which later mature into naïve B cells that are fully mature but have not encountered an antigen.[6] Finally, when naïve B cells encounter their cognate antigens in secondary lymphoid organs such as the spleen, lymph nodes, and Peyer's patches, these cells become activated and mature into memory B cells (which have encountered antigen and can survive for extended periods) and plasma cells (which produce soluble antibodies) (Figure 2). Subsequent exposure to the same antigen will activate memory B cells and trigger a larger and more rapid antibody response.[6,8]

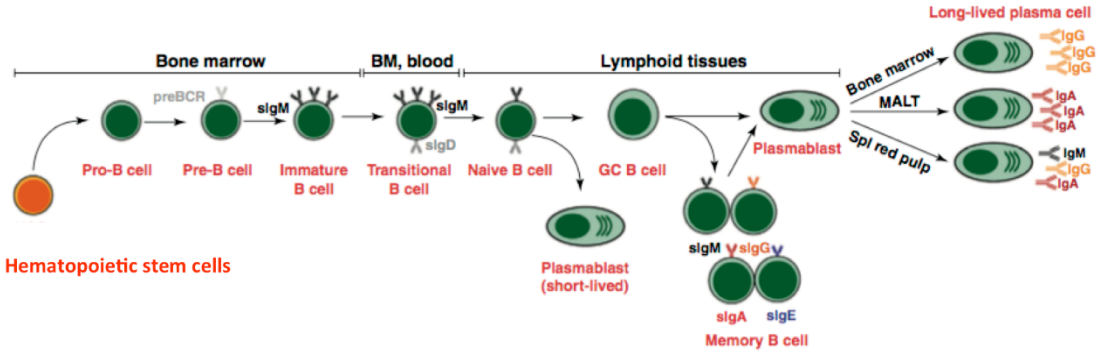

Figure 2. B cell development and differentiation from hematopoietic *stem cells* to plasma cells. BCR, B cell receptor; BM, bone marrow; GC, germinal center; MALT, mucosa-associated lymphoid tissue; sIg, surface Immunoglobulin; Spl, Spleen. Adapted from Tangye, 2011.[9]

1.2.1.1 B cell classification systems

B cell subsets of different maturational stages, from transitional to memory B cells, have been identified in peripheral blood using the mature B (Bm)1-Bm5 classification system. This classification system has been shown to be effective for the identification of alterations in the proportions of peripheral blood B cell subsets in patients with autoimmune diseases (e.g., Lupus or Sjögren's syndrome)[10-12] and in those undergoing therapy (e.g., with biological agents), by assessing the depletion and repopulation of B cells.[13] Recently, highly successful new therapeutic approaches based on changes in B cell subsets, such as depletion, have been highly successful for the treatment of a variety of leukemic and autoimmune diseases.[14]

The Bm1-5 classification system used to identify the development of mature B cells by flow cytometry is based on the expression of IgD and CD38 phenotypic markers. According to this system, cells are characterized as transitional B cells (Bm2': $IgD^{+}CD38^{Hi}$), naïve B cells (Bm2: $IgD^{+}CD38^{+}$), unswitched memory B cells (Bm1: $IgD^{+}CD38^{-}$), and switched memory B cells (Bm5: $IgD^{-}CD38^{+/-}$), which are subsequently divided into post-germinal memory B cells (early Bm5: $IgD^{-}CD38^{+}$) and resting memory (late Bm5: $IgD^{-}CD38^{-}$) B cells, and plasmablasts (Bm3 + Bm4: $IgD^{-}CD38^{Hi}$).[10-12,15] However, this division is arbitrary and suggests a chronological relationship that has never been properly validated.[10-12]

The different maturational B cells subsets also can be identified in peripheral blood using another classification system centered on the expression of IgD and CD27.[16] This classification is based on the concept that CD27 is a universal marker of the human memory B cells and allows the distinction between memory cells ($CD27^+$) and the naïve B cells (IgD^+CD27^-). Memory B cells are then divided into switched memory (IgD^-CD27^+) and unswitched memory/marginal zone-like (IgD^+CD27^+) B cell subsets. Yet another subset of B cells are termed double-negative memory cells (IgD^-CD27^-).[17]

None of these two classification systems is complete and definitive (Figure 3) because the Bm1 compartment includes an important proportion (60%) of the memory B IgD^+CD27^+ subset, compared with 10% of the Bm2 compartment, which contains the essential pool of naïve B cells; the Bm2' compartment corresponds to the transitional B cells $CD24^{Hi}CD38^{Hi}$; and the IgD^-CD27^- B cell subset includes a population of $CD27^-$ memory B cells that are late Bm5 B cells.[16,17]

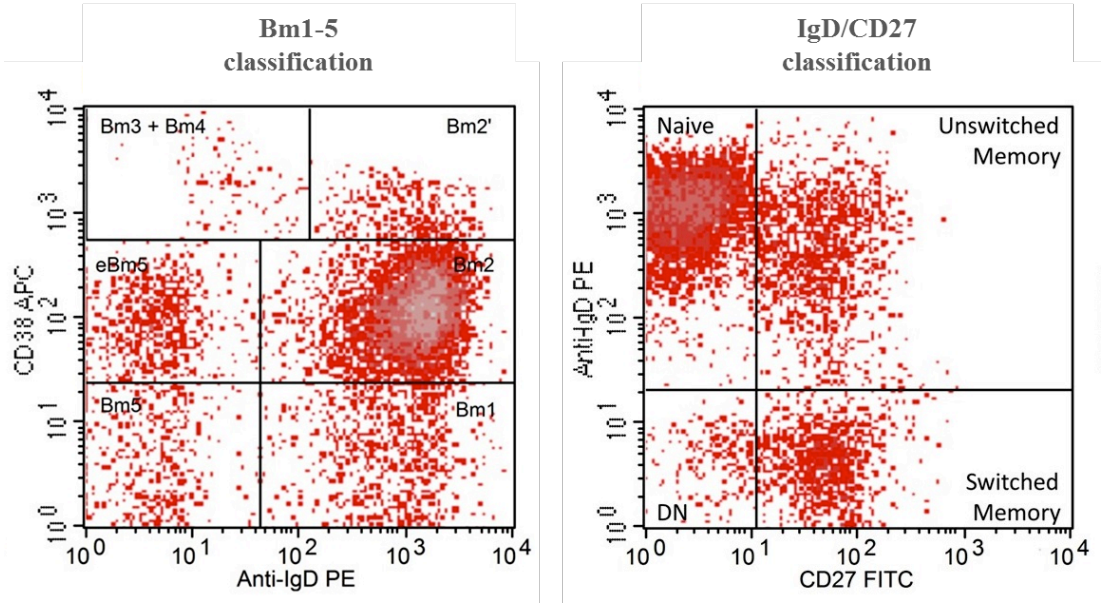

Figure 3. Bm1-5 and IgD/CD27 classification systems of peripheral blood mature B cell subsets according to their differential expression of IgD/CD38 and IgD/CD27.

1.2.2 Regulatory B cells

In recent studies,[15,18] B lymphocyte functions have been reconsidered, and it is now accepted that these cells play much more diverse functions than antibody production alone. In addition to their humoral activity, specific subsets of B cells have regulatory functions (regulatory B cell, Breg), although this notion remains controversial. They can down regulate cellular immune responses and inhibit excessive tissue-specific inflammation. Bhan's group first introduced the term "Bregs" based on evidence obtained in chronic colitis in 1997.[19]

Breg cells have been thoroughly investigated during the last decade, mostly in studies involving murine models of chronic inflammation and autoimmune diseases.[20] The presence of Breg cells in humans was later confirmed in other studies using in vitro models.[21,22]

According to recent studies, Breg can inhibit pro-inflammatory responses by secreting the anti-inflammatory cytokine IL-10, one of the most important anti-inflammatory cytokines capable of down regulating inflammation, avoiding an exacerbated inflammatory response.[18,23] Breg cells were first reported to be IL-10-producing B cells in mice and termed B10 cells.[23] Hence, most of the work investigating Breg has focused on IL-10-producing B cells. However, B cells can exert regulatory functions independently of IL-10 production.[24] Breg can regulate distinct profiles of inflammation, including T helper (Th) type 1 (Th1), Th type 2 (Th2), and Th type 17 (Th17) cells (Figure 4).[24] There is a growing body of evidence demonstrating that Breg cells are key players in different conditions, such as rheumatoid arthritis, autoimmune diabetes, autoimmune encephalomyelitis and lupus.[25]

Regulatory functions have been attributed to different B cell subsets, and despite some controversy over this issue, some progress has been achieved in the characterization of Breg.[26] The inability to identify a Breg-specific transcription factor, together with the phenotypic heterogeneity of Breg, supports the idea that Breg are not lineage-specific and that they may expand in response to inflammation when immunosuppression is necessary.[27] It remains unclear whether the B cell

regulatory function is a specific role of a particular B cell subset or whether it is a reflection of their stage of maturation.

Transitional B cells represent an essential developmental stage of B cell maturation,[20] and the discovery of Breg has generated interest in their potential relationship with transitional B cells because of their phenotypic and functional similarities. Although almost all human B cells have the ability to produce IL-10, there is evidence that Breg cells are the most effective IL-10-producing B cells and consistently found within the $CD24^{Hi}CD27^{+}$ and $CD24^{Hi}CD38^{Hi}$ cell populations, which are similar to $CD24^{Hi}CD38^{Hi}$ transitional B cells.[12,18,23] Furthermore, links between the expansion of $CD24^{Hi}CD38^{Hi}$ transitional B cells and immune regulation were noted in the favorable clinical outcomes of patients with inflammatory and autoimmune diseases (e.g., more prolonged clinical responses).[28]

However, the biology of transitional B cells in humans remains controversial. A recent study reported a new developmental pathway for human transitional B cell maturation.[29] Simon et al. demonstrated that the transitional B cell subsets identified within $CD24^{Hi}CD38^{Hi}$ cells display differential regulatory abilities. This study revealed that $CD27^{+}$ transitional B cells suppress the production of pro-inflammatory cytokines and have the capacity to produce high levels of IL-10. Although the capacity to produce IL-10 is frequently used as a marker for human Bregs, it was demonstrated that this property is not restricted to a specific B cell subset. Indeed, IL-10 was shown to be produced by transitional B cell subsets within the $CD24^{Hi}CD27^{+}$ cells. Therefore, immune regulation by B cells might not be confined to a specific B cell subset and does not occur in a functionally restricted manner.[29] Instead, different B cell subsets seem to have different regulatory properties that are influenced by their microenvironment. Nevertheless, Simon et al. provides primary evidence that transitional B cells are not a homogeneous Breg cell population but rather represent a complex combination of subsets from which different Breg cells arise.

Moreover, beyond the IL-10-mediated suppressive effects, strong evidence supports the dependence of the immunosuppressive effects of Breg cells on interactions with other immune cells and other regulatory cell lineages. For example, the maturation and function of human dendritic cells are regulated by B

lymphocytes.[22] Moreover, B cells require signals from T cells to induce B cell regulatory functions, which in turn suppress T cell proliferative responses.[30]

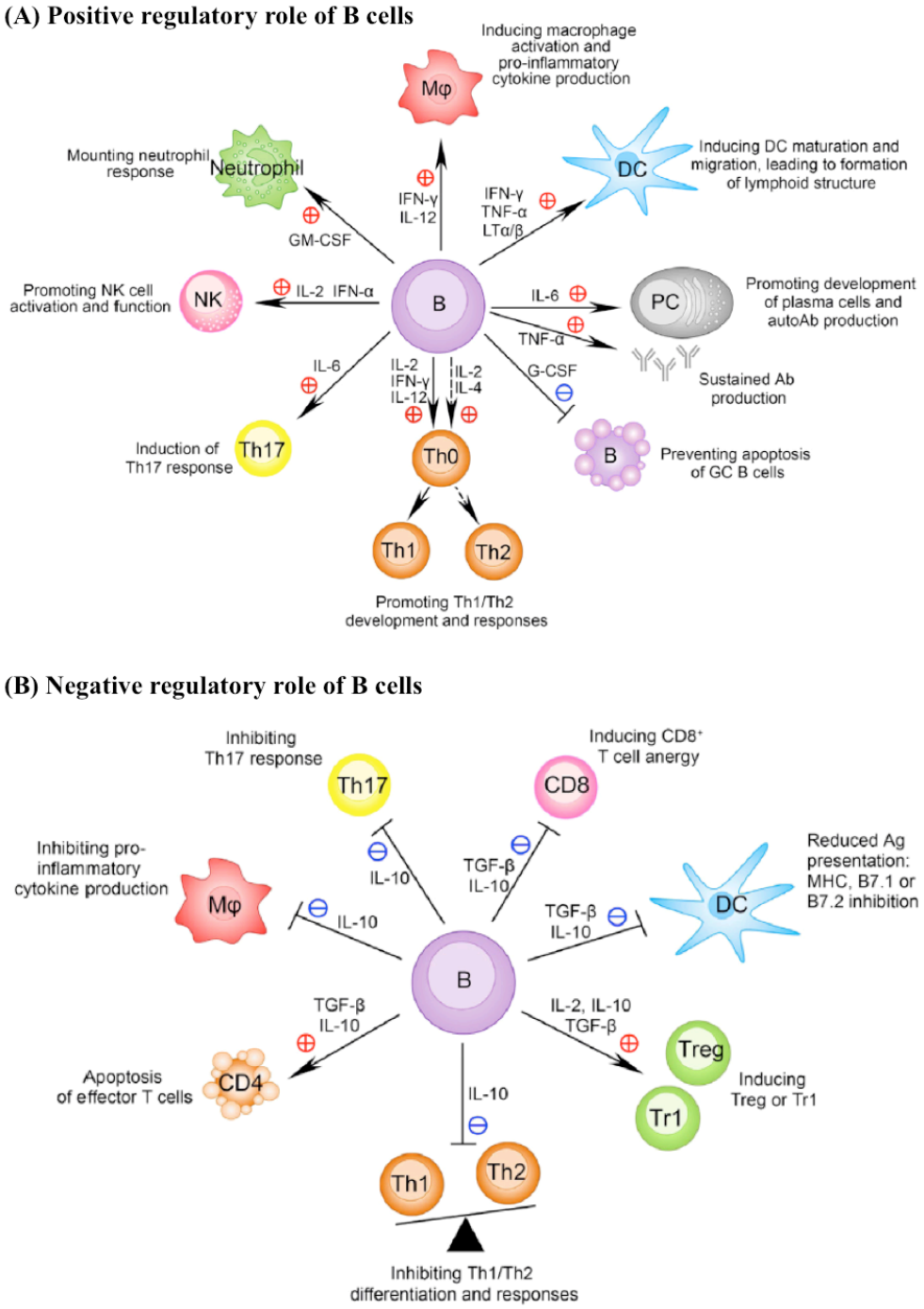

Figure 4. Positive (A) and negative (B) regulatory role of B cells.
DC, dendritic cell; GC, germinal center; G-CSF, granulocyte-colony stimulation factor; GM-CSF, granulocyte-macrophage colony-stimulation factor; IFN, interferon; IL, interleukin; Mφ, macrophage; NK, natural killer; PC, plasma cell; TGF, transforming growth factor; Th, T helper cell; Tr1, type 1 regulatory T cell Foxp3$^-$; Treg, regulatory T cell. Adapted from Bao & Cao, 2014.[31]

1.2.3 T cells

Following their development in the thymus, T cells are considered to be naïve until they first encounter a cognate peptide antigen. This encounter takes place in the secondary lymphoid organs and entails interactions with dendritic cells that present the cognate antigen on their cell surface in association with major histocompatibility complex (MHC) molecules. For $CD4^+$ T cells, T cell receptor (TCR) signaling is stimulated upon engagement of peptide antigen:MHC class II complexes; for $CD8^+$ T cells, T cell receptor signaling is stimulated upon engagement of peptide antigen:MHC class I complexes. If dendritic cells also supply sufficient co-stimulation, the responding T cell suffers clonal expansion and then differentiates into one of the several T cell subsets, as specified by the set of cytokines encountered during antigen exposure (Figure 5). These subsets are Th1, Th2, Th17, effector $CD4^+$ T cells, regulatory $CD4^+$ T (Treg) cells or even cytotoxic T lymphocytes, which are usually effector $CD8^+$ T cells. Once activated, an effector T cell exits the secondary lymphoid organs and then homes via the blood to the peripheral tissues.[32] The T cells subsets are defined by the set of transcription factors they express to maintain their differentiated state, as well as by the set of cytokines they produce, which mediate their effector responses.[33-35]

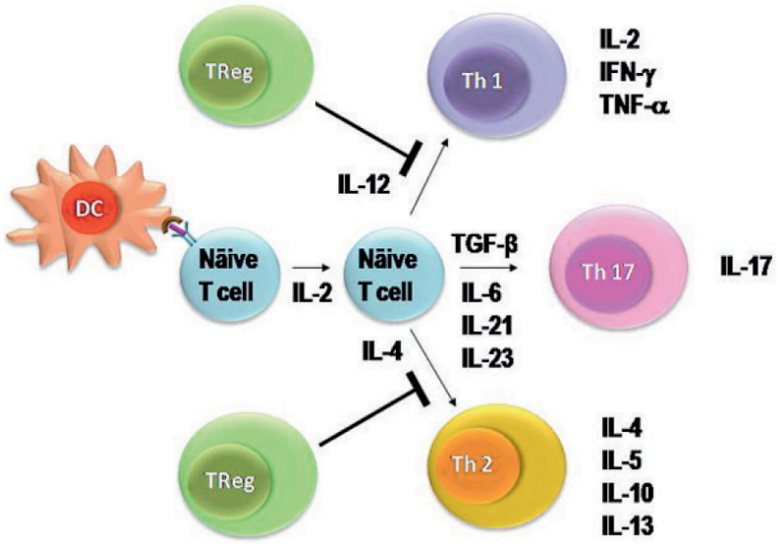

Figure 5. Naïve T cell differentiation into Th1, Th2 or Th17 cells, and the respective cytokine profiles. DC, dendritic cell; IL, interleukin; IFN, interferon; TGF, transforming growth factor; Th, T helper cell; TNF, tumor necrosis factor; TReg, regulatory T cell. Adapted from Zen et al., 2010.[36]

1.2.4 Regulatory T cells

Regulatory T cells can be classified into thymic (naturally occurring) and extrathymic (inducible or peripheral) Treg cells.[37] The former differentiate in the thymus, similar to other T cells, and constitute the majority of Treg, which are capable of recognizing both self and non-self-antigen.[38] The latter are a smaller population of Treg, which differentiate from peripheral T cells (naïve $CD4^+$ T cells) or are produced by ex vivo stimulation with antigens in the presence of TGFβ and retinoic acid.[38] The thymic-derived Treg are predominantly associated with the preservation of immune homeostasis and the prevention of autoimmunity, and they are not involved in maternal-fetal tolerance. Extrathymic Treg cells have been shown to play an important role in establishing tolerance at interfaces, such as the mucosal surfaces of the lung and gut, where the immune system comes into contact with the exterior and is chronically exposed to allergens and other foreign antigens; and maintaining an allogeneic pregnancy by inducing maternal-fetal tolerance (Figure 6).[37]

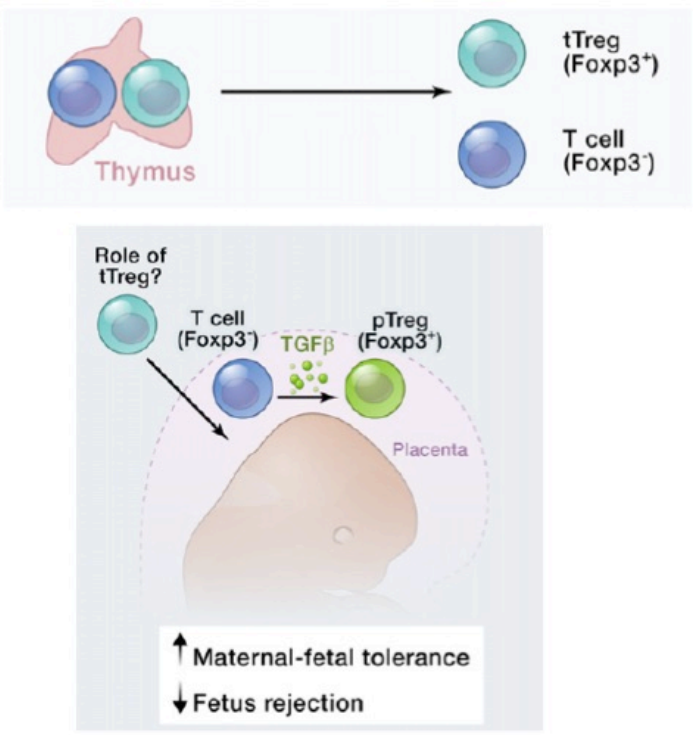

Figure 6. Role of peripheral regulatory T cells in maternal-fetal immunotolerance. The thymus exports naïve T cells (not expressing the transcription factor Foxp3, $Foxp3^-$) and thymus-generated Tregs (tTreg). Peripheral Tregs (pTreg) differentiate from peripheral $Foxp3^-$ T cells in response to TGFβ. Adapted from Gobert & Lafaille, 2012.[37]

As mentioned above, Treg are a specialized $CD4^+$ T cell subpopulation that is involved in the establishment of immune tolerance against self-antigens and antigens encountered in the foreign graft,[2,39] because they are potent suppressors of inflammatory immune responses. Additionally, Treg prevent maternal T cell activation against fetal cells, and this protection of the fetus from the maternal immune system has been widely reported in both mice and humans.[40-42]

Although numerous Treg-specific markers have been proposed for mice and humans, expression of the transcription factor Forkhead box p3 (Foxp3) is still the most consistent marker, and this protein seems to be essential for the development and maintenance of Treg and for the function of these immunosuppressive cells.[43,44] The Treg population has been defined as $CD4^+CD25^{Hi}CD127^{-/dim}Foxp3^+$, although the literature varies when considering markers for the exact phenotype of a Treg population.[45] In humans, detection of the $CD4^{Dim}CD25^{Hi}$, $CD4^+CD25^{Hi}Foxp3^+$ and $CD4^+CD25^{Hi}CD127^{-/dim}$ T cell populations remains amongst the most common analytical strategies used to identify Treg subsets.[42,46-48]

2 Immunological Aspects of Pregnancy

Pregnancy in humans is accompanied by profound changes and adaptations in both endocrine and immune systems. These adaptations are necessary for the maternal immune system to accept and tolerate a semi-allogeneic fetus expressing foreign antigens.[49] However, in some cases, these changes may influence the course of pre-existing pathologies, as observed in the case of autoimmune diseases.[50] Simultaneously, mother and fetus must be properly protected against potential infections.[51,52] To accomplish this goal, the immune machinery must be strictly regulated during the different stages of pregnancy.[53] As described previously, this regulation is considered to be promoted by several types of immune cells and by an array of anti-inflammatory and pro-inflammatory cytokines.[2,3] In addition, abundant evidence has highlighted the role of female sex hormones in shaping the immune system toward the transient state of tolerance necessary for the maintenance of pregnancy, in addition to their accepted functions in coordinating and controlling the anatomical modifications associated with pregnancy.[54] Our knowledge of the unique immunological relationship between the mother and the fetus is now substantially advanced, but there are still missing pieces of the puzzle.

In general terms, discussions concerning the nature of the immune response to pregnancy have centered on the extent to which the innate and adaptive immune compartments, and their component elements of the immune response, are essential participants in the process of maternal immune suppression or tolerance of the embryo/fetus.[2] For example, macrophages, DCs, and NK cells, which are central cellular components of the innate immune system, have been described to be important in pregnancy.[55] However, the participation of components of the adaptive immune system is more difficult to ascertain because it is a more advanced system of defense involving a repertory of lymphocytes that can express a wide range of receptor systems against specific foreign entities.

2.1 Mechanisms of Maternal-Fetal Immunotolerance

2.1.1 Maternal-Fetal Immunotolerance Initiated by the Trophoblast

Much of what is known about maternal-fetal immunology has been derived from research designed to explain how the fetal and placental semi-allograft avoids immunological attack by the mother. There are numerous mechanisms underlying immune tolerance in the placenta, and many of these depend on the interaction between maternal and fetal cells (Table 1).

First, during the immunosuppression mechanism that occurs via paracrine signaling, macrophages from the decidua up-regulate indoleamine 2,3-dioxygenase (IDO), which metabolizes the amino acid tryptophan into L-kynurenine. This tryptophan depletion at the maternal-fetal interface prevents effector T cell and NK cell activation and promotes Treg cell activation.[56,57]

Immunosuppression may also act via exosomes produced by the syncytiotrophoblast carrier molecules, namely Fas ligand (FasL), whereas the active form has the capacity to induce apoptosis in activated immune cells.[58] Trophoblast cells induce apoptosis of activated immune cells through the expression of FasL on its surface, and this FasL expression is supported by locally produced anti-inflammatory cytokines.[58] The Fas/Fas ligand system is an excellent example of a mechanism that supports life and of the function of the reproductive tissues, by enabling death via apoptosis.

In another mechanism, the expression of MHC molecules on trophoblast cells is repressed (the trophoblast does not present alloantigens) as a strategy to avoid recognition and destruction by maternal immune cells.[56] As a result, peripheral blood lymphocytes from pregnant women demonstrate a reduced capacity to develop into effector cytotoxic T lymphocytes.

In the mechanism of immune evasion, expression of MHC proteins at the maternal–fetal interface is strictly regulated during pregnancy,[56] and the human leukocyte antigens (HLAs) that are expressed in fetal membranes are tolerogenic rather than immunogenic. MHC class I genes are subdivided into classes Ia and Ib.

MHC class Ia is further subdivided into HLA-A, B, and C, and class Ib is subdivided into HLA-E, F, and G. HLA class II (HLA-D) genes are not translated in human trophoblast cells. The lack of MHC class II molecules protects the fetus from the presentation of fetal antigens to maternal immune cells. Human trophoblast cells express one MHC class Ia (HLA-C) and all MHC class Ib molecules. In the human placenta, fetal trophoblast cells lack expression of MHC class Ia (i.e., HLA-A and B) molecules, which are responsible for the rejection of allografts in humans. In normal pregnancies, decidual T cells recognize fetal HLA-C at the maternal-fetal interface but are prevented from inducing a destructive immune response.[56]

HLA-G seems to be an essential immunosuppressive factor during pregnancy and has been identified as a naturally occurring tolerance-inducing molecule.[59] HLA-G was originally discovered on the extravillous cytotrophoblast at the maternal-fetal interface, and its expression in the amniotic membrane may influence the maternal immune system during the inhibition of $CD8^+$ cytotoxic T cell and peripheral and decidual NK cell activities, the allogeneic proliferative response of $CD4^+$ T cells and cell cycle progression of alloreactive T cells.[59]

Table 1. Mechanisms of Immunological Tolerance Initiated by the Trophoblast

Mechanism	Function
Immunosuppression via paracrine signaling	• IDO secretion: inhibits T cell responses via tryptophan depletion • VIP secretion: promotes differentiation of regulatory T cells • Soluble HLA-G secretion: protects against NK-cell-mediated cytotoxicity
Cell-cell interaction	• FasL expression: induces apoptosis upon ligation of Fas expressed on activated leukocytes
Induction of maternal tolerance	• Trophoblast shedding: exposes the maternal circulation to paternal antigens shed from trophoblast cells • Fetal microchimerism: exposes the maternal circulation to paternal antigens by directing the trafficking of fetal cells
Immune evasion	• Lack of surface MHC II: induces escape recognition by $CD4^+$ helper T cells • Lack of HLA-A and HLA-B: induces escape recognition by $CD8^+$ cytotoxic T cells • Expression of HLA-C, HLA-E and HLA-G: decreases recognition by the immune system because these MHC isotypes exhibit low to undetectable levels of polymorphism

Note: FasL, Fas ligand; HLA, human leukocyte antigen; IDO, indoleamine 2,3-dioxygenase; MHC, major histocompatibility complex; VIP, vasoactive intestinal peptide.
Adapted from Hsiao & Patterson, 2012.[60]

2.1.2 Maternal Innate Immune Response During Pregnancy

Implantation requires a receptive endometrium, a so-called healthy uterine "milieu" that allows invasion of the blastocyst and rapid growth of the placenta while supporting the transformation of uterine into decidual cells.[2] This process is facilitated by hormonal changes and, importantly, by the immune cell populations and their cytokines.[61]

The immune cells that are relevant for implantation are already present in the uterus before pregnancy. The most studied uterine immune cells are the uterine NK (uNK) cells, comprising 70% of the human decidual leukocytes,[62] uterine dendritic cells (uDCs),[63-66] uterine mast cells (uMCs),[67,68] and uterine macrophages (comprising about 20% of the human decidual leukocytes),[57,69] which are crucial for modulating trophoblast function (Table 2).

The specific immune cells that are present in the uterus fluctuate in number, potentially due to a hormonal influence.[61] This phenomenon applies to uNK cells and macrophages, but other cells are likely to be attracted from the periphery to the uterus in response to the hormonal changes in early pregnancy, as shown for uMCs and macrophages.[61] For uDCs, it is still uncertain whether they live in the uterus or are attracted by pregnancy hormones, or both.[2]

Additionally, to act as the first line of defense, the cells of the innate immune system that are present in the uterus acquire an additional role. They dynamically contribute to the establishment of pregnancy either by remodeling the tissue or by releasing angiogenic factors that contribute to spiral artery remodeling.[2]

Table 2. Function of Uterine Innate Immune Cells During Pregnancy

Cell type	Role	Reference
Natural killer cells	Promote decidual angiogenesis and spiral artery remodeling, important for implantation.	Ratsep et al., 2015[62]
Dendritic cells	Promote T helper type 2 cell response, preparation of the uterus for implantation, early antigen presentation to T cells and regulatory T cells; mediators of communication between the trophoblast and regulatory T cells.	Bachy et al., 2008[63], Plaks et al., 2008[64], Zenclussen et al., 2010[65], Du et al., 2014[66]
Mast cells	Promote angiogenesis at the maternal—fetal interface; modulate spiral artery remodeling; support implantation and fetal growth.	Woidacki et al., 2013[67], Schmerse et al., 2014[68]
Macrophages	Protect the growing fetus from microorganisms, aid placental development by promoting trophoblast invasion and spiral artery remodeling, and contribute to the parturition process.	Fest et al., 2007[69], Tang et al., 2015[57]
Neutrophils	Support angiogenesis.	Amsalem et al., 2014[70]

2.1.3 Maternal Adaptive Immune Response During Pregnancy

After pregnancy is established, multiple immune system mechanisms must coexist to guarantee maternal-fetal immunotolerance (Table 3). This state of immunological tolerance may influence the course of pre-existing pathologies (e.g., autoimmune diseases)[50] and may also increase fetal-maternal susceptibility to infections.[71] Indeed, a decrease in B cells function with a loss of responsiveness to mitogens and infectious agents during the course of a normal human pregnancy has been reported.[72]

B cells play a role in pregnancy because of their humoral activity.[73] The generation of asymmetric antibodies occurs in normal pregnancy, protecting the fetus and placental antigens against maternally derived symmetric (antipaternal) antibodies at the maternal-fetal interface.[74] Additionally, these asymmetric antibodies prevent immunological attack by maternal NK cells and cytotoxic lymphocytes. Their secretion seems to be partially hormone-regulated, and their absence has been associated with pregnancy failure.[75]

Beyond the commonly recognized role of B lymphocytes in antibody production, B cells can also act as antigen-presenting cells to induce T cell immune responses. However, B cell functions can be regulated by cytokines. For example, B cell-activating factor (BAFF), a member of the TNF family of cytokines that is produced by both innate immune cells and non-hematopoietic cells, is a key regulator of B cell maturation and function. Signals induced when BAFF interact with its main receptor on B cells are pivotal for the survival and differentiation of immature transitional B cells into mature naïve cells.[76] Circulating BAFF levels have been positively associated with the proportions of memory B cells, and inversely related to the proportions of immature/naive B.[77] Excessive BAFF-mediated survival of peripheral immature B cells contributes to the emergence and maturation of autoreactive B cells,[76] and high levels of BAFF are associated with autoimmune disorders.[78]

The physiological and pathophysiological function of BAFF in pregnancy is uncertain, and it is not known whether BAFF plays any role in mediating the immune bond between mother and infant during the postpartum period.[79] Still, there is accumulating evidence that BAFF could be a suitable candidate for the role of a pivotal autocrine and paracrine regulator of immune processes in the human placenta and breast, leading to specific regulatory effects on placental cell viability and differentiation.[80]

Table 3. Function of Adaptive Immune Cells During Pregnancy

Cell type	Role	Reference
B cells	Protection of the fetus against maternally derived symmetric antibodies; fight danger signals at the maternal-fetal interface.	Zenclussen et al., 2001[74]
Regulatory B cells	Involved in maternal-fetal immune tolerance through IL-10; play a role in protecting pregnancy from the adverse effects of inflammatory stress during gestation; positively influence pregnancy by blocking Th1 cell immune responses.	Zenclussen et al., 2001[74]; Fettke et al., 2014[81]; Muzzio et al., 2014[82]
T cells	Protection of the placenta from immune rejection and facilitation of embryo implantation.	Nancy & Erlebacher, 2014[32]
Regulatory T cells	Contribution to an adequate uterine environment that guarantees implantation; early tolerance of paternal antigens expressed by the fetus.	Guerin et al., 2011[83]; Teles at al., 2013[84]

2.1.3.1 B cell subsets and regulatory B cells in pregnancy

Numerous studies,[85-95] both prospective and cross-sectional, have reported data for circulating B cells during normal human pregnancy, among which the majority have described a lower total number or frequency compared with the levels in the postpartum period or in healthy non-pregnant women (Table 4). Most studies[87-93,95] have only investigated total B cells, as defined by the expression of CD20 or CD19, and older studies[85,86,94] used either the expression of Ia (human leucocyte antigen – antigen D related complex, HLA-DR) or surface immunoglobulin to identify B cells. A few studies[88-91] have reported the frequency of B cell subsets expressing CD5, with the majority describing a lower frequency or lower total numbers of this subset during pregnancy, at delivery, or early in the postpartum period. One study[88] reported lower CD21 and CD23 frequencies at delivery. However, peripheral blood B cells have not been characterized in human pregnancy while considering the different

maturational stages, from transitional to memory B cells (using CD38 and immunoglobulin IgD as differentiation markers).

Table 4. Peripheral Blood B Cell Populations During Human Pregnancy and the Postpartum Period

B cell marker	Variation in B cell numbers (period)	*N*	Reference
Ig^+	decrease in % (pregnancy)	32	Christiansen et al., 1976[94]
Ia^+	no change in % and decrease in absolute numbers (pregnancy)	5	Moore et al., 1983[85]
Ig+	decrease both in % and in absolute numbers (pregnancy)	77	Valdimarsson et al., 1983[86]
$CD20^+$	no change in % and decrease in absolute numbers (pregnancy); increase in % (postpartum)	94	Iwatani et al., 1988[87]
$CD20^+CD5^+$ $CD20^+CD19^+$ $CD20^+CD21^+$ $CD20^+CD23^+$	decrease in absolute number (postpartum)	23	Delgado et al., 1994[88]
$CD20^+CD5^+$	decrease in % (pregnancy); increase in % (postpartum)	18	Bhat et al., 1995[89]
$CD19^+$ $CD19^+CD5^+$	decrease in absolute numbers (pregnancy); increase in absolute numbers (postpartum)	5	Matthiesen et al., 1996[90]
$CD19^+$ $CD19^+CD5^+$	decrease in absolute numbers (pregnancy); increase in absolute numbers (postpartum)	106	Watanabe et al., 1997[91]
$CD19^+$	decrease in % (pregnancy)	92	Kuhnert et al., 1998[92]
$CD19^+$	decrease both in % and in absolute numbers (pregnancy)	34	Mahmoud et al., 2001[93]
$CD19^+$	decrease in absolute numbers (pregnancy); increase in absolute numbers (postpartum)	50	Kraus et al., 2012[95]

Note. N, number of healthy pregnant women.

There is a growing body of evidence demonstrating that Breg are key players in different situations in which the immune system is hyperactivated.[24,96] Breg counts increase in the first trimester of pregnancy, suppressing the unwanted immune responses of maternal effector T cells and protecting against pregnancy loss.[15] These findings suggest that B cells with regulatory functions may be important for a better understanding of the mechanisms characterizing pregnancy and delivery.

While the phenotype and function of regulatory T cells have been extensively studied,[97] additional studies are needed to investigate the mechanisms underlying the activation and expansion of Breg and other B cell subsets during pregnancy.

2.1.3.2 T cell subsets and regulatory T cells in pregnancy

Physiological pregnancy has been compared to a state of quiescent systemic inflammation, and parturition has been compared to an immunological reaction that results in the recruitment of immune cells to the systemic circulation and the maternal-fetal interface.[98] This entire process may have an effect on the maturation and activation profiles of T cells in the peripheral blood of pregnant women.[99,100]

T cells are abundant in the human decidua, comprising approximately 10-20% of decidual leukocytes,[49] of which 30% to 45% are $CD4^+$ T cells and 45% to 75% are $CD8^+$ T cells.[32] The main function of T cells in the decidua, particularly of the $CD4^+$ Treg cells, is generally thought to be the promotion of fetal tolerance.

During a successful pregnancy, the normal profile is Th2-type immunity, which is more accentuated at the maternal-fetal interface.[49] In addition, Th2 cytokines down regulate Th1-type reactivity. Labor is often linked to a pro-inflammatory state with reversal back to a Th1 rather than a Th2 profile. Currently, to explain this immunotolerance in pregnancy, the Th1/Th2 paradigm has been expanded into the Th1/Th2/Th17/Treg paradigm. In this new paradigm, Th17 cells, which produce IL-17, are mediators of inflammation along with Th1 cells, while Treg cells, which are potent suppressors of inflammatory immune responses, may be important for the induction of antigen-specific tolerance.[101]

Th17 cells are present in decidua and are increased in the peripheral blood of normal pregnancies, playing a role in controlling the maternal-fetal relationship and development of the placenta.[102] Practically all effector T cells generated in response to an immunogenic antigen undergo apoptosis following the completion of antigen clearance. Still, a few of these antigen-experienced cells survive and differentiate into memory T cells, which are capable of establishing rapid responses after a second contact with the antigen;[32] however, their role in pregnancy has not yet been investigated.

Recent reports have suggested that another heterogeneous population of T cells might also have an important function in fetal-maternal immunological tolerance and in the regulation of the Th1/Th2 balance: the $CD3^{+}CD56^{+}CD16^{+}$ natural killer T cells-like (NKT-like). This subset simultaneously expresses T cell and NK cell surface markers, and has been found in peripheral blood and in human decidua.[103] Nevertheless, published data regarding the role of circulating levels of NKT-like cells in embryo implantation have been contradictory.[104,105]

Regarding T cells with regulatory functions, several studies have reported their expansion in the decidua of normal pregnant women. This expansion is most vigorous during early pregnancy and decreases as the end of term approaches, suggesting that the immune regulatory processes that occur at the maternal-fetal interface are dynamic and provide immune protection for the fetus.[41] However, the current set of data does not determine whether Treg cells act locally within the uterus, systemically, or both.[101]

Owing to the bidirectional cell trafficking between mother and fetus, antigen presentation and recognition can occur in any compartment of the maternal immune system.[106] Given the well-known immunosuppressive functions of Treg cells, a new model suggests a hypothetical scenario in which Treg cells expand within the decidua as a result of the presentation of placental antigens by decidual antigen-presenting cells.[107] This phenomenon occurs to limit effector T cell activity at the maternal-fetal interface to permit tolerance to the fetus and to prevent rejection via classical immune mechanisms. Paternal antigens are first encountered in the vaginal lumen, where maternal dendritic cells meet paternal antigens present in the seminal fluid. After this first step, dendritic cells take up paternal antigen and migrate to

lymph nodes draining in the uterus. DCs present paternal antigens to Treg cells, and Treg cell proliferate prior to the implantation. These paternal antigen-specific Treg cells migrate to the pregnant uterus (maternal-fetal interface) guided by chemokines and human chorionic gonadotropin (hCG), modulate the phenotype and function of immune cells and generate a friendly environment for embryo implantation.[101,107]

Changes in Treg numbers during human pregnancy occur not only in the decidua but also in the maternal peripheral blood.[108,109] Treg subsets in the peripheral blood have been explored in normal human pregnancy and during the postpartum period in numerous studies (Table 5); however, the results have been highly inconsistent, with some reports showing a decrease[110] or an increase[42,111-115] during the 1st trimester, a decrease[110,116] or an increase[42,112,114,115,117] during the 2nd trimester, or a decrease[41,42,110,112-115] or an increase[48,109,113,117] during the 3rd trimester; others have shown either no changes[113] or a decline[115,117] during labor and a decrease[42,112,115] or an increase[118] in the postpartum period. These discrepancies can be attributed to differences in study design because some studies did not include the postpartum period,[41,110,113,114,116,117] and other studies did not obtain samples from some women at all time points.[42,110,112-115,118] Distinct reports of Treg patterns may also be attributed to differences in sample size, laboratory techniques, or the use of different and sometimes non-specific markers to identify Treg subsets.

While the phenotype and function of regulatory T cells have been extensively examined,[97] additional studies are needed to investigate the mechanisms underlying the activation and expansion of Breg and other B cell subsets in human pregnancy.

Table 5. Peripheral Blood Regulatory T Cell Populations During Human Pregnancy and the Postpartum Period

Regulatory T cell marker	Variation in Treg numbers					*N*	Reference
	1st T	2nd T	3rd T	L	PP		
$CD4^+CD25^+$ $CD4^+CD25^+CTLA\text{-}4i^+$	peak	↗	↘			7	Heikkinen et al., 2004[41]
$CD4^+CD25^+$	↗	peak	↘		↘	63	Somerset et al., 2004[112]
$CD4^+CD25^{bright}$	↗					19	Sasaki et al., 2004[111]
$CD4^+CD25^{bright}$			↗			40	Sasaki et al., 2007[109]
$CD4^+CD25^+Foxp3^+$		↘	↘			188	Steinborn et al., 2008[114]
$CD4^+CD25^{bright}$	peak	↗	↗			32	Seol et al., 2008[113]
$CD4^+CD25^{high}$ $CD4^+CD127^{low}CD25^+$ $CD4^+Foxp3^+$			↗			47	Santner-Nanan et al., 2009[48]
$CD4^{dim}CD25^{high}Foxp3^+$		↗				38	Mjosberg et al., 2009[116]
$CD4^+CD25^+Foxp3^+$	↗	peak	↘	↘	↘	139	Xiong et al., 2010[115]
$CD4^+CD25^+C127^{low+/-}$ $Foxp3^+HLA\text{-}DR^+$	↘	↘	↘			82	Kisielewicz et al., 2010[110]
$CD4^+CD25^+Foxp3^+CD127^-$					↗	208	Wegienka et al., 2011[118]
$CD4^+CD25^+$ $CD4^+CD25^+CD127^-$	↗	↗	↘		↘	90	Xiong et al., 2013[42]
$CD4^+CD25^{high}CD127^{low}$ $Foxp3^+$		↗	↗	↘		20	Areia et al., 2015[119]

Note. Treg, regulatory T cell; T, trimester; L, labor; PP, postpartum; N, number of healthy pregnant women. ↗, increase; ↘, decrease.

2.2 Endocrine and Immune System Interaction During Pregnancy

Pregnancy is associated with changes in the concentrations of several hormones, including estradiol, estriol, progesterone, human chorionic gonadotropin, corticosteroids, and prolactin. These hormonal changes contribute to the immunological shifts that occur during pregnancy, and there is increasing evidence that immune-endocrine interactions generate a complex network of immune regulation that ensures fetal survival within the maternal uterus (Figure 7).[120,121]

Human chorionic gonadotropin initially maintains progesterone levels by inducing its production by the corpus luteum. After the luteal-placental shift, the placenta (trophoblast cells) takes over progesterone production, which is then secreted into the maternal circulation. The placenta also synthesizes estrogens from fetal and maternal precursors and secretes them into the fetal and maternal circulations. These pregnancy hormones are critically involved in the successful establishment, maintenance, and termination of pregnancy.[61] They suppress damaging maternal alloresponses while promoting tolerance pathways (reduction of the antigen-presenting capacity of dendritic cells, monocytes, and macrophages, and blockage of NK cells, T and B cells). Pregnancy hormones also promote the proliferation of pregnancy-supporting uterine NK cells, retention of tolerogenic DCs, and efficient induction of Treg cells. Moreover, they are involved in the recruitment of mast cells and Treg cells into the maternal-fetal interface, contributing to a local accumulation of pregnancy-protective cells.[36,61]

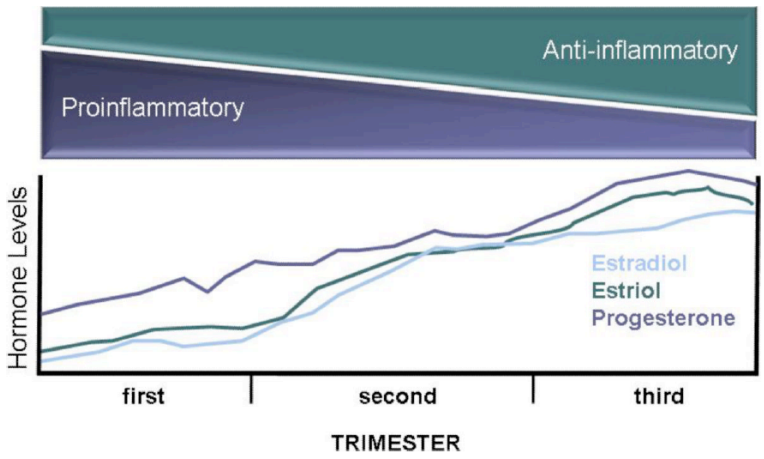

Figure 7. Pro-inflammatory and anti-inflammatory responses during pregnancy. Adapted from Robinson & Klein, 2012.[120]

Progesterone exerts its immune regulatory functions via mediators such as progesterone-induced blocking factor (PIBF). PIBF has several anti-abortive effects in vivo and a pregnancy-protective role: it enhances asymmetric antibody production by B cells, causes a Th2/Th1 shift to increase the production of Th2 cytokines, and inhibits the cytotoxicity of NK cells.[122] Recently, membrane progesterone receptors (e.g., mPRα) were found in human peripheral blood T lymphocytes,[123] specifically in Tregs,[119] suggesting that their levels change during preterm labor and that human labor may develop as a consequence of a decline in $mPR\alpha^+$ Treg cells. This decline reduces progesterone anti-inflammatory activity by permitting Treg cell inflammatory responses associated with cervical ripening, membrane rupture and uterine contractions.[117,119]

During pregnancy, estrogens affects different immune cell populations (their numbers and functions) and thereby contributes to fetal tolerance. Estrogens enhance IL-10, IL-2, and IFN-γ production, and inhibit TNF-α secretion by peripheral blood mononuclear cells; stimulate antibody production by B cells, and decrease apoptosis of DC and macrophages.[36] These effects are mediated via the binding of estrogens to its intracellular receptors, estrogen receptor alpha (ERα) and beta (ERβ), expressed in various lymphoid tissue cells as well as in lymphocytes, macrophages, and dendritic cells, which in turn modulate the expression of many genes.[61]

The effects of pregnancy-related hormones on Treg expansion have been investigated. While one study in mice[124] have shown that neither estrogen nor progesterone can stimulate Treg expansion, others[125,126] have indicated the opposite, showing that estrogens expand the Treg compartment and increase Foxp3 expression. A recent study suggested that high estrogen levels might induce an increase in Treg in the peripheral blood, influencing immune functions in pregnant women.[42] These results confirmed an important role of estrogen in immunomodulation during pregnancy.

The maternal and fetal immune system activation that occurs with labor[127-130] is a consequence not only of the increased production of cytokines in the uterine milieu,[131] but also due the increase in cortisol levels in the maternal circulation and in the umbilical cord blood from neonates.[132] In humans, circulating glucocorticoids increase as gestation progresses, peaking during labor.[133] Although the precise

function of cortisol in pregnancy is not well defined, it is known that this stress hormone, with potent anti-inflammatory and immunosuppressive effects, inhibits the synthesis of pro-inflammatory molecules, including cytokines, suppresses pro-inflammatory cytokine production by term human placental cells in vitro, and has the capacity to suppress both innate and adaptive immune responses.[134,135]

2.3 Changes in Immune Responses and Pregnancy Complications

Cells of the innate immune system are present in the uterus at fecundation, and their presence is fundamental for the initiation of tissue remodeling that ensures implantation and later to support the adaptation of the maternal spiral arteries. The presence of these essential immune cells and the influx of new ones at peri-implantation dictate its success. In animal models, the specific depletion of macrophages, dendritic cells, and mast cells disrupts implantation.[2]

The human maternal-fetal interface is normally colonized by a wide variety of bacteria, suggesting that effector T cells within the decidua have specificity for placental antigens and might have a positive function in controlling infection.[32] This function is very finely adjusted so that the response is sufficient to prevent problems related to the progression to pregnancy, such as acute infectious chorioamnionitis, and it is not so robust that the attendant inflammation required to fight the infection can lead to pregnancy complications, such as preterm labor secondary to chorioamnionitis.[32] The existence of intra-decidual mechanisms to limit maternal T cell responses against the placenta, which alter the dynamics of the decidual $CD8^+$ T cell response by changing the $CD8^+$ T cell repertoire and increasing the influx of $CD8^+$ T cells to the decidual tissue, alter MHC expression profiles of infected trophoblast cells and increase the production of pro-inflammatory cytokines and chemokines. These processes might also explain why the decidua is susceptible to infection by certain organisms such as *Listeria monocytogenes* and cytomegalovirus.[136]

From the adaptive immune system, regulatory T cells are critical for early pregnancy establishment. Their depletion before implantation negatively affects the

uterine environment, provoking inflammation and fibrosis in the uterus, which impedes implantation.[84] Interestingly, primary unexplained infertile women have reduced expression of endometrial Foxp3, supporting the relevance of Treg for implantation.[137]

A composed state of immune cell populations in the uterus during initial pregnancy is of importance not only for successful implantation but also for the quality of placentation. It is well-known that a deficient immune response at the onset of pregnancy can result in abnormal implantation and poor placentation, eventually ending in miscarriage, fetal growth restriction, or pre-eclampsia.[2] Traditionally, the state of immunological fetal tolerance has been explained by the predominantly Th2-type immunity found in normal pregnancies; in contrast, a predominantly Th1-type immunity was used to explain pathological pregnancies, such as those characterized by recurrent spontaneous abortion, intrauterine growth restriction, and pre-eclampsia.[138-143]

A decline in maternal Treg populations could cause failure of immunological tolerance to the fetus and has been associated with obstetrical complications, such as miscarriage, pre-eclampsia, and preterm labor.[109,115,143-146] Finally, disproportions between Th17 and Treg cells during the proliferative phase of the menstrual cycle has been recently considered as a cause for abortion in women with recurrent spontaneous abortion.[147,148]

Modifications of the immune environment during pregnancy have systemic consequences that may modify the course of some diseases.[84] The decrease in activity of pro-inflammatory cytokines and NK cells, inflammatory macrophages, and Th1 cells, together with the elevated Treg cell activity and production of anti-inflammatory cytokines, decreases the severity of diseases caused by inflammatory responses (i.e., arthritis and multiple sclerosis) and increases the severity of diseases that are relieved by inflammatory responses (such as influenza and malaria) (Figure 8).[120]

Unlike the protective effect proposed for asymmetric antibodies, the production of natural autoantibodies facilitates pregnancy complications. In patients with autoimmune disorders, it is possible that B cells secrete more autoantibodies that

compromise the pregnancy outcome.[73] In this regard, antiphospholipid antibodies have been associated with venous or arterial thrombosis and obstetric complications, such as recurrent miscarriage, fetal growth restriction, pre-eclampsia, placental abruption, premature delivery or fetal death.[149] In pre-eclampsia, B cells can become autoreactive and secrete antibodies against angiotensin receptor 1.[150] Emphasizing the idea of the involvement of B cells in the pathophysiology of pre-eclampsia, some authors have demonstrated that B cell depletion in a rat model of pre-eclampsia correlates with lower levels of antibodies against angiotensin receptor 1 and reduced symptoms of pre-eclampsia.[151]

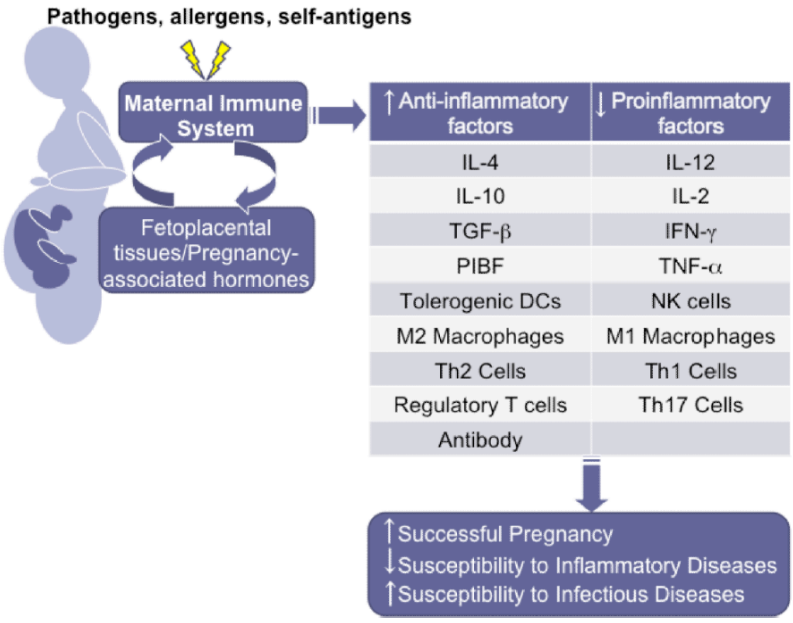

Figure 8. Immunological changes in pregnancy and outcome of diseases. Adapted from Robinson & Klein, 2012.[120]

The mechanism responsible for the syndrome remains unknown, but pre-eclampsia has the "fingerprints" of an immune response. It occurs mainly in first pregnancies, when the mother has had fewer opportunities to develop tolerance against paternal antigens, and inflammation is a major part of its pathogenesis. In 2012, it was reported that memory-type fetal antigen-specific Treg cells are present in a second gestation and that these Treg cells induce fetal antigen-specific tolerance.[152] This important finding explained the epidemiological paradigm in which the first conception and first pregnancy with a new partner or a long interval

between births are risk factors for pre-eclampsia.[153] Consequently, the proper development of regulatory T cells is likely to play a role in preventing this disorder. Some authors have found that women with pre-eclampsia not only have a lower percentage of Treg cells but also a higher percentage of Th17 cells, as compared with normal pregnant women.[48] Recent evidence has shown that the polymorphism *Fas*-1377G>A in one of the most important apoptotic pathways, the Fas/FasL system, is associated with decreased *Fas* and *FasL* expression, a predominance of Th1 immunity, inflammation and trophoblast damage, and an elevated risk of pre-eclampsia.[154]

Inflammation at the maternal-fetal interface is one of most well recognized causes of preterm birth. During labor, the maternal immune system is activated, resulting in increased absolute counts and percentages of peripheral blood leukocytes,[128-130] mainly due to the increase in absolute counts and percentages of neutrophils and the decrease in absolute counts and percentages of peripheral blood lymphocytes.[127] Therefore, excessive levels of pro-inflammatory cytokines or reduced levels of anti-inflammatory cytokines can explain the subsequent events leading to preterm birth. The number of Th17 cells is increased in the chorioamniotic membrane of cases of preterm delivery with chorioamnionitis.[155] These findings suggest that Th17 cells promote inflammation at the maternal-fetal interface in preterm delivery. Recent data have revealed the presence of membrane progesterone receptors (mPRs) in human Treg cells, which may explain the actions of progesterone on preterm birth and strengthen the rational use of this hormone for this type of obstetric complication.[117,119]

CHAPTER II

Objectives

General Objective

The main goal of the present research was to characterize the populations of B cells and T cells, specifically their regulatory subsets, in the peripheral blood of healthy pregnant women from the 3rd trimester to the postpartum compared to non-pregnant women.

Specific Objectives

In detail, the specific objectives were as follows:

- Analyze the variation of the absolute counts and percentages of specific B cell subsets (maturational stages) and regulatory B cell subsets (defined as $CD24^{Hi}CD27^{+}$, $CD24^{Hi}CD38^{Hi}$ and IL-10 producing B cells) in the peripheral blood of pregnant women, from late pregnancy to the postpartum (**Chapter III**);

- Investigate the variation of the absolute counts and percentages of the regulatory T cell subsets (defined as $CD4^{Dim}CD25^{Hi}$, $CD4^{+}CD25^{Hi}Foxp3^{+}$, and $CD4^{+}CD25^{Hi}CD127^{-/dim}$) in the peripheral blood of pregnant women, from late pregnancy to the postpartum (**Chapter IV**);

- Assess the effect of labor on the absolute counts and percentages of T cell subsets (maturational and activation profiles), regulatory T cell subsets, and regulatory B cell subsets in the peripheral blood of healthy women on the day of delivery (**Chapter V**).

CHAPTER III

Characterization of B Cells in Healthy Pregnant Women from Late Pregnancy to Post-Partum: A Prospective Observational Study

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Characterization of B cells in healthy pregnant women from late pregnancy to post-partum: a prospective observational study

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Abstract

Background: B cells play a role in pregnancy due to their humoral and regulatory activities. To our knowledge, different maturational stages (from transitional to memory) of circulating B cell subsets have not yet been characterized (cell quantification and phenotype identification) in healthy pregnant women. Thus, the objective of our study was to characterize these subsets (as well as regulatory B cells) from late pregnancy to post-partum and to compare them with the circulating B cells of non-pregnant women.

Methods: In all of the enrolled women, flow cytometry was used to characterize the circulating B cell subsets according to the expression of IgD and CD38 (Bm1-Bm5 classification system). Regulatory B cells were characterized based on the expression of surface antigens (CD24, CD27, and CD38) and the production of IL-10 after lipopolysaccharide stimulation.

Results: Compared to the absolute counts of B cells in the non-pregnant women ($n = 35$), those in the pregnant women ($n = 43$) were significantly lower ($p < 0.05$) during the 3rd trimester of pregnancy and on delivery day (immediately after delivery). The percentages of these cells on delivery day and at post-partum were significantly lower than those in the non-pregnant women.
In general, the absolute counts and percentages of the majority of the B cell subsets were significantly lower in the 3rd trimester of pregnancy and on delivery day than in the non-pregnant women. However, these counts and percentages did not differ significantly between the post-partum and the non-pregnant women.
The most notable exceptions to the above were the percentages of naïve B cells (which were significantly higher in the 3rd trimester and on delivery day than in the non-pregnant women) and of $CD24^{hi}CD38^{hi}$ regulatory B cells (which were significantly higher in the post-partum than in the non-pregnant women).

Conclusion: According to our study, the peripheral B cell compartment undergoes quantitative changes during normal late pregnancy and post-partum. Such findings may allow us to better understand immunomodulation during human pregnancy and provide evidence that could aid in the development of new strategies to diagnose and treat pregnancy-associated disturbances. Our findings could also be useful for studies of the mechanisms of maternal responses to vaccination and infection.

Keywords: B cell subsets, Flow cytometry, Human pregnancy, Obstetrics

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Background

The immune system of pregnant women tolerates a genetically foreign fetus. This physiological adaptation is thought to be promoted by an array of anti-inflammatory and pro-inflammatory cytokines that are produced by T and B cells [1]. This state of immunological tolerance may influence the course of pre-existing pathologies (e.g., autoimmune diseases) [2] and may also increase fetal-maternal susceptibility to infection [3]. Furthermore, a decrease of function of B cells with loss of responsiveness to mitogens and infectious agents during the course of normal human pregnancy has also been reported [4].

B cells have a role in pregnancy because of their humoral activity (i.e., the production of protective antibodies against paternal antigens during pregnancy and the production of auto-antibodies that may lead to pregnancy complications) [5]. Normally, B cells leave the bone marrow and enter the circulation as immature transitional B cells, which later mature into naïve B cells. Finally, when naïve B cells encounter their cognate antigens in secondary lymphoid organs, these cells become activated and mature into memory B cells and plasma cells [6, 7]. B cell subsets of different maturational stages, from transitional to memory B cells, have been identified in peripheral blood using the mature B (Bm)1-Bm5 classification system. This classification system has proven to be effective in the identification of disturbances in the proportions of peripheral blood B cell subsets in patients with autoimmune diseases (e.g., Lupus or Sjögren's syndrome) [8–10] and in those undergoing therapy (e.g., with biological agents), by assessing the depletion and repopulation of B cells [11].

Furthermore, it has been suggested that, in addition to their humoral activity, specific B cells can also have a regulatory function although this is still controversial. According to recent studies, regulatory B cells (Bregs) can inhibit pro-inflammatory responses by secreting the anti-inflammatory cytokine IL-10 [12, 13]. Breg counts increase in the first trimester of pregnancy, suppressing unwanted immune responses of maternal effector T cells, protecting against pregnancy loss [14]. While the phenotype and function of regulatory T cells has been extensively studied [15], further studies are needed to investigate the mechanisms behind the activation and expansion of Bregs and other B cell subsets in pregnancy.

These regulatory functions have been attributed to different B cell subsets, and despite some controversy, great progress has been made in the characterization of Bregs. The inability to identify a Breg-specific transcription factor, together with the phenotypic heterogeneity of Bregs, supports the idea that Bregs are not lineage specific and that they may expand in response to inflammation when immunosuppression is necessary [16]. It remains unclear whether the regulatory B cell function is a specific role of a particular subset or whether it is a reflection of their maturation stage. Although the expression of IL-10 has been a valuable tool in defining populations of Bregs, $CD24^{hi}CD27^{+}$ and $CD24^{hi}CD38^{hi}$ are the most frequently characterized phenotypes in humans [9, 12, 13].

Several studies [17–27] both prospective and cross-sectional have reported on circulating B cells during normal human pregnancy with majority describing lower total numbers and/or frequency when compared to levels post-partum or in healthy non-pregnant. Most studies [17, 19–24, 27] have only investigated total B cells as defined by the expression of CD20 or CD19 with older studies [18, 25, 26] using either expression of Ia (HLA-DR) or surface immunoglobulin. A few studies [17, 19, 24, 27] have reported on the frequency of B cell subsets expressing CD5 with majority describing lower frequency or lower total numbers of this subset during pregnancy, at delivery or early in the postpartum period. One study [19] reported lower CD21 and CD23 frequencies at delivery. However, peripheral B cells have not been characterized in human pregnancy while considering the different maturational stages, from transitional to memory B cells (using CD38 and immunoglobulin IgD as differentiation markers). Consequently, the objective of our study was to characterize these specific peripheral blood B cell subsets (transitional, naïve, unswitched memory, post-germinal, and resting memory B cells as well as plasmablasts) and Bregs ($CD24^{hi}CD27^{+}$, $CD24^{hi}CD38^{hi}$ and IL-10 regulatory B cells) from late pregnancy to post-partum and compare them with those in non-pregnant women.

Methods

Study population

This prospective observational study followed healthy pregnant women over time to characterize (i.e., cell quantification and phenotype identification) their peripheral blood B cell subsets from late pregnancy to post-partum. This characterization of B cells in the pregnant women was also compared with the characterization of single samples of peripheral blood B cells from a control group of healthy non-pregnant women to investigate changes associated with pregnancy.

Sequential non-laboring healthy women with singleton pregnancies who were attending an outpatient clinic (routine obstetrical care) during the 3rd trimester were recruited for participation. None of the pregnancies had complications prior to recruitment. Furthermore, all of the fetuses exhibited appropriate growth (as measured by uterine fundal height and by ultrasound performed after 28 weeks of gestation).

Sequential non-pregnant women who were attending an outpatient clinic were also recruited (healthy controls). These were asymptomatic women who were attending their annual routine well-woman exams.

For all of the women, the exclusion criteria were a history of diabetes, hypertension, or autoimmune disease and smoking during the 6 months prior to peripheral blood sample collection. Additional exclusion criteria for the pregnant women included prenatal use of any medication (other than vitamins and iron supplements) and ongoing complications in the pregnancy. Non-pregnant women taking oral contraceptives were also excluded, as these drugs affect circulating B cells [28].

All of the women were recruited at the *Hospital CUF Descobertas* in Lisbon (Portugal) between July 2013 and March 2014. The Ethics Committee of this hospital approved the study protocol. All of the recruited women provided written informed consent before the start of the study.

Study visit procedures

Three visits were planned for the pregnant women: visit 1 was planned for the 3rd trimester of pregnancy (3rd trimester); visit 2, for the day of delivery; and visit 3, for post-partum (at least 6 weeks after delivery). A single visit was planned for the non-pregnant controls.

To characterize B cell subsets from late pregnancy to post-partum, peripheral blood samples were collected from all of the pregnant women at each planned visit: the "3rd trimester" sample was collected at visit 1, the "on delivery day" sample was collected at visit 2 (immediately after delivery, within 15 min after placental expulsion and oxytocin administration), and the "post-partum" sample was collected at visit 3. A peripheral blood sample was collected from the non-pregnant women at the planned visit, which took place during the follicular phase of their menstrual cycle because hormone status during the luteal phase is similar to that during pregnancy [29].

The baseline data collected for all women at the time of enrollment included demographics (age and ethnicity), anthropometrics [body mass index (BMI)], obstetric history, and systolic and diastolic blood pressures. The data collected for the pregnant women on the day of delivery included gestational age, type of analgesia and/or anesthesia, and mode of delivery. The data collected for the newborns included gender, weight, and 1-min and 5-min Apgar scores.

Flow cytometry analysis and laboratory measurements

Peripheral blood samples were collected into EDTA-coated and heparinized tubes. These samples were analyzed by four-color flow cytometry (BD FACSCalibur, BD Biosciences, San Jose, CA, USA) to characterize B cell subsets and their maturation profiles. MultisetTM and CellQuest 3.3TM (BD Biosciences) software were used for both acquisition and analysis.

To obtain absolute counts of B cells ($CD19^+$), a single-platform strategy was used. EDTA samples were assayed using a lyse-no-wash technique, with a BD IMK Kit with BD Trucount™ Tubes (BD Biosciences). The assay was performed according to the manufacturer's instructions. In brief, 50 μL of blood were incubated for 15 min in the dark, at room temperature, with the monoclonal antibodies provided in the kit, in Trucount™ tubes containing a calibrated number of microbeads for counting purposes. Red blood cells were then lysed with the lysing solution (also provided with the BD IMK Kit), for 15 min and finally samples were acquired. The cells were gated on CD45/SSC, and a minimum of 2500 lymphocyte events were acquired. Multiset software provided percentage and absolute counts of B cells using the number of microbeads in each Trucount™ tube, along with the number of microbead and lymphocyte events acquired in each tube.

To study the surface B cell markers, a modified lyse-wash protocol was used. EDTA samples were washed twice in phosphate-buffered saline (PBS) to lower background staining. The washed cells were then stained with a panel of monoclonal antibodies (mAbs) that were conjugated with different fluorochromes: anti-CD19 PerCPCy5.5 (clone HIB19, Biolegend), anti-CD24 PE (clone ML5, Biolegend), anti-CD27 FITC (clone O323, Biolegend), CD38 APC (clone HIT2, Biolegend), and anti-IgD PE (clone IA6-2, BD Pharmingen). Red blood cells were incubated for 15 min at room temperature in the dark. The red cells were then lysed with BD FACS lysing solution (BD Biosciences) according to the manufacturer's instructions. After a wash step with PBS, events were acquired. For the characterization of IL-10-producing Bregs, heparin samples were incubated for 5 h at 37 °C in a 5 % CO_2 atmosphere with phorbol 12-myristate 13-acetate (PMA) (50 ng/mL, Sigma Aldrich), calcium ionophore (1 μg/mL, Sigma Aldrich), and lipopolysaccharide (LPS) (10 μg/mL, Sigma Aldrich) in the presence of Brefeldin A (1.0 μg/ml, BD Pharmingen) [13, 30]. After the stimulation, the red blood cells were lysed via the addition of BD FACS lysing solution and were stained for surface markers with anti-CD3 FITC (clone SK7, BD Biosciences), anti-CD19 PerCPCy5.5 (clone HIB19, Biolegend), and anti-CD8 APC (clone SK1, Biolegend) mAbs. The Cytofix-Cytoperm kit (BD Pharmingen) was used for cell fixation and permeabilization according to the manufacturer's instructions. To assess the cytoplasmic expression of IL-10 in the B cells, a final intracellular staining step with an anti-IL-10 PE mAb (clone JES3-19F1, Biolegend) was performed before cell acquisition. A minimum of 2000 B cells (CD19+) were acquired in all tubes (gate in CD19/SSC). The analysis strategies are presented in Figs. 1 and 2. The flow cytometry results are presented as a percentage of total B cells and as absolute cell counts (cells/μL).

The Bm1-5 classification system used to identify the development of mature B cells was based on the expression

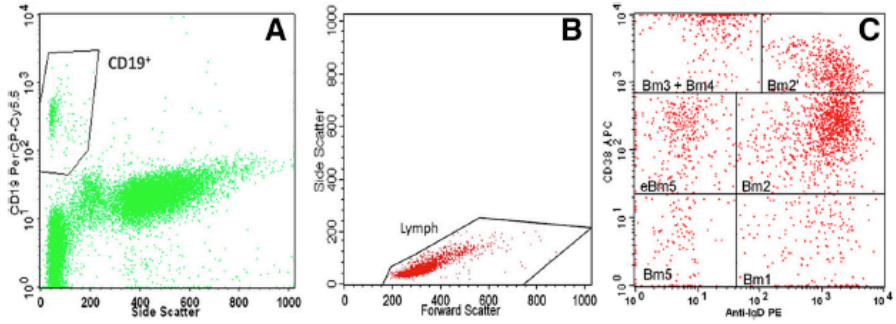

Fig. 1 Identification of B cell subsets according to Bm1-5 classification system. **a** and **b** Gating strategy for $CD19^+$ B cells using an initial CD19/SSC plot and refinement of the gate using a plot of FSC vs SSC. **c** Bm1-5 classification from double staining for IgD and CD38 (unswitched memory Bm1: IgD^+CD38^-; naïve Bm2: IgD^+CD38^+; transitional Bm2': IgD^+CD38^{hi}; plasmablasts Bm3 + Bm4: IgD^-CD38^{hi}; post-germinal memory/early eBm5: IgD^-CD38^+; and resting memory/late Bm5: IgD^-CD38^-)

of IgD/CD38 phenotypic markers. The cells were characterized as follows: transitional B cells (Bm2': IgD^+CD38^{hi}), naïve B cells (Bm2: IgD^+CD38^+), unswitched memory B cells (Bm1: IgD^+CD38^-), and switched memory B cells (Bm5: $IgD^-CD38^{+/-}$) and were subsequently divided into post-germinal memory B cells (early Bm5: IgD^-CD38^+), resting memory (late Bm5: IgD^-CD38^-) B cells, and plasmablasts (Bm3 + Bm4: IgD^-CD38^{hi}) [8–10, 14]. Bregs were evaluated in three different populations: $CD24^{hi}CD27^+$, $CD24^{hi}CD38^{hi}$ and IL-10-producing B cells.

Our laboratory measurement included both absolute counts and percentages of total B cells and the different B cell subsets, as we feel that the two types of data are complementary. Percentages were measured as these allow interpreting the relative fluctuations in distinct B cell subsets from pregnancy to post-partum. Absolute counts were also measured and reported, although we are aware that pregnancy is characterized by variable degrees of hemodilution, and that changes in these counts may not reflect true variations in the total numbers of circulating cells.

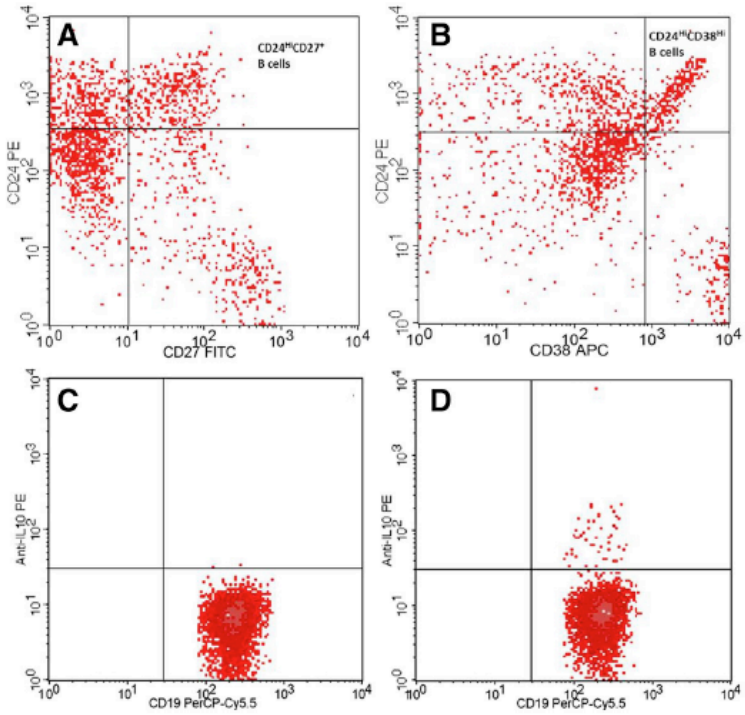

Fig. 2 Identification of regulatory B cell subsets. **a** and **b** Gating strategy for $CD24^{hi}CD27^+$ (**a**) and $CD24^{hi}CD38^{hi}$ (**b**) Bregs; **c** and **d** IL-10-producing $CD19^+$ B cells ($CD19^+$ B cells were analyzed for the expression of IL-10 after a 5-h incubation period without stimulation (**c**) and with stimulation (**d**) with phorbol 12-myristate 13-acetate, calcium ionophore and lipopolysaccharide)

Statistical analysis

If the baseline data were normally distributed, they were presented as means (±standard deviations); otherwise, these data were presented as medians and ranges. Categorical variables were described as absolute and relative frequencies and were expressed as percentages.

Cell counts and percentages were presented as medians and ranges. If normally distributed, 2 independent groups were compared using Student's t-tests; otherwise, Mann–Whitney U tests were used. If normally distributed, pairs of samples were compared using paired Student's t-tests; otherwise, Wilcoxon signed-rank tests were used. For normally distributed data, comparisons between more than 2 groups were performed using ANOVA I; otherwise, Kruskal-Wallis tests were used. Statistical significance was defined by a *P*-value <0.05. The *P*-values for the comparisons of B cells between the non-pregnant women and pregnant women at different visits, as well as for the comparisons of the B cells of the pregnant women between visits, were adjusted for multiplicity using the Benjamini and Yekutieli method [31]. All of the data were analyzed using R software, version 3.12 for Windows.

Results

Baseline characteristics

A total of 78 women were enrolled in the study (43 pregnant and 35 non-pregnant). The characteristics of these women and of their newborns are presented in Table 1. The mean BMI of the non-pregnant women was 21.5 (±2.8) Kg/m^2, while for the pregnant, it was 26.2 (±2.8) Kg/m^2. All of the women were normotensive [mean systolic blood pressure for the non-pregnant was 119.8 (±10.5) mmHg, while for the pregnant, it was 115.7 (±9.3) mmHg; mean diastolic blood pressure for the non-pregnant was 74.7 (±7.4) mmHg, while for the pregnant, it was 67.4 (±7.4) mmHg]. Among the non-pregnant women, the median number of weeks since the last pregnancy (regardless of whether the pregnancies were interrupted or resulted in a live birth) was 169 (23–449). The median gestational age in the 3rd trimester of pregnancy was 33.0 (31–35) weeks, while it was 39.0 (37–41) weeks on the day of delivery. The pregnant group was significantly younger ($p = 0.016$) and included significantly more nulliparous women ($p < 0.001$) than the non-pregnant group. All of the pregnant women, regardless of the mode of delivery, received regional analgesia and/or anesthesia. No general anesthesia was administered to these women. All of the pregnant women were discharged from the hospital 2 days after a vaginal delivery or 3 days after a cesarean section. Final post-partum measurements were carried out a median of 45 (41–58) days after delivery.

Table 1 Characteristics of the women enrolled in the study and of their newborns

	Non-pregnant women (*n* = 35)	Pregnant women (*n* = 43)
Age in years, median (range)	35.0 (20–40)	32.0 (25–41)*
Ethnicity, *n* (%)		
White	35 (100)	42 (97.8)
Black	0	1 (2.2)
Gestational age in weeks, median (range)		
3rd trimester		33.0 (31–35)
Day of delivery		39.0 (37–41)
Parity, *n* (%)		
Nulliparous	5 (14.3)	24 (55.8)*
Primiparous	14 (40)	18 (41.9)
Multiparous	16 (45.7)	1 (2.3)
Mode of delivery, *n* (%)		
Vaginal		18 (41.8)
Cesarean		25 (58.2)
Elective cesarean[a]		14 (55.6)
Intrapartum cesarean[b]		11 (44.4)
Newborns		
Birth weight in grams, mean (± SD)		3265.0 (±393.5)
Gender, *n* (%)		
Male		22 (51)
Female		21 (49)
APGAR score, median (range)		
1-min Apgar score		9 (6; 10)
5-min Apgar score		10 (9; 10)
5-min Apgar score less than 7		0 (0)

Note: *SD* standard deviation; [a]preformed prelabor; [b]performed in labor; *statistically significant differences ($p < 0.05$) between pregnant and non-pregnant

Characterization of the B cell population (CD19$^+$)

The characterization of the B cell population for all of the enrolled women is presented in Fig. 3. The median absolute numbers (259 [110–485]) and percentages (12 [6–20]) of these cells in the non-pregnant were within the expected normal ranges of our protocol (absolute count: 80–616 cells/μL; percentage: 5–22 %). The absolute counts of B cells at delivery were significantly lower ($p < 0.05$) than those at post-partum and in the non-pregnant women. Furthermore, the absolute counts of these cells during the 3rd trimester were also significantly lower ($p < 0.05$) than those in the non-pregnant. The percentages of B cells at delivery and at post-partum were significantly lower than those in the pregnant women during the 3rd trimester of pregnancy and in the non-pregnant.

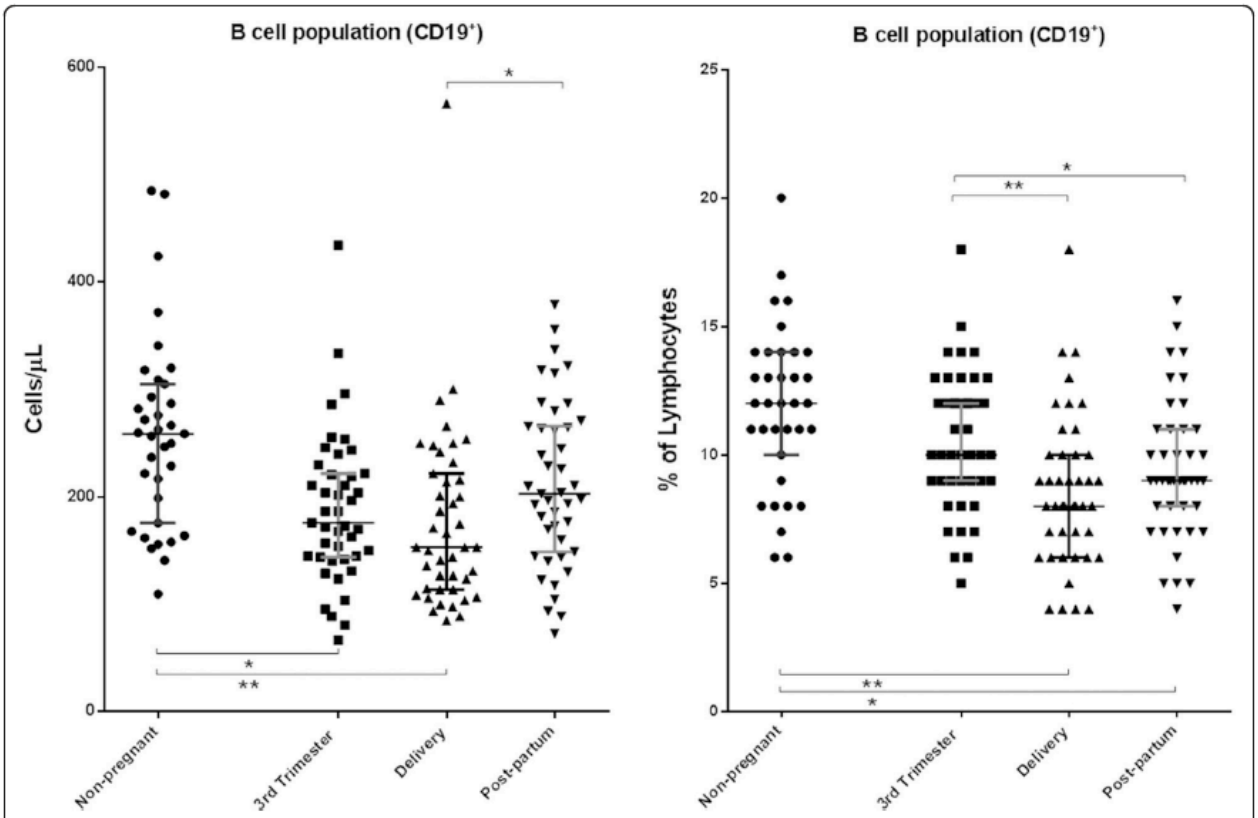

Fig. 3 B cell population in peripheral blood samples. Delivery, within 15 min after placental expulsion; Post-partum, at least 6 weeks after delivery; The *bottom line* represents the 25th percentile, the *top line* represents the 75th percentile, and the *middle line* represents the median. * $p < 0.05$; ** $p < 0.001$

Characterization of maturational stages of B cells

The characterization of specific B cell subsets (transitional, naïve, unswitched memory, post-germinal, and resting memory B cells as well as plasmablasts) for all of the enrolled women is presented in Fig. 4 (absolute counts) and Fig. 5 (percentages). The absolute counts of transitional B cells, unswitched memory B cells, resting memory B cells, and plasmablasts during the 3rd trimester of pregnancy and on delivery day were significantly lower ($p < 0.05$) than the corresponding counts in the non-pregnant. The absolute counts of naïve and of post-germinal memory/early B cells did not significantly differ ($p \geq 0.05$) between the pregnant and non-pregnant women at any of the study visits. The absolute counts of all of the B cell subsets, excluding naïve B cells, were significantly higher ($p < 0.05$) at post-partum compared to those during the 3rd trimester of pregnancy and on delivery day.

The percentages of transitional B cells in the 3rd trimester of pregnancy and on delivery day were significantly lower ($p < 0.05$) than those in the non-pregnant and post-partum women. Conversely, the percentages of naïve B cells in the 3rd trimester of pregnancy and on delivery day were significantly higher ($p < 0.05$) compared those in both the non-pregnant and post-partum women. No significant differences ($p < 0.05$) in the percentages of unswitched memory, post-germinal memory, and resting memory B cells as well as of plasmablasts were identified between the pregnant and non-pregnant women at any of the study visits. However, the percentages of unswitched memory and resting memory B cells and plasmablasts were significantly lower ($p < 0.05$) during the 3rd trimester compared to post-partum. Furthermore, the percentages of plasmablasts were also significantly lower ($p < 0.05$) on delivery day compared to post-partum.

Characterization of Breg

The characterization of Breg is presented in Fig. 6. The absolute counts of IL-10 regulatory B cells and $CD24^{hi}CD38^{hi}$ Bregs during the 3rd trimester of pregnancy and on delivery day were significantly lower ($p < 0.05$) than those in the post-partum women. Additionally, the absolute counts of $CD24^{hi}CD38^{hi}$ Bregs were also significantly lower ($p < 0.05$) during the 3rd trimester of pregnancy and on delivery day compared to the corresponding counts in the non-pregnant women. The absolute counts of $CD24^{hi}CD27^{+}$

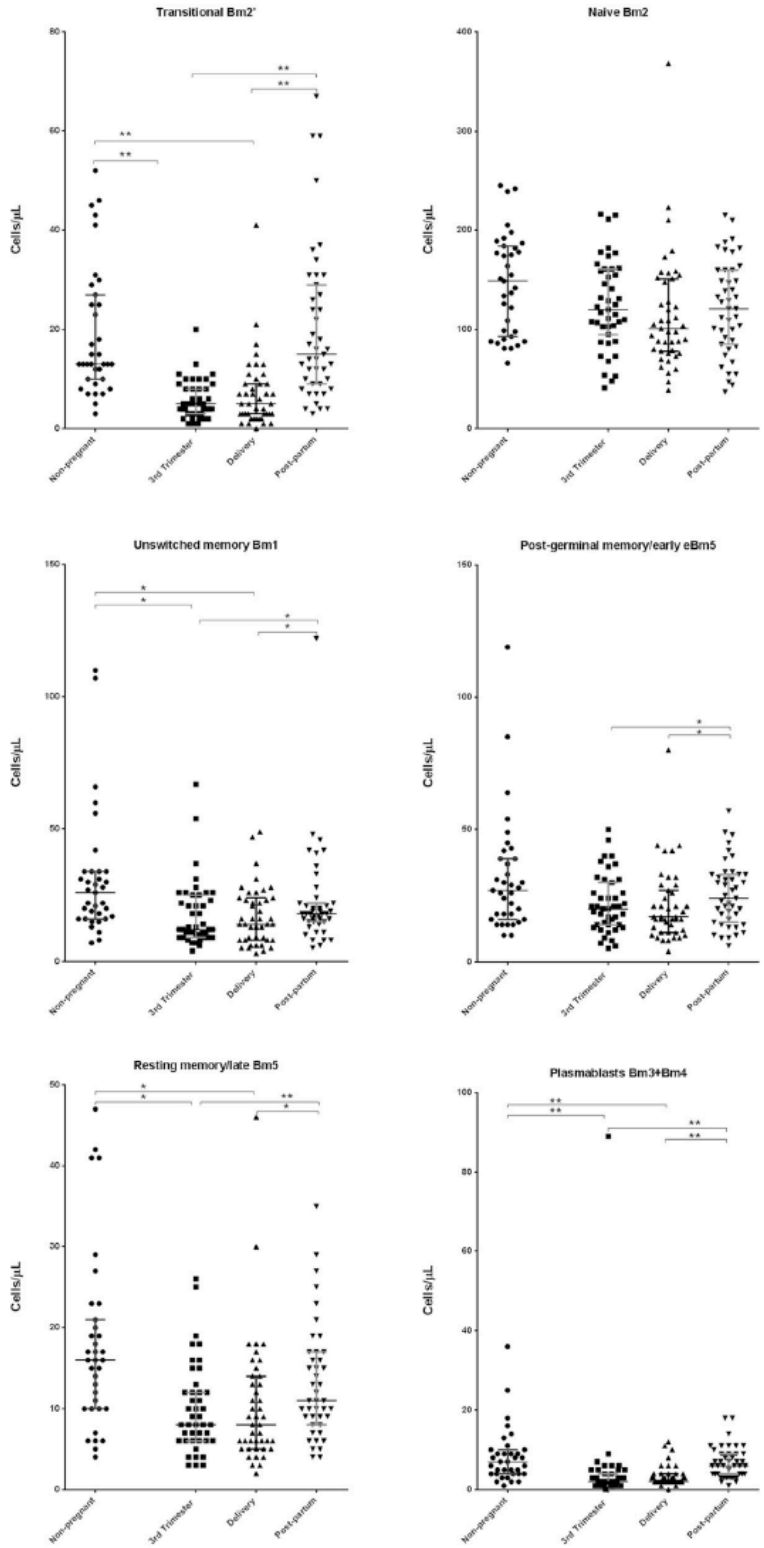

Fig. 4 Maturational stages of B cells (absolute counts) in peripheral blood samples according to Bm1-5 classification. Bm1-5 classification, IgD/CD38 cell surface markers; Delivery, within 15 min after placental expulsion; Post-partum, at least 6 weeks after delivery; Non-pregnant women. The *bottom line* represents the 25th percentile, the *top line represents* the 75th percentile, and the *middle line* represents the median. * $p < 0.05$; ** $p < 0.001$

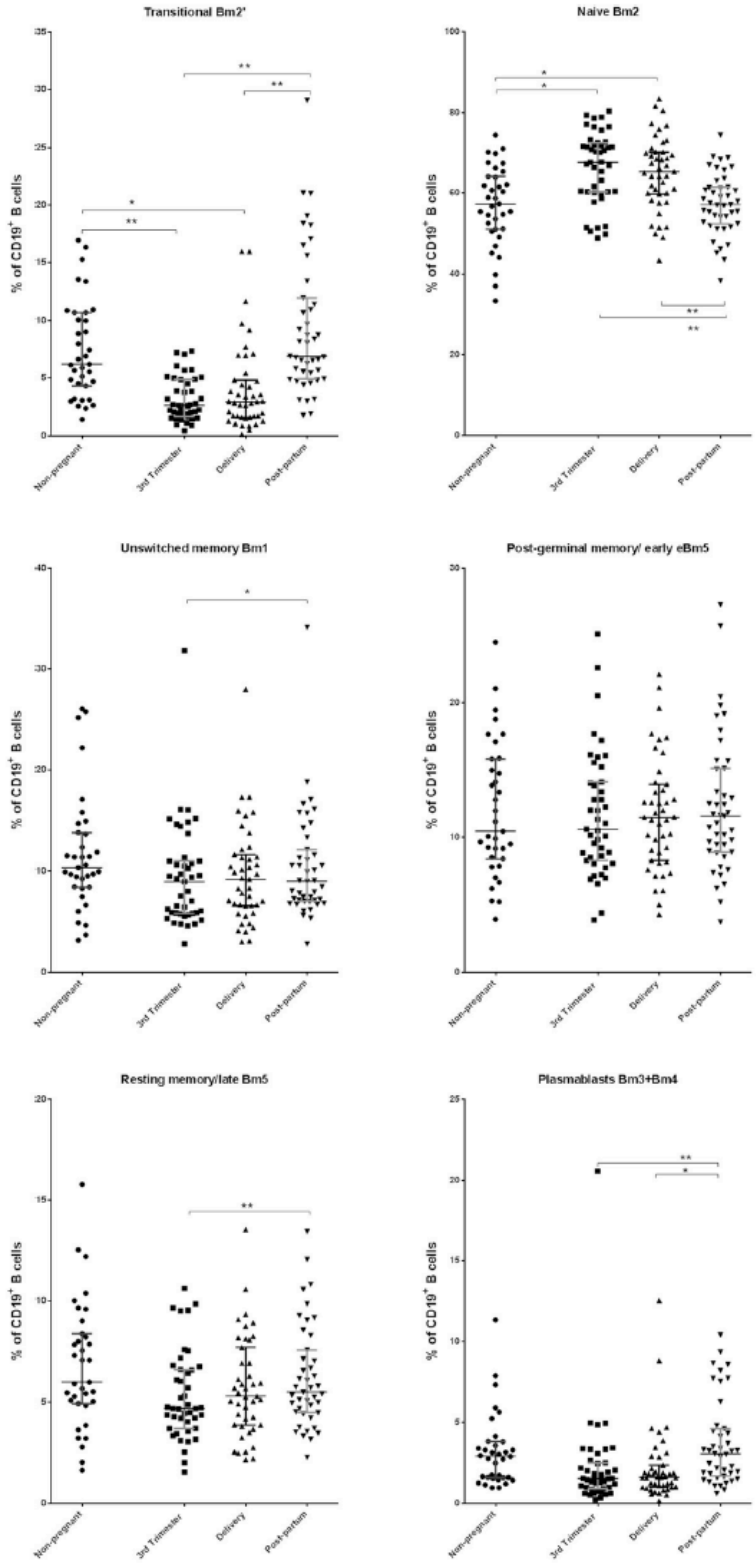

Fig. 5 Maturational stages of B cells (percentages) in peripheral blood samples according to Bm1-5 classification. Bm1-5 classification, IgD/CD38 cell surface markers; Delivery, within 15 min after placental expulsion; Post-partum, at least 6 weeks after delivery; Non-pregnant women. The *bottom line* represents the 25th percentile, the *top line* represents the 75th percentile, and the *middle line* represents the median. * $p < 0.05$; ** $p < 0.001$

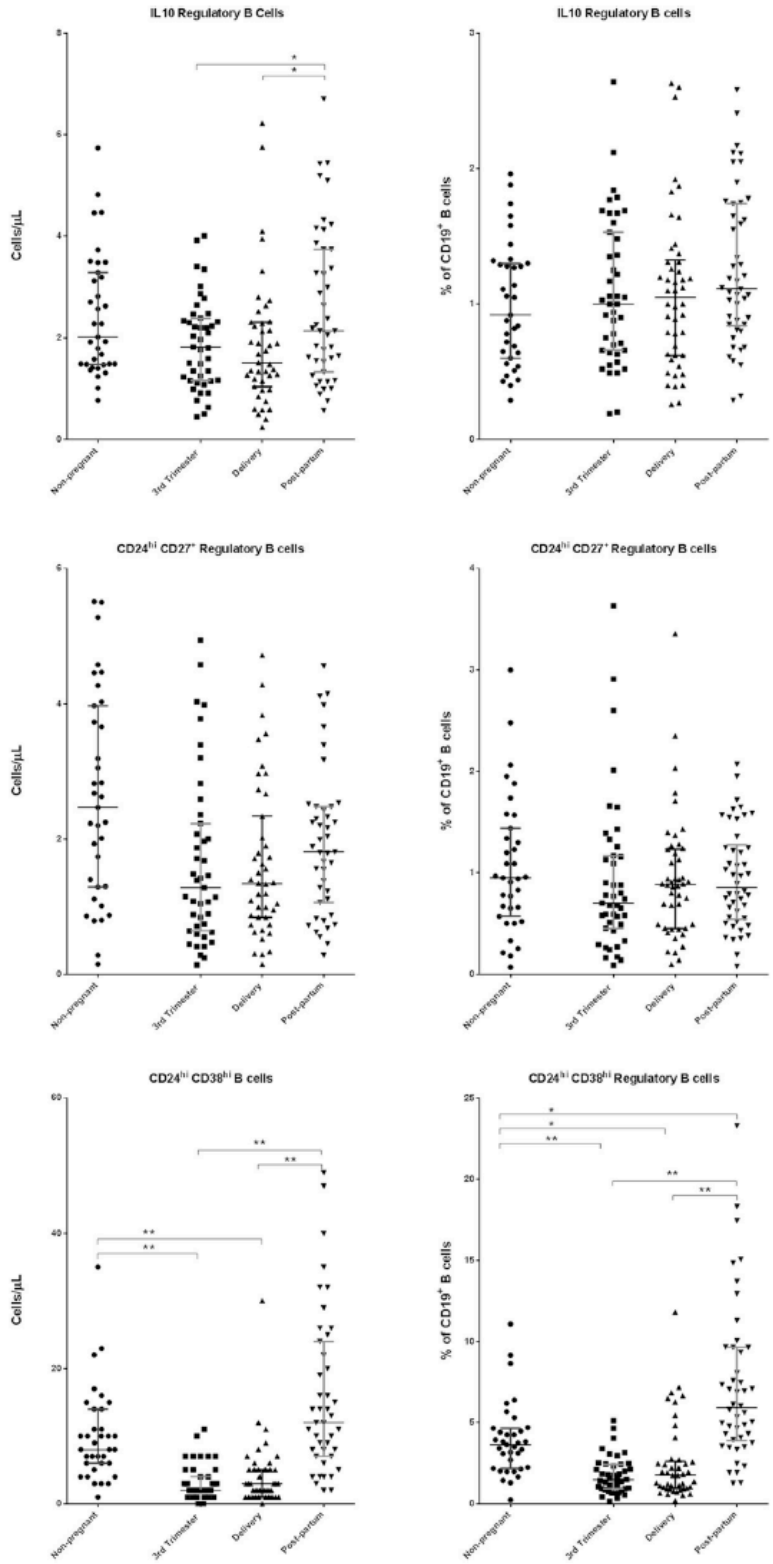

Fig. 6 Regulatory B cells in peripheral blood samples (absolute counts and percentages). Delivery, within 15 min after placental expulsion; Post-partum, at least 6 weeks after delivery; Non-pregnant women. The *bottom line* represents the 25th percentile, the *top line* represents the 75th percentile, and the *middle line* represents the median. * $p < 0.05$; ** $p < 0.001$

Bregs did not significantly differ between the pregnant and non-pregnant women or between study visits.

The percentages of $CD24^{hi}CD38^{hi}$ Bregs during the 3rd trimester of pregnancy and on delivery day were significantly lower ($p < 0.05$) than those in the non-pregnant and post-partum women. Furthermore, the percentages of these cells at post-partum were significantly higher ($p < 0.05$) than those in the non-pregnant women. The percentages of IL-10 regulatory B cells and of $CD24^{hi}CD27^{+}$ Bregs did not significantly differ between the pregnant and non-pregnant women or between study visits.

Discussion

According to our study, late-stage pregnancy (between the 3rd trimester and delivery) is associated with peripheral blood B cell lymphopenia. Indeed, the absolute counts and percentages of most B cell subsets in the 3rd trimester of pregnancy and on delivery day were significantly lower compared to the corresponding counts and percentages in the non-pregnant. However, these differences did not significantly differ between the post-partum and non-pregnant women, suggesting that at this later time point, the absolute counts and percentages of most B cell subsets revert (or at least partially revert) to normal values. The most notable exceptions to this observation were identified for naïve B cells (whose percentages were significantly higher during the 3rd trimester and on delivery day than in the non-pregnant) and for $CD24^{hi}CD38^{hi}$ Bregs (whose percentages were significantly higher in the post-partum compared to the non-pregnant women).

To our knowledge, this is the first study to characterize the circulating B cell compartment in pregnancy while taking into account the maturational stages of the different B cell subsets. Furthermore, the study was conducted prospectively, from the 3rd trimester of pregnancy to post-partum, in a sample of 43 pregnant women.

The pregnancy-associated B lymphopenia that we identified in our study has already been described in animal models [32, 33] and in humans [17–27]. According to Medina et al. [32], B lymphopoiesis in bone marrow is selectively reduced during normal pregnancy because of hormonal influences. Furthermore, Muzzio et al. [33] demonstrated that B lymphopoiesis is reduced in late pregnancy, when estradiol levels are high. Cellular migration is another mechanism that can contribute to B cell lymphopenia. Studies of animal models have shown that monocytes and other immune cells migrate to the uterus during the later stages of pregnancy due to changes in the expression of chemokines [34, 35]. Furthermore, small populations of B cells have been identified in the decidua, suggesting leucocyte recruitment into the maternal-fetal interface [36]. The biological meaning of this suppression of B lymphopoiesis in normal pregnancy is uncertain but is probably related to the physiological immune tolerance.

In this study, we found that the absolute counts of the majority of the B cell subsets were significantly lower in the 3rd trimester of pregnancy than in the non-pregnant women, suggesting pregnancy-associated B lymphopenia (though most pregnant women still presented values within normal ranges). The increased blood volemia observed during pregnancy could in part explain these observations. In fact, pregnant presented also decreased absolute lymphocyte counts, along with decreased absolute counts of T and NK cells, compared to non-pregnant (data not shown). Nonetheless, B cell percentages in pregnant also seem to decrease during pregnancy, which does not happens with either T cells or NK cells.

Compared to the percentages of peripheral blood naïve B cells in the non-pregnant women, we found higher values during the 3rd trimester and on delivery day, but no differences were observed in absolute counts. This relative increase in naïve B cells may be a consequence of decreased differentiation of B cells into memory cells and/or plasmablasts. In fact, Muzzio et al. [33] reported the expansion of naïve B cells in pregnant mice. The high levels of progesterone present in late pregnancy may potentially explain this, as high progesterone levels inhibit B cell activation in mice [37]. Our results may also be explained by the mobilization of more differentiated B subsets from peripheral blood to other body tissues.

Normal pregnancy has been compared to a state of quiescent systemic inflammation, while parturition has been likened to an immunological reaction that results in the recruitment of immune cells not only to the maternal-fetal interface but also to the systemic circulation [38]. The results of our study support this idea, as we identified higher counts and percentages of $CD24^{hi}CD38^{hi}$ Bregs post-partum relative to during the 3rd trimester and on delivery day. This observation may represent a regulatory mechanism for the suppression of immune cell activation events and may also explain the increased susceptibility to infections that occurs during the post-partum period and the altered clinical outcomes of some autoimmune diseases.

Interestingly, we found that while the majority of B cell subsets increased to levels closer to those of the non-pregnant (or to normal values) from the third trimester of pregnancy to post-partum, the percentages of $CD24^{hi}CD38^{hi}$ Bregs were significantly higher in the post-partum compared to the non-pregnant women. Because B lymphopoiesis is under endocrine regulation during pregnancy [32, 33], our results may be explained by the decline of hormonal levels that typically occurs post-partum, which may have lead both to lymphopoiesis recovery and to B cell activation. As hypothesized by Medina et al. [32], this result may be of clinical utility to increase lymphocyte formation in transplanted patients and in those with immunodeficiencies.

Unlike for the $CD24^{hi}CD38^{hi}$ Bregs, no significant differences between the non-pregnant and the pregnant or from the 3rd trimester of pregnancy to post-partum were identified for IL-10 Bregs (cell percentages) or for $CD24^{hi}CD27^{+}$ Bregs (cell percentages and counts). This heterogeneity of Breg subsets has been reported in other studies with humans [39]. Furthermore, $CD24^{hi}CD27^{+}$ Bregs, an activated memory subset, are more mature than transitional $CD24^{hi}CD38^{hi}$ Bregs; thus, it is more likely for them to develop into antibody-producing cells that no longer possess a regulatory function [40].

The differences between pregnant and non-pregnant women identified for age, and parity are not likely to bias our results. In fact, among all of the women who were included in our study, counts and percentages of B cell subsets were not significantly associated with age, as demonstrated by the non-significant Spearman correlation coefficients between these variables (see Additional file 1). Furthermore, in the vast majority of cases, there were no statistically significant differences in counts and percentages of B cell subsets among women, despite parity (see Additional file 2). Finally, in the vast majority of cases, we have also not found significant associations between counts (and percentages) of B cell subsets and gestational age at the 3rd trimester of pregnancy (see Additional file 3), gestational age at delivery (see Additional file 4), length of time post-partum until the collection of the final blood samples (see Additional file 5), and time since last pregnancy in the non-pregnant (see Additional file 6).

Previous studies have identified differences in the counts and percentages of B cells (total and subsets) between neonates and individuals of up to 50 years of age [41]. However, to the best of our knowledge, there are no data regarding B cell variation in women over short periods of time, such as that of our study.

Although the pregnant women received analgesia and/or anesthesia, which may cause temporary changes in maternal blood pressure, this is unlikely to cause important changes in B cell counts because regional administration is generally associated with low plasma levels of these drugs. Ideally, samples collected before pregnancy would have been compared with samples collected during pregnancy in the same individuals; however, this would have been very difficult for us from a practical point of view. The fact that several of the changes that were observed during the 3rd trimester of pregnancy seem to be reversed during the post-partum period suggests that comparisons with non-pregnant women were adequate.

In future research, it is important to investigate whether B cell subset characterization could help to identify risk markers for the development of obstetric complications in pregnant women with or without autoimmune diseases. In this context, it would also be important to clarify the role of B-cell activating factor (BAFF), an essential survival factor for transitional B cells, and of CD23, a B-lymphocyte differentiation marker.

Conclusion

According to our study, the characteristics of peripheral B cell compartment differ significantly between pregnant and non-pregnant women and vary over time from late pregnancy to post-partum. Such findings may allow us to recognize normal fluctuations in B cell subsets to better understand immune regulation during human pregnancy and to identify new strategies for the diagnosis and treatment of pregnancy-associated disturbances as well as the mechanisms of maternal responses to vaccination and infection.

Additional files

Additional file 1: Correlation coefficients between counts (and percentages) of B cell subsets and age, both for pregnant and non-pregnant women. [a]Healthy non-pregnant women; [b]Third trimester of pregnancy; [c]Within 15 min after placental expulsion; [d]At least 6 weeks after delivery; [e]Spearman correlation coefficient. (XLSX 11 kb)

Additional file 2: Comparison of counts (and percentages) of B cell subsets between nulliparous, primiparous, and multiparous women, both for pregnant and non-pregnant women. Values presented as median (Interquartile range); Three classes were compared using one-way ANOVA, or Kuskal-Wallis tests; Two classes were compared with t-Student or Wilcoxon tests; There was only one multiparous women among the pregnant, and this woman was excluded from this analysis. [a]Non-pregnant women; [b]Third trimester of pregnancy; [c]Within 15 min after placental expulsion; [d]At least 6 weeks after delivery; [e]Kuskal-Wallis test; f Wilcoxon test; * $p < 0.05$. (XLSX 46 kb)

Additional file 3: Correlation coefficients between counts (and percentages) of B cell subsets and gestational age at the 3rd trimester of pregnancy. Corr. Coef., Spearman correlation coefficient. (XLSX 9 kb)

Additional file 4: Correlation coefficients between counts (and percentages) of B cell subsets and gestational age at delivery. Corr. Coef., Spearman correlation coefficient. (XLSX 9 kb)

Additional file 5: Correlation coefficients between counts (and percentages) of B cell subsets and length of time post-partum until the collection of the final blood samples. Corr. Coef., Spearman correlation coefficient. (XLSX 9 kb)

Additional file 6: Correlation coefficients between counts (and percentages) of B cell subsets and time since last pregnancy, in the non-pregnant women. Corr. Coef., Spearman correlation coefficient. (XLSX 9 kb)

Abbreviations

Bm, mature B cells; Bregs, regulatory B cells

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Authors' contributions

JL had the original research idea. All of the authors designed the study and created the study protocol. JL recruited the patients and collected the data. CM and GN analyzed the blood samples using flow cytometry. All of the authors contributed to the data analysis and interpretation. JL drafted the

manuscript, and all of the authors revised it and intellectually contributed. All of the authors approved the final version of the manuscript.

Competing interests
The authors declare that they have no competing interests.

Ethics approval and consent to participate
All procedures were performed in accordance with the Declaration of Helsinki and were approved by the ethics committee of the *Hospital CUF Descobertas* in Lisbon (Portugal).
Informed consent to participate in the study was obtained from all of the recruited women before the start of the study.

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CHAPTER IV

Regulatory T Cells Show a Dynamic Behavior in Late Pregnancy, Delivery and Postpartum

Regulatory T cells show dynamic behavior during late pregnancy, delivery and the postpartum period

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Abstract

Regulatory T cells (Tregs) are critical immunomodulators during early pregnancy by preventing maternal T cell activation against fetal cells. However, how populations of maternal Tregs vary during and after pregnancy in humans is still unclear. Therefore, we investigated Treg subsets in the peripheral blood of pregnant women from late pregnancy through the postpartum period. To accomplish this, the following circulating Treg subsets were analyzed in 43 healthy pregnant women and 35 non-pregnant women by flow cytometry during the 3rd trimester, on the day of delivery, and postpartum: $CD4^{Dim}CD25^{Hi}$, $CD4^{+}CD25^{Hi}Foxp3^{+}$, and $CD4^{+}CD25^{Hi}CD127^{-/dim}$. Additionally, the expression levels of the transcription factor Foxp3 in $CD4^{Dim}CD25^{Hi}$ Treg were analyzed. We have found that $CD4^{Dim}CD25^{Hi}$ Treg subset significantly decreased in the pregnant women on the day of delivery relative to the 3rd trimester ($p < 0.05$), and that all Treg subsets significantly increased postpartum compared to the 3rd trimester and the day of delivery ($p < 0.05$). Moreover, the Foxp3 expression ratios within the $CD4^{Dim}CD25^{Hi}$ Treg subset decreased during pregnancy and until delivery compared to those measured in the non-pregnant women and significantly increased postpartum compared to the 3rd trimester and the day of delivery ($p < 0.05$). Thus, despite their established role in offering immunoprotection to the fetus in early pregnancy, the number of circulating Tregs also varies from late pregnancy to the postpartum period. Our results offer an explanation for the possible effects of pregnancy on the clinical outcomes of some autoimmune diseases or the increased susceptibility to infections during the postpartum period.

Keywords

Regulatory T cells, immunology, Foxp3, immunomodulation, pregnancy

Background

The survival of a semiallogeneic fetus is a unique immunological challenge during pregnancy. Regulatory T cells (Tregs) are a specialized T cell subpopulation involved in preventing autoimmunity and graft rejection,[1] as they are potent suppressors of inflammatory immune responses. Additionally, Tregs prevent maternal T cell activation against fetal cells, and this protection of the fetus from the maternal immune system has been widely reported in both mice and humans.[2-4] A reduction in maternal Treg populations could cause failure of immunological tolerance to the fetus and has been associated with obstetrical complications, such as miscarriage, preeclampsia, and preterm labor.[5-10]

Although numerous Treg-specific markers have been proposed for mice and humans, the transcription factor Foxp3 is still the most consistent marker, and this protein seems to be essential for the function of these immunosuppressive cells.[11,12] In humans, the detection of $CD4^{Dim}CD25^{Hi}$, $CD4^{+}CD25^{Hi}Foxp3^{+}$ and $CD4^{+}CD25^{Hi}CD127^{-/dim}$ T cell populations remains one of the most common analytical strategies used to identify Treg subsets.[3,13-15]

Treg expansion in the decidua of normal pregnant women is most robust during early pregnancy and decreases as term approaches, suggesting that the immune regulatory processes that occur at the maternal—fetal interface are dynamic and provide immune protection for the fetus.[4,16] Variations in Treg numbers during human pregnancy occur not only at the decidua but also in the maternal peripheral blood.[6,17]

Treg subsets in peripheral blood have been explored in normal human pregnancy and during the postpartum period in several studies; however, the results have been highly variable, with some reports showing a decrease[18] or an increase[3,10,19-22] in the first trimester, a decrease[18,23] or an increase[3,10,19,20,24] in the second trimester, or a decrease[3,4,10,18-20,22] or an increase[24] in the 3rd trimester and others showing either no changes[22] or a decline[10,24] during labor and a decrease[3,10,19] or an increase[25] in the postpartum period. These discrepancies can be attributed to differences in study design, as some studies do not include the postpartum period,[4,18,20,22-24] and in others not all women contribute samples at all time points.[3,10,18-20,22,25] Contrasting reports

of Treg patterns may also be attributed to differences in sample size or the use of different and sometimes non-specific markers for Tregs subsets. Therefore, the currently available data on variations in maternal Treg subsets during pregnancy need re-evaluation. Consequently, the aim of the current study was to characterize the evolution of Tregs subsets (defined as $CD4^{Dim}CD25^{Hi}$, $CD4^{+}CD25^{Hi}Foxp3^{+}$ and $CD4^{+}CD25^{Hi}CD127^{-/dim}$ T cells) and the expression of Foxp3 within $CD4^{Dim}CD25^{Hi}$ Treg subset in the peripheral blood of normal pregnant women from late pregnancy through the postpartum period and to compare these measurements with those taken from non-pregnant women.

Materials and Methods

Study population

This was a prospective observational study that followed healthy pregnant women over time to characterize (through cell quantification and phenotype identification) peripheral blood Tregs ($CD4^{Dim}CD25^{Hi}$, $CD4^{+}CD25^{Hi}Foxp3^{+}$ and $CD4^{+}CD25^{Hi}CD127^{-/dim}$ T cells) and Foxp3 expression in $CD4^{Dim}CD25^{Hi}$ Tregs from late pregnancy through the postpartum period. Furthermore, changes in the above parameters were compared between the pregnant women and non-pregnant women to identify associations with pregnancy.

We recruited sequential non-laboring pregnant women with singleton pregnancies who were attending routine obstetrical care while in the 3^{rd} trimester of pregnancy. Furthermore, we also recruited sequential non-pregnant women who were attending routine annual well-woman exams.

Pregnant women were recruited if their fetuses exhibited appropriate growth (as measured by uterine fundal height and by an ultrasound performed after 28 weeks of gestation) and if they had no pregnancy complications (either prior or after study inclusion).

The following exclusion criteria were applied to all of the women: diabetes, hypertension, autoimmune or vascular disease, infection (human immunodeficiency virus, syphilis, or hepatitis B or C), or smoking during the 6 months prior to study

inclusion. Furthermore, pregnant women were excluded if they had a pre-term delivery (< 37 weeks of gestation), signs of infection, if they required labor induction, or if they required administration of prenatal medication (other than vitamins and iron supplements). We also excluded non-pregnant women who were taking oral contraceptives, as these drugs may influence peripheral blood lymphocytes.[26]

All of the women were recruited between July 2013 and March 2014 from *CUF Descobertas Hospital* in Lisbon (Portugal). The ethics committee of the hospital approved our study protocol, and all the recruited women provided written informed consent before study inclusion.

Study procedures

To characterize peripheral blood Tregs and Foxp3 expression from late pregnancy through the postpartum period, 3 blood samples were collected: the first was collected during the 3rd trimester of pregnancy, the second was collected on the day of delivery (within 15 minutes after placental expulsion and oxytocin administration), and the last was collected during the postpartum period (at least 6 weeks after delivery). A single peripheral blood sample was taken from the non-pregnant women at a planned visit, which occurred during the follicular phase of the menstrual cycle.

Demographics, anthropometrics [body mass index (BMI)], obstetric history, and systolic and diastolic blood pressures were collected as baseline data for all the women. Gestational age (at baseline and at delivery), type of analgesia and/or anesthesia, and mode of delivery were collected only for the pregnant women. Gender, birth weight, and 1-minute and 5-minute Apgar scores were collected for the newborns.

Flow cytometry analysis and laboratory measurements

Blood samples (anticoagulated with EDTA) were evaluated by multicolor flow cytometry in a BD FACS Calibur (BD Biosciences, San Jose, CA) equipped with two lasers (488 nm air-cooled argon-ion laser and 635 nm red diode laser). All samples were processed within 24 hours of collection.

A BD Multitest™ IMK kit (BD Biosciences) was used to obtain absolute counts of lymphocyte subsets and to characterize these subsets according to the manufacturer's instructions.

For Treg phenotyping, a panel of monoclonal antibodies (mAbs) including anti-CD3-FITC (clone SK7, BD Biosciences), anti-CD25 PE (clone BC96, Biolegend, San Diego, CA), anti-CD4 PerCP Cy5.5 (clone SK3, Biolegend) and anti-CD127 Alexa Fluor 647 (clone A019D5, Biolegend) antibodies was employed with a lyse-wash protocol using BD FACS lysing solution (BD Biosciences). The Human FoxP3 Buffer Set (BD Pharmingen, San Jose, CA) was also used to characterize Foxp3 expression. In brief, cells were lysed, washed and then incubated with the surface mAbs anti-CD4 and anti-CD25 (referred to above). Staining with Anti-Foxp3 Alexa Fluor 488 (clone 259D/C7, BD Pharmingen) was performed after fixing and permeabilizing the cells with the reagents supplied in the Human FoxP3 Buffer Set kit. A minimum of 10,000 CD4 T cells was acquired per tube, and data analysis was performed using CellQuest software (BD Biosciences).

The gating strategies used are described in Figure 1. In brief, CD4 T cells were gated within the lymphocyte cluster using a combined Boolean gating strategy. Bivariate dot plots of CD25 versus CD127, CD25 versus Foxp3, and CD25 versus CD4 were further used to identify $CD4^{Dim}CD25^{Hi}$, $CD4^{+}CD25^{Hi}Foxp3^{+}$, and $CD4^{+}CD25^{Hi}CD127^{-/dim}$ regulatory subsets, which were quantified as percentages of total CD4 T cells. Fluorescence Minus One (FMO) tubes and internal cell populations were used as negative controls to assess CD25, CD127 and Foxp3 positivity.[27,28]

The expression of the transcription factor Foxp3 was assessed within the $CD4^{Dim}CD25^{Hi}$ Treg subset using the geometric mean values of mean fluorescence intensity (MFI) units, which were converted into a ratio (MFI of Foxp3 in $CD4^{Dim}CD25^{Hi}$ Treg / MFI of Foxp3 in $CD4^{-}$ lymphocytes) as previously described to reduce the impact of day-to-day variation.[29]

Statistical analysis

Data normality was assessed using the Shapiro-Wilk test. Baseline data were presented as means (±standard deviations) if normally distributed or as medians and ranges if not normally distributed. Categorical variables were described using absolute and relative frequencies. Treg counts and percentages were presented as medians and ranges. If normally distributed, 2 independent groups were compared using Student's t-tests; otherwise, Mann–Whitney U tests were used. If normally distributed, paired data were compared using paired Student's t-tests; otherwise, Wilcoxon signed-rank tests were used. For normally distributed data, comparisons between more than 2 groups were performed using ANOVA I; otherwise, Kruskal-Wallis tests were used. Statistical significance was defined by a P-value < 0.05. The P-values for the comparisons of Treg subsets and Foxp3 expression levels at different time points were adjusted for multiplicity using the Benjamini and Yekutieli method.[30] All of the data were analyzed using R software, version 3.12 for Windows.

Results

Baseline characteristics

Our study included a total of 78 healthy women (43 pregnant and 35 non-pregnant). The demographic and clinical data collected for all the participants and their newborns are presented in Table 1. The mean BMI was 21.5 (±2.8) Kg/m^2 for the non-pregnant women and 26.2 (±2.8) Kg/m^2 for the pregnant women. All the women had normal blood pressures [mean systolic blood pressure: 119.8 (±10.5) mmHg for the non-pregnant women and 115.7 (±9.3) mmHg for the pregnant women; mean diastolic blood pressure: 74.7 (±7.4) mmHg for the non-pregnant women and 67.4 (±7.4) mmHg for the pregnant women]. The median time since the last pregnancy (regardless of whether the pregnancies were interrupted or resulted in a live birth) for the non-pregnant women was 169 (23-449) weeks. For the pregnant women, the median gestational age at the 3rd trimester of pregnancy was 33.0 (31-35) weeks, and at the delivery day it was 39.0 (37-41) weeks. The group of pregnant

women was significantly younger ($p < 0.05$) and included significantly more nulliparous women ($p < 0.05$) than the group of non-pregnant women.

All vaginal deliveries were spontaneous and received epidural analgesia during labor. All cesarean sections were performed with regional anesthesia. All women were discharged from the hospital 2 days after a vaginal delivery or 3 days after a cesarean section. Postpartum evaluations were undertaken at a median of 45 (41-58) days after delivery.

Characterization of Tregs

The Treg characterization results for all of the enrolled women are presented in Figure 2. Relative to during the 3rd trimester, on the day of delivery, a significant decrease in the percentage of $CD4^{Dim}CD25^{Hi}$ Tregs ($p < 0.05$) was found, but no other significant differences were identified for percentages of Tregs between any of the other study visits. However, postpartum, there was a significant increase ($p < 0.05$) in the absolute counts of Tregs ($CD4^{Dim}CD25^{Hi}$, $CD4^{+}CD25^{Hi}Foxp3^{+}$, and $CD4^{+}CD25^{Hi}CD127^{-/dim}$) compared with the values obtained during the 3rd trimester and on the day of delivery. The absolute counts and percentages of $CD4^{Dim}CD25^{Hi}$ Tregs, $CD4^{+}CD25^{Hi}Foxp3^{+}$ Tregs, and $CD4^{+}CD25^{Hi}CD127^{-/dim}$ Tregs did not significantly differ ($p \geq 0.05$) between the non-pregnant and pregnant women at any of the study visits.

Foxp3 expression in $CD4^{Dim}CD25^{Hi}$ Tregs

The ratios for Foxp3 expression in $CD4^{Dim}CD25^{Hi}$ Tregs for all of the enrolled women are presented in Figure 3. This ratio was significantly lower ($p < 0.001$) during the 3rd trimester and on the day of delivery in the pregnant women compared to the non-pregnant women. Postpartum, the ratios of Foxp3 expression in $CD4^{Dim}CD25^{Hi}$ Tregs significantly increased ($p < 0.001$) compared to the values measured during the 3rd trimester and on the day of delivery, reaching expression levels similar to those observed in the control group of non-pregnant women. No statistically significant differences ($p \geq 0.05$) were identified for this ratio between the 3rd trimester and the day of delivery.

Discussion

In the present study, we investigated whether Treg subsets varied in the peripheral blood of normal women between late pregnancy and the postpartum period. We found that percentages of $CD4^{Dim}CD25^{Hi}$ Tregs in pregnant women decreased significantly on the day of delivery compared to those measured during the 3rd trimester ($p < 0.05$). This variation was not observed in the other tested subsets of Tregs ($CD4^{+}CD25^{Hi}Foxp3^{+}$ and $CD4^{+}CD25^{Hi}CD127^{-/dim}$ T cells), which can be explained by the heterogeneity of Treg populations. For example, Loewendorf et al[31] demonstrated that defining Tregs as $CD4^{+}CD25^{Hi}CD127^{-/dim}$ underestimates the number of CD4 T cells that express Foxp3.

Our results are consistent with those of previous human studies showing that Treg subsets in peripheral blood decrease on the day of delivery. These studies[10,24] suggest that Tregs play a role in the immunological changes that occur before labor and that human labor may be initiated as an effect of the decrease in Tregs. Furthermore, estrogen and progesterone appear to impact Treg populations in humans. Xiong et al[3] showed that Treg subsets in peripheral blood are positively correlated with estrogen levels, a relationship that might play an important immunomodulatory role during pregnancy. Additionally, another study recently demonstrated that membrane progesterone receptor alpha (mPRα) is present on Tregs, suggesting an association between the immune system and the initiation of human labor; notably, in the referenced study, the highest Treg values were measured during the 3rd trimester, and these values decreased on the day of delivery.[32]

In the present study, we used three analytical strategies to characterize Treg substes ($CD4^{Dim}CD25^{Hi}$, $CD4^{+}CD25^{Hi}Foxp3^{+}$ and $CD4^{+}CD25^{Hi}CD127^{-/dim}$ T cells) and found similar patterns overall, namely, that significantly higher counts of Tregs were present in circulating blood in the postpartum period compared to the 3rd trimester and the day of delivery ($p<0.05$). These results are consistent with the conclusions of Wegienka et al[25] but are inconsistent with earlier studies.[3,10,19] The increase in absolute Treg counts suggests that an immunosuppressed environment, which allows maternal-fetal tolerance in pregnancy, is also present postpartum. It has

been suggested that maternal immunological activation starting after delivery and progressing with gestational age is related to the progressive fetal-maternal cell trafficking that occurs during pregnancy.[33] According to our findings, we hypothesize that Treg expansion is a potential physiological mechanism for downregulating the activation of maternal immunological events (i.e., causing less susceptibility to systemic inflammatory responses) that occur during the postpartum period.

We also studied Foxp3 expression levels in $CD4^{Dim}CD25^{Hi}$ Tregs. We found that during pregnancy and until delivery Foxp3 expression was lower within the $CD4^{Dim}CD25^{Hi}$ Tregs in the pregnant women compared to the non-pregnant women. It has been reported that progesterone and 17β-estradiol reduce Foxp3 expression in Tregs in mid-pregnancy.[23] Our data are consistent with these findings, which probably extend through the 3rd trimester and delivery. Postpartum, Foxp3 expression increased significantly compared to the 3rd trimester and the day of delivery, reaching levels similar to those measured in the control group of non-pregnant women. This confirms that pregnancy causes modulation of Foxp3 expression.

Furthermore, postpartum, we observed fluctuations in Foxp3 expression that showed a similar pattern to one previously described for $CD24^{Hi}CD38^{Hi}$ regulatory B cells in healthy human pregnancies, namely, absolute counts and percentages increased significantly compared to those measured during the 3rd trimester of pregnancy and on the day of delivery.[34] These results are concordant with recent studies in human cell lines demonstrating that regulatory B cells play an important role in Treg differentiation by increasing Foxp3 expression in these cells.[35,36]

Our study included only women with a normal singleton pregnancy between 37 and 41 weeks of gestation to obtain a relatively homogenous sample and reduce potential bias. In addition, all the enrolled pregnant women contributed samples at all of the study's time points (3rd trimester, delivery day and postpartum). Preferably, samples from additional time points, such as during the first and second trimesters of pregnancy, would be included, as well as the monitoring of women from before becoming pregnant through the postpartum period. However, these additional time points were beyond the scope of the current study.

We also accounted for the phase of the menstrual cycle during which the samples were collected from the control group. Peripheral blood samples were collected from the non-pregnant women during the follicular phase of the menstrual cycle, as hormone status during the luteal phase is comparable to that during pregnancy.[37] However, a previous study[38] reported that Treg subsets expand during the follicular phase, reaching a peak immediately before ovulation (to induce immune tolerance to facilitate implantation), and then decline in the subsequent luteal phase. This may have contributed to the absence of significant differences in Treg numbers between the non-pregnant and pregnant groups. Additionally, because crucial immunological events are more likely to occur at the maternal—fetal interface, it is important that future studies compare Tregs in both the peripheral blood and the decidua from the same healthy pregnant women.

In the current study, we performed complementary measurements of both percentages and absolute counts of different Treg subsets. The use of percentages allows for the interpretation of the relative fluctuations in Treg cell subsets from late pregnancy through the postpartum period. Although absolute counts were also measured, pregnancy is characterized by variable degrees of hemodilution, during which changes in total numbers of circulating Tregs may not necessarily reflect variations in these subsets. Further functional and epigenetic experiments are required to identify T cells expressing Foxp3 as Tregs because activated T cells without regulatory function can also present a Foxp3-positive phenotype.[39]

In conclusion, we found that $CD4^{+}CD25^{Hi}$ Treg subset decrease significantly on the day of delivery compared to those measured during the 3rd trimester in healthy pregnant women. We also showed significant increases in circulating Treg subsets and in Foxp3 expression in $CD4^{Dim}CD25^{Hi}$ Treg subset in the postpartum period compared to during the 3rd trimester and on the day of delivery. Our results support that Tregs, despite their established immunoprotective role for the fetus in early pregnancy, present dynamic behavior during late pregnancy and in the postpartum period. The observed postpartum increases in Treg subsets could explain the increased susceptibility to infections that occurs during this period and the possible interference of pregnancy with the clinical outcomes of some autoimmune diseases. We suggest that variations in Treg numbers from late pregnancy through the

postpartum period are reflective of pregnancy's effect on the maternal immune system and may permanently affect the immune status of a woman and have implications for future pregnancies.

Author Contributions

Jorge Lima conceived of the original research idea, while all of the authors designed the study and created the study protocol. Jorge Lima recruited the patients and collected the data. Catarina Martins and Glória Nunes analyzed the blood samples using flow cytometry. Luís-Miguel Borrego supervised all the work and the research protocol. All of the authors contributed to data analysis and interpretation. Jorge Lima drafted the manuscript, and all of the authors revised it and contributed to it intellectually. All of the authors have approved the final version of the manuscript.

Declaration of Conflicting Interests

The authors declare that they have no conflicts of interest.

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Ethical Approval

All procedures performed in studies involving human participants were conducted in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This article does not contain any studies with animals performed by any of the authors.

Informed Consent

Informed consent was obtained from all individual participants included in the study.

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Table 1 – Demographic and clinical characteristics of non-pregnant women and pregnant women and their newborns

	Non-pregnant woman (n=35)	**Pregnant women (n=43)**
Age in years, median (range)	35.0 (20-40)	32.0 (25-41)*
Parity: nulliparous, n (%)	5 (14.3)	24 (55.8)*
Gestational age in weeks, median (range)		
3rd trimester		33.0 (31-35)
Day of delivery		39.0 (37-41)
Postpartum evaluation in days, median (range)		45 (41-58)
Mode of delivery, n (%)		
Vaginal		18 (41.8)
Cesarean		25 (58.2)
Newborns		
Birth weight in grams, mean (± standard deviation)		3,265.0 (±393.5)
Gender: Male, n (%)		22 (51)
APGAR score, median (range)		
1-minute Apgar score		9 (6-10)
5-minute Apgar score		10 (9-10)

*Statistically significant differences ($p<0.05$) identified between the healthy pregnant women and the healthy non-pregnant women

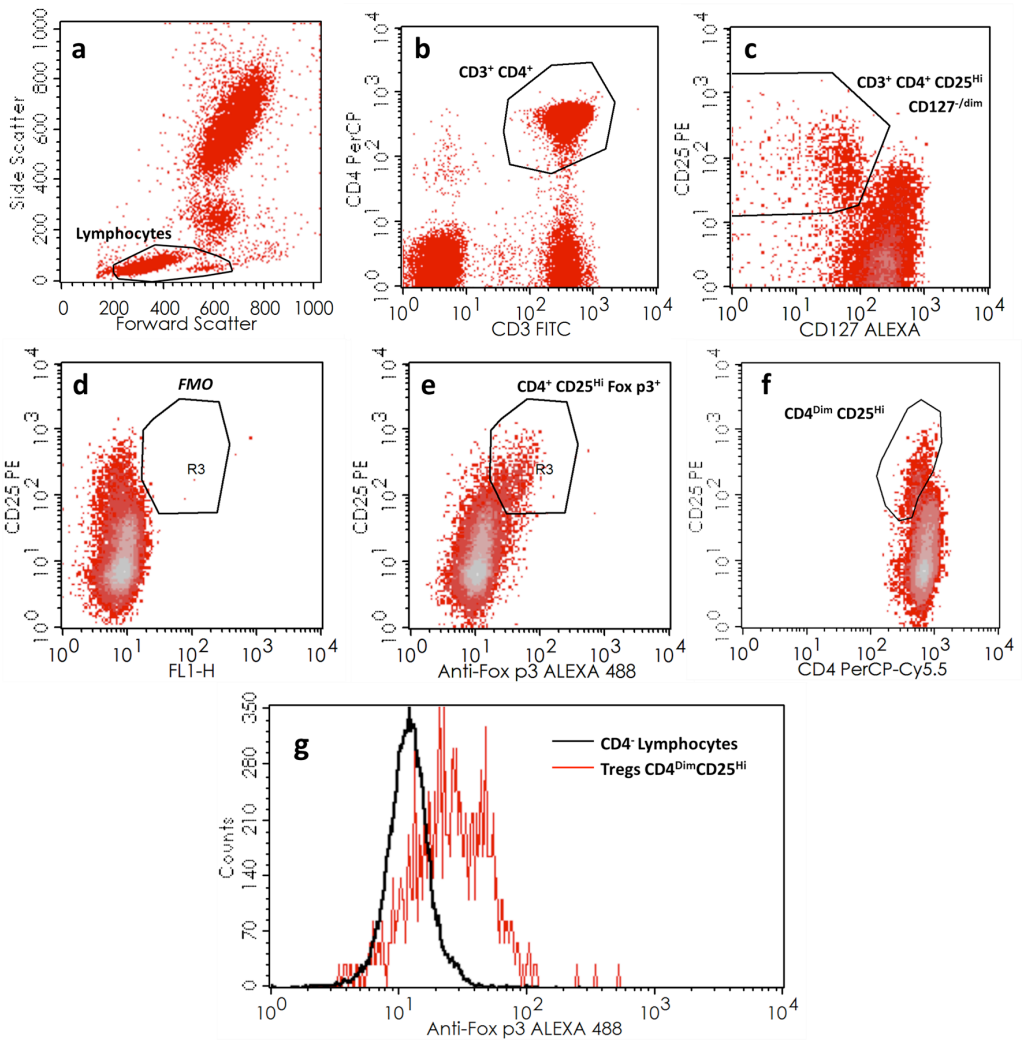

Figure 1 - Gating strategy for the identification of Tregs. **a.** Lymphocyte gate on FSC/SSC. **b.** Identification of CD4 T cells within lymphocyte populations ($CD3^{+}CD4^{+}$ T cells). **c.** Identification of $CD4^{+}CD25^{Hi}CD127^{-/dim}$ regulatory T cells. **d** and **e.** Identification of $CD4^{+}CD25^{Hi}Foxp3^{+}$ regulatory T cells with dot plots of Fluorescence Minus One (FMO) (d) and Foxp3 (e) tubes. **f.** CD4 vs. CD25 dot plot showing the identification of $CD4^{Dim}CD25^{Hi}$ regulatory T cells. **g.** Histogram for the evaluation of Foxp3 expression in $CD4^{Dim}CD25^{Hi}$ regulatory T cells (red line), overlaid on that for the expression of Foxp3 within $CD4^{-}$ lymphocytes (black line). Tregs, regulatory T cells.

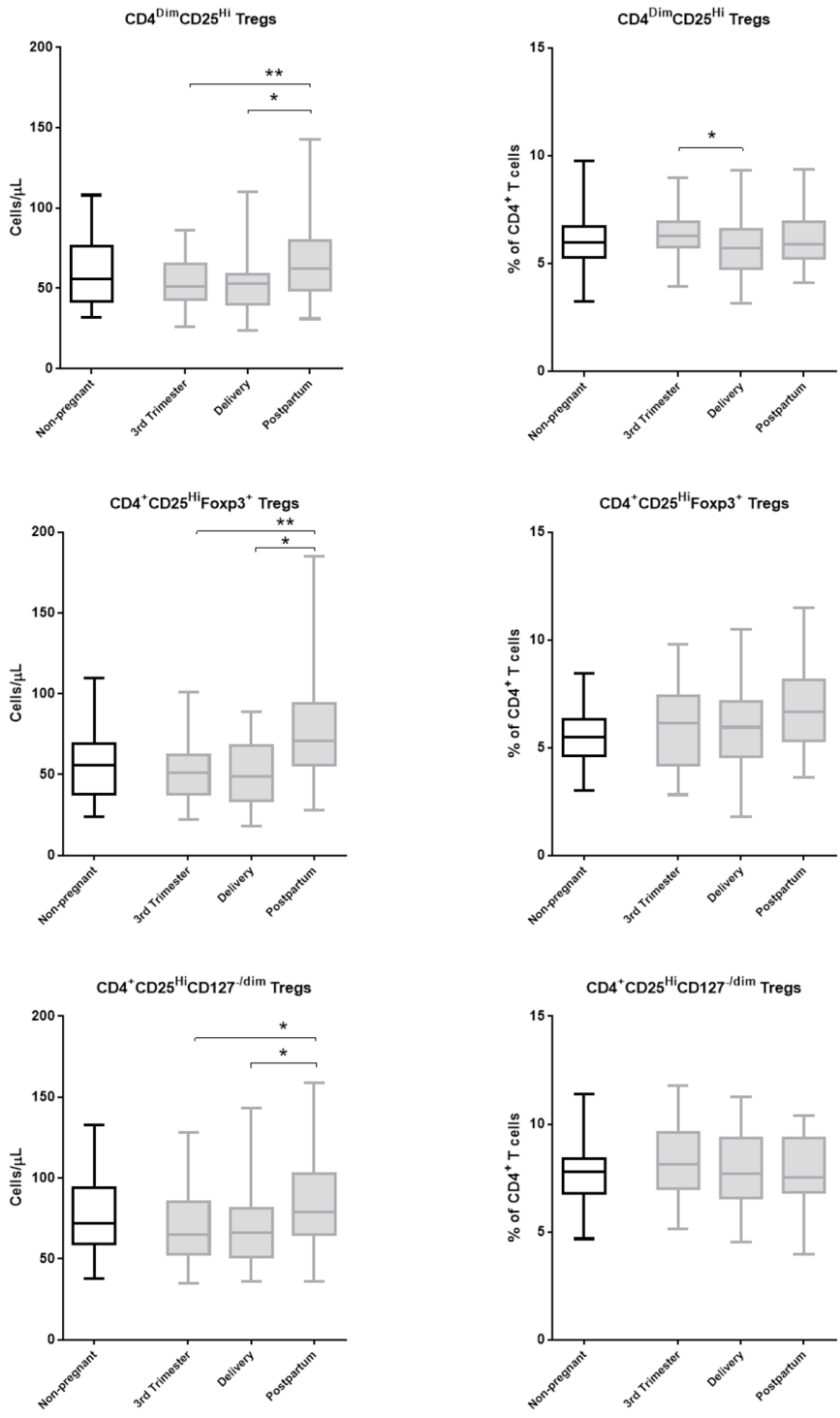

Figure 2 - Absolute counts and percentages of Tregs in peripheral blood samples. Delivery, within 15 minutes after placental expulsion; postpartum, at least 6 weeks after delivery; Tregs, regulatory T cells. The bottom of the box represents the 25th percentile, the top of the box represents the 75th percentile, the horizontal line inside the box represents the median, the bottom whiskers represent the minimum value, and the top whiskers represent the maximum value. * $p<0.05$; ** $p<0.001$

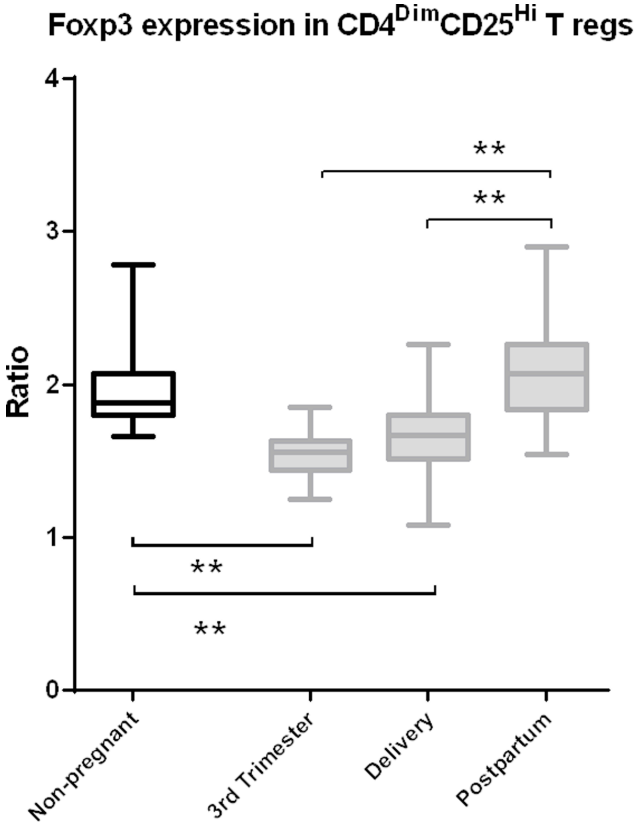

Figure 3 - Foxp3 expression in $CD4^{Dim}CD25^{Hi}$ Tregs. Delivery, within 15 minutes after placental expulsion; postpartum, at least 6 weeks after delivery; Tregs, regulatory T cells. The bottom of the box represents the 25th percentile, the top of the box represents the 75th percentile, the horizontal line inside the box represents the median, the bottom whiskers represent the minimum value, and the top whiskers represent the maximum value. * $p<0.05$; ** $p<0.001$

CHAPTER V

Impact of Labor on Peripheral Blood Maternal T-Cell Subsets and on Regulatory T and B Cells

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Impact of Labor on Peripheral Blood Maternal T-Cell Subsets and on Regulatory T and B Cells

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Abstract

Purpose: Labor is thought to positively influence immune system development in the offspring, but studies investigating the impact of different modes of delivery on maternal immune system cells are scarce. Therefore, the aim of this study was to investigate the effect of labor on maternal peripheral blood T-cell subsets and on the recently described regulatory T and B cells. **Methods:** Cross-sectional study comparing the absolute counts and percentages of peripheral blood T-cell subsets (maturation and activation profiles) and regulatory T and B cells between healthy pregnant women who delivered their newborns via elective cesarean (no labor; n = 14) and those who had a spontaneous vaginal delivery (after labor; n = 18). The cells were characterized using flow cytometry. **Results:** We found that compared to the women who had elective cesareans, those who had spontaneous vaginal deliveries had significantly ($P < .05$) lower absolute counts of B cells (median [cells/μL]: 146 [interquartile range, IQR = 49] vs 192 [IQR = 65]) and natural killer-like T (NKT-like) cells (median [cells/μL]: 154 [IQR = 125] vs 224 [IQR = 117]) in the peripheral blood. No further significant differences, particularly in regulatory T and B cells, were identified between the study groups. **Conclusion:** Labor does not seem to have a major impact on maternal peripheral blood T-cell subsets or regulatory T and B cells.

Keywords

labor, T-cell subsets, regulatory cells, immunology, flow cytometry

Background

The immune system of a healthy pregnant woman undergoes a transformation that allows it to tolerate fetal alloantigens, thus permitting the development of the fetus in the maternal uterus. It is plausible that several types of immune cells might contribute to this maternal–fetal immunotolerance. Physiological pregnancy has been compared to a state of quiescent systemic inflammation, and parturition has been compared to an immunological reaction that results in the recruitment of immune cells to the systemic circulation and the maternal–fetal interface.[1] This entire process may have an effect on the maturation and activation profiles of T cells in the peripheral blood (PB) of pregnant women.[2,3]

Traditionally, this state of immunological fetal tolerance has been explained by the predominantly Th2-type immunity found in normal pregnancies; in contrast, a predominantly Th1-type immunity was used to explain pathological pregnancies, such as those characterized by recurrent spontaneous abortion, intrauterine growth restriction, and preeclampsia.[4-9] Currently, this immunotolerance is explained by both Th2-type immunity and the roles of regulatory T cells (Tregs). Tregs are potent suppressors of inflammatory immune responses. Furthermore, they play a crucial role in immune tolerance, and their role in maintaining pregnancy has widely been reported in both humans and mice.[10,11] A decrease in maternal Tregs could cause the fetus to be rejected, and such decreases have been reported in conditions related to preterm labor and to immunological tolerance failure, such as miscarriage and preeclampsia.[9,12-15]

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Recent reports have suggested that another heterogeneous population of T cells might also have an important function in fetal–maternal immunological tolerance and in the regulation of Th1/Th2 balance. The $CD3^+CD56^+CD16^+$ natural killer-like T (NKT-like) cell subset simultaneously expresses T cell and natural killer cell surface markers and has been found in PB and in human decidua.[16] Published data regarding the role of the circulating levels of NKT-like cells in embryo implantation have been contradictory.[17,18]

Recent studies[19,20] show that in addition to their humoral activity, specific subsets of B cells have a regulatory function (regulatory B cells [Bregs]). Their counts increase during the first trimester of pregnancy, and they have the capacity to inhibit proinflammatory responses through the secretion of the anti-inflammatory cytokine interleukin 10, which protects against pregnancy loss.[20] These findings suggest that these cells with regulatory functions may be important for better understanding the mechanisms characterizing pregnancy and delivery.

Natural labor involves physiological processes that may not occur in pregnant women who deliver their newborns via elective cesarean. For example, during labor, the maternal immune system is activated, resulting in increased absolute counts and percentages of PB leukocytes[21-23] mainly because of the increase in the absolute counts and percentages of neutrophils and the decrease in the absolute counts and percentages of PB lymphocytes.[24]

The number of cesarean deliveries has dramatically increased worldwide over the past decades. Although a cesarean delivery can benefit the well-being of the mother and the child in certain situations, it is not a risk-free procedure; potential risks include infection, thrombosis, abnormal placentation, hysterectomy, and even maternal death, and cesarean deliveries are associated with more maternal complications compared to vaginal deliveries. Earlier studies have shown that offspring delivered via prelabor cesarean have a higher risk of immune disorders later in life,[25,26] and early changes in the subpopulations of lymphocytes can be detected in neonates soon after birth.[27] Data related to the PB T-cell subsets in mothers have been scarce,[27,28] and neither of the published studies of this topic investigated the effect of labor on maternal Tregs and Bregs. The results of such investigations would be clinically important because they could provide further knowledge regarding the impact of different modes of delivery on maternal health and the maternal immune system. Therefore, the objective of our study was to investigate the effect of labor on the maternal circulating T-cell subsets, Tregs, and Bregs.

Materials and Methods

Study Population

This was a cross-sectional study comparing the characteristics (ie, cell quantification and phenotype identification) of the PB T-cell subsets, Tregs, and Bregs between women who gave birth vaginally (ie, normal spontaneous vaginal birth) and by elective cesarean (ie, any surgical operation for delivering a baby through the abdominal wall without labor and with a duration of ruptured membranes lasting 5 minutes or less). The details of these modes of delivery were recorded in the patients' obstetric records.

An experienced practitioner decided whether to opt for vaginal delivery or elective cesarean. The hospital's procedure for managing vaginal deliveries and elective cesareans did not change over the course of our study. As part of this procedure, each newborn was evaluated at birth by a pediatrician.

Our study included a sample of the healthy pregnant women ($n = 32$) attending the outpatient clinic of our hospital. Healthy pregnant women were defined as women with asymptomatic, uncomplicated third trimester singleton pregnancies. These women received regular antenatal care and had appropriate fetal growth (based on uterine fundal height and ultrasounds performed after 28 weeks of gestation).

We excluded all women who did not have a term delivery ($\geq$37 weeks of gestation); did not give birth to a newborn whose birth weight was above the 10th percentile for gestational age; had a labor induction, diabetes, hypertension, atherosclerosis, autoimmune disease, vascular disease, or renal disease; had spontaneous rupture of the fetal membranes 48 hours before labor; had any clinical or laboratory signs of infection; used prenatal medications other than vitamins, folic acid, and iron supplements; or smoked during the 6 months prior to the PB sample collection.

All of the women were recruited between July 2013 and March 2014 at Hospital CUF Descobertas in Lisboa (Portugal). The ethics committee of this hospital approved the study protocol. All of the procedures involving human participants were performed in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. All of the women provided written informed consent prior to being included in the study.

Study Procedures

On the delivery day, PB samples were collected from all pregnant within 15 minutes after placental expulsion and oxytocin administration. Flow cytometry was then used to characterize the T-cell subsets, Tregs, and Bregs. Finally, these characterizations were compared between the pregnant who gave birth vaginally and those who gave birth via elective cesarean. The samples collected after the elective cesareans were used to represent a state with no labor, whereas the samples taken from vaginal deliveries were used to represent the state after the completion of labor. Labor was defined as the presence of regular uterine contractions that occurred at a frequency of at least 2 every 10 minutes and that were associated with cervical changes that led to vaginal delivery.

The following data were collected from all of the women: maternal age, ethnicity, body mass index (BMI), parity, systolic and diastolic blood pressure, gestational age at delivery, mode of delivery, and prolonged maternal hospital stay. The following data

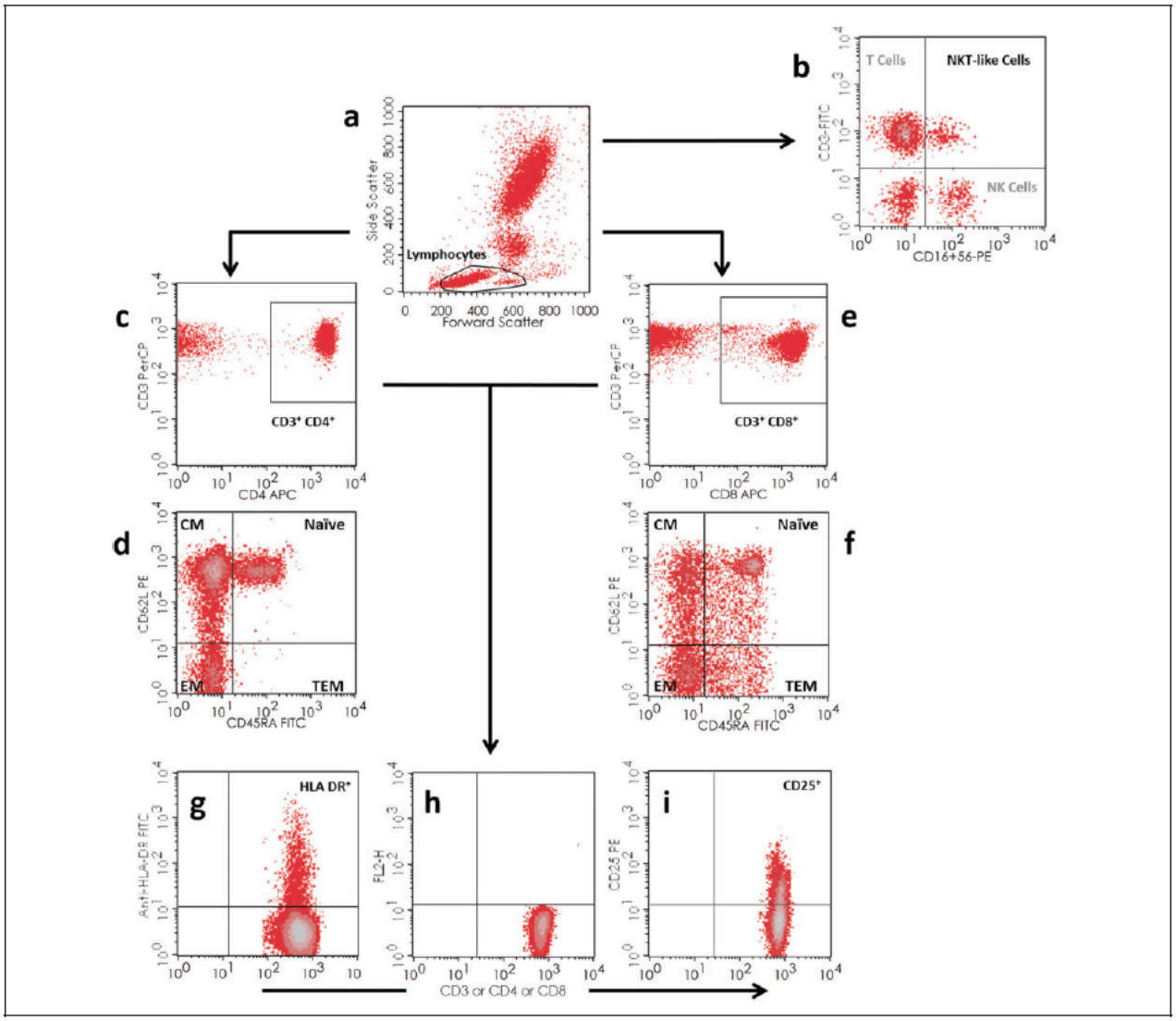

Figure 1. Gating strategy for the identification T-cell subsets. A, Lymphocyte gate (FSC/SSC). B, Identification of $CD3^+CD16^+/CD56^+$ NKT-like cells. C-F, Naïve and memory subsets of $CD4^+$ and $CD8^+$ T cells: $CD4^+$ T cells were identified within the lymphocyte gate by the coexpression of CD3 and CD4; $CD8^+$ T cells were similarly identified by the coexpression of CD3 and CD8. $CD4^+$ and $CD8^+$ T-cell subsets were classified as represented in the CD45RA/CD62L dot plots: Naïve—$CD45RA^+CD62L^+$; Central Memory, CM—$CD45RA^-CD62L^+$; Effector Memory, EM—$CD45RA^-CD62L^-$; Terminally Differentiated Effector Memory, TEM—$CD45RA^+CD62L^-$. G-I, Activation markers (HLA DR and CD25) were evaluated in $CD3^+$, $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells. Fluorescence minus one (FMO) tubes (dot plot H) were used to assess CD25 positivity.

were collected from the newborns: weight at birth, sex, APGAR score, arterial cord pH <7.0, and intensive care unit admission.

Flow Cytometry Analysis and Laboratory Measurements

A BD FACS Calibur (BD Biosciences, San Jose, California) equipped with 2 lasers (a 488-nm air-cooled argon-ion laser and a 635-nm red-diode laser) was used to characterize the PB lymphocytes of the pregnant included in this study. To obtain absolute cell counts, PB was assayed using the BD Multitest IMK kit (with CD3, CD4, CD8, CD16, CD19, CD45, and CD56) in BD Trucount tubes (all from BD Biosciences) following the manufacturer's instructions. Then, all of the samples were treated with a prevalidated panel of monoclonal antibodies (mAbs) that included the following fluorescence-conjugated mAbs acquired from Biolegend: CD3 FITC (clone SK7), CD3 PerCP (clone UCHT1), CD4 PerCP Cy5.5 and CD4 APC (clone SK3), CD8 PE Cy7 and CD8 APC (clone SK1), CD19 PerCP Cy5.5 (clone HIB19), CD24 PE (clone ML5), CD25 PE (clone BC96), CD27 FICT

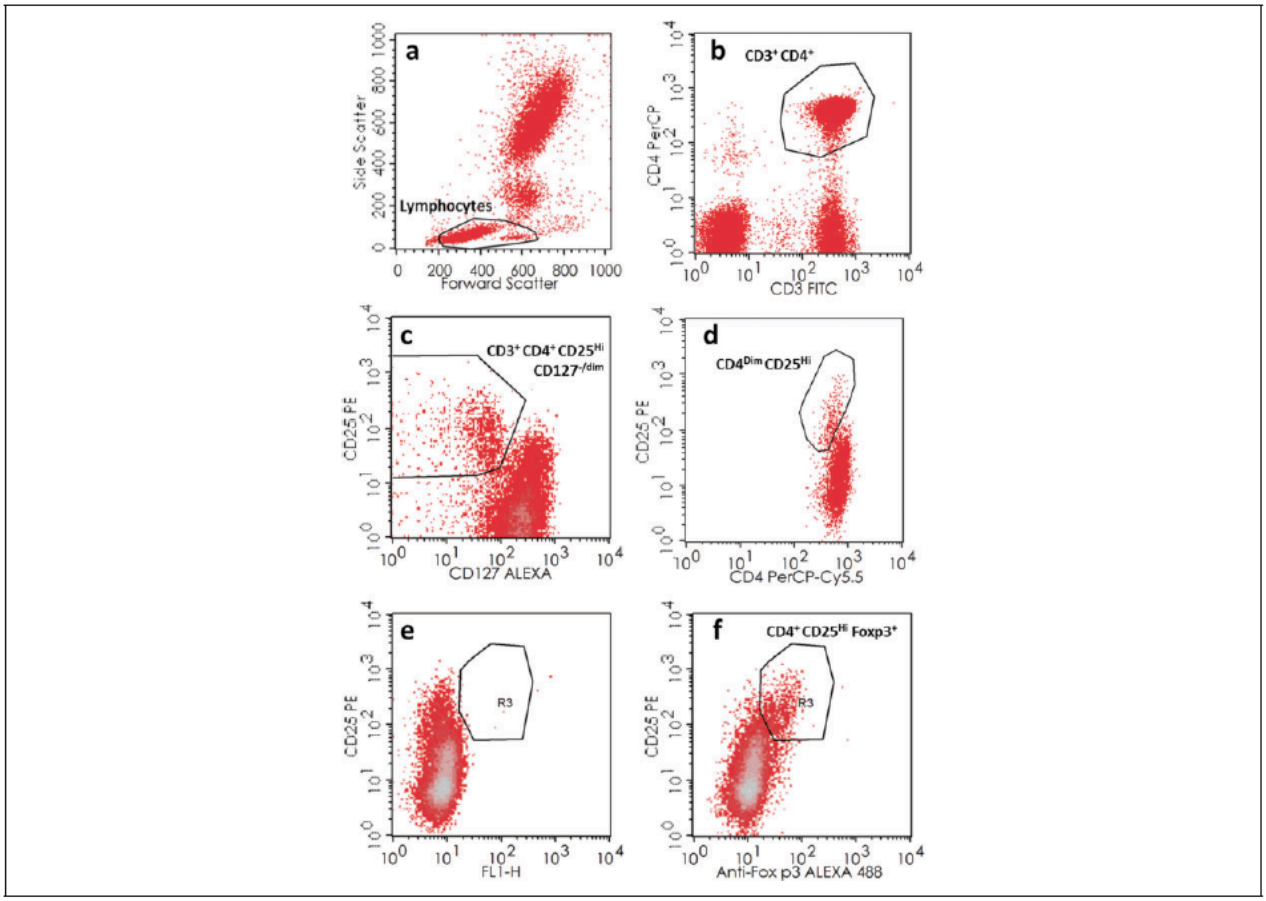

Figure 2. Gating strategy for the identification of regulatory T cells. Three analytical strategies were used to characterize Treg cells: $CD4^{Dim}CD25^{Hi}$, $CD4^{+}CD25^{Hi}CD127^{-/dim}$, and $CD4^{+}CD25^{Hi}Foxp3^{+}$ T cells. A-B, $CD4^{+}$ T cells were recognized as the $CD3^{+}$ $CD4^{+}$ (or $CD4^{+}$) cells within the lymphocyte gate. C, Dot plot exemplifying the identification of $CD4^{+}CD25^{Hi}CD127^{-/dim}$ regulatory T cells. D, CD4 versus CD25 dot plot showing the identification of $CD4^{+(dim)}CD25^{Hi}$ regulatory T cells. E-F, Identification of $CD4^{+}CD25^{Hi}Foxp3^{+}$ regulatory T cells with dot plots of fluorescence minus one (FMO; E) and Foxp3 (F) tubes.

(clone O323), CD38 APC (clone HIT2), CD45RA FITC (clone HI100), CD62L PE (clone DREG-56), CD127 Alexa647 (clone A019D5), CD152 (CTLA-4; clone L3D10), and anti-HLA DR FITC (clone L243). For surface antigen characterization, PB was processed with a simple lyse-wash protocol using BD FACS lysing solution (BD Biosciences) to lyse the red blood cells. The Human FoxP3 Buffer Set (BD Pharmingen, San Jose, California) was used to evaluate Foxp3. Following the manufacturer's recommendations, the cells were lysed with BD FACS lysing solution (BD Biosciences) and then incubated with anti-CD4 and -CD25 (see earlier) mAbs for surface antigen staining. After the fixation and permeabilization steps, the cells were incubated with Anti-Foxp3 Alexa488 (clone 259D/C7; BD Pharmingen). CellQuest software (BD Biosciences) was used for both acquisition and analysis. A minimum of 10 000 T cells was acquired in the T-cell panels, and a minimum of 2000 B cells was acquired in the B-cell panels. The data analysis and gating strategies are described in Figures 1 to 3.

Statistics

The normality of data was assessed using the Shapiro-Wilk test. Normally distributed data were compared using *t* tests and summarized as mean and standard deviation, and nonnormally distributed data were compared using Mann-Whitney *U* tests and reported as the median with the interquartile range (IQR). Count data were compared using Fisher exact test. Statistical significance was defined by a P value $<.05$. All data were analyzed using *R* software, version 3.12 for Windows.

Results

Our study included 32 pregnant who gave birth to their newborns at term either via spontaneous vaginal delivery (n = 18) or via elective cesarean (n = 14). As Table 1 shows, these women did not differ significantly in terms of age, ethnicity, BMI, systolic and diastolic blood pressure, gestational age, and prolonged maternal hospital stay. However, there were

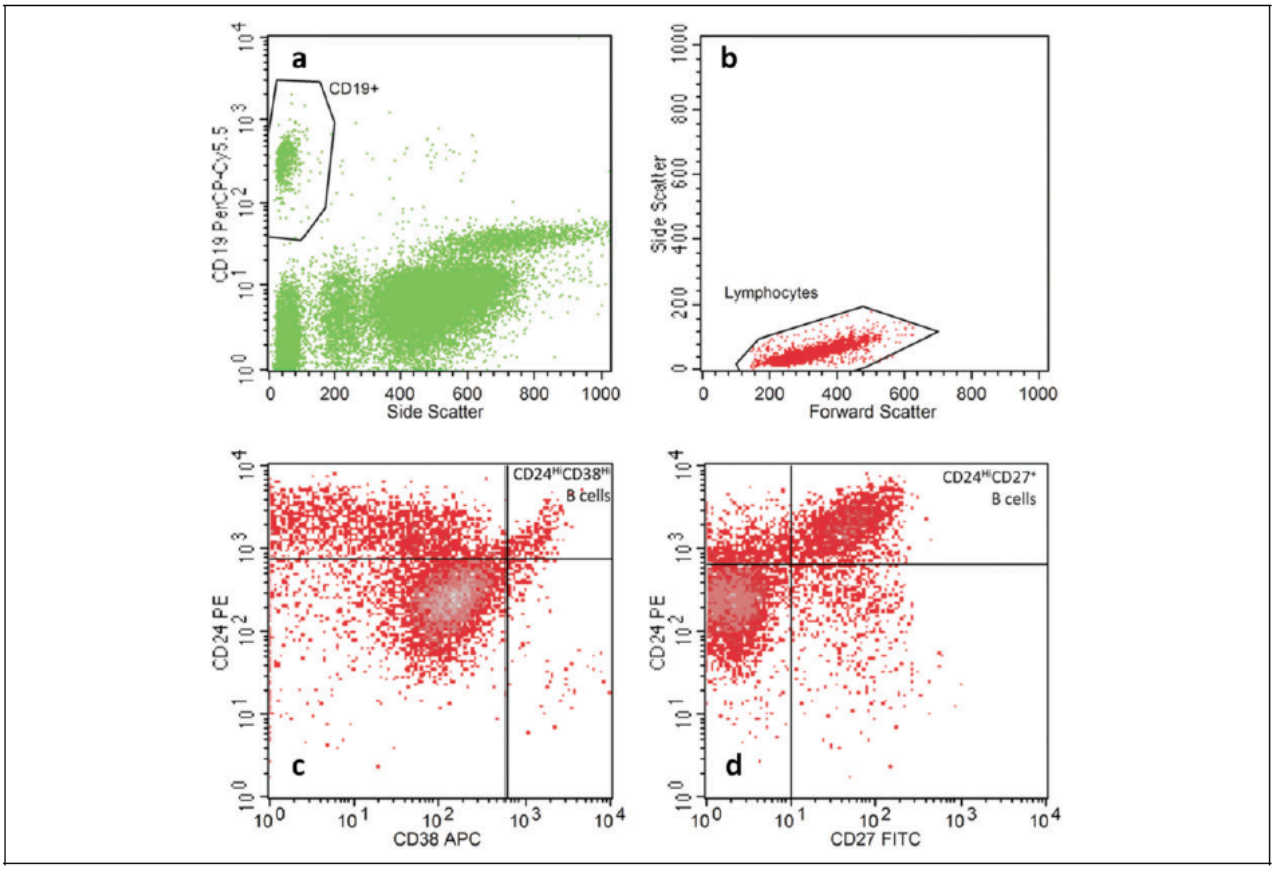

Figure 3. Gating strategy for the identification of regulatory B cells. Considering the lack of consensus regarding the Bregs phenotype, 2 analytical strategies were used to characterize Bregs: $CD24^{hi}CD27^{+}$ and $CD24^{hi}CD38^{hi}$. A-B, B cells were identified as $CD19^{+}$ cells (a) in the lymphocyte population, gated according to forward and side scatter (B). C-D, Identification of regulatory B-cell subsets according to their expression of CD24, CD27, and CD38 ($CD24^{Hi}CD38^{Hi}$ Bregs and $CD24^{Hi}CD27^{+}$ Bregs).

significantly ($P < .05$) more nulliparous pregnant who delivered their newborns vaginally (n = 12) than via elective cesarean (n = 1). Among the spontaneous vaginal deliveries, the median time for dilation from 4 to 10 cm was 6 hours (95% confidence interval, 4.8-9.3 hours). Regardless of the mode of delivery, all of the pregnant received regional analgesia and/or anesthesia. None of the women received general anesthesia.

All 18 of the vaginal deliveries were spontaneous (without forceps or vacuum) and received epidural analgesia during labor. All elective cesareans (n = 14) were made with regional (combined spinal epidural) anesthesia. The indications for the elective cesareans were previous cesarean section (n = 12; 86%) and breech presentation (n = 2; 14%).

Tables 2 to 5 present a comparison of the PB T-cell subsets, Tregs, and Bregs between women who had spontaneous vaginal deliveries and those who had elective cesarean births. Compared to women who had elective cesareans (no labor), those who delivered spontaneously and vaginally had significantly ($P < .05$) lower circulating absolute counts of B cells (median [cells/μL]: 146 [IQR = 49] vs 192 [IQR = 65]) and NKT-like cells (median [cells/μL]: 154 [IQR = 125] vs 224 [IQR = 117]).

Furthermore, no significant differences in T cells, $CD4^{+}$ and $CD8^{+}$ T cells, the maturation profiles (naïve, central memory, effector memory, and terminally differentiated effector memory) of $CD4^{+}$ and $CD8^{+}$ T cells, and the activation profiles of $CD3^{+}$, $CD4^{+}$, and $CD8^{+}$ T cells (markers for T-cell activation included HLA-DR and CD25) were identified between the study groups.

Additionally, no significant differences in Tregs and Bregs were identified between the pregnant with spontaneous vaginal deliveries and those who underwent elective cesareans.

Discussion

In this study, we investigated whether labor has an effect on maternal PB T-cell subsets, Tregs, and Bregs. We found that compared to elective cesareans (no labor), spontaneous vaginal deliveries (after the completion of labor) were associated with significantly lower median absolute counts of B cells and

Table 1. Demographics and Clinical Variables.[a]

Pregnant Women	Spontaneous Vaginal Delivery (n = 18)	Elective Cesarean (n = 14)	*P*
Maternal age in years, mean (SD)	32.7 (3.3)	34.5 (3.4)	>.05[b]
Ethnicity, No			
White	18	14	
BMI in Kg/m^2, mean (SD)	26.1 (2.6)	25.9 (2.4)	>.05[b]
Parity, No. (%)			<.001[c]
Nulliparous	12 (66.7)	1 (7.0)	
Parous	6 (33.3)	13 (93.0)	
Systolic blood pressure in mm Hg, mean (SD)	115.2 (9.9)	114.5 (9.5)	>.05[b]
Diastolic blood pressure in mm Hg, mean (SD), (range)	65.9 (7.5) (54.0-85.0)	67.2 (6.9) (57.0-79.0)	>.05[b]
Gestational age in weeks at delivery, median (range)	38.5 (38.0-41.0)	38.0 (38.0-40.0)	>.05[d]
Prolonged maternal hospital stay[e], No.	0	0	
Offspring			
Weight at birth in grams, mean (SD)	3340.8 (402.6)	3208.2 (349.5)	>.05[b]
Gender, No. (%)			
Male	10 (55.6)	6 (42.9)	
Female	8 (44.4)	8 (57.1)	
APGAR score, median (range)			
1-minute Apgar score	9.5 (6-10)	9 (8-10)	
5-minute Apgar score	10 (9-10)	10 (10-10)	
5-minute Apgar score < 7	0	0	
Arterial cord pH < 7.10, No.	0	0	
Intensive care unit admission, No.	0	0	

Abbreviations: BMI, body mass index.
[a]SD, standard deviation.
[b]*t* test.
[c]Fisher exact test for count data.
[d]Wilcoxon rank test.
[e]More than 2 days after vaginal delivery or more than 3 days after cesarean.

Table 2. Comparison of Maternal Peripheral Blood Lymphocytes Subsets Between Women Who Gave Birth by Spontaneous Vaginal Delivery and by Elective Cesarean.[a]

	Spontaneous Vaginal Delivery	Elective Cesarean	*P*
Lymphocytes, cells/μL, mean (SD)	1958 (690)	2240 (852)	.146
T cells, cells/μL, mean (SD)	1412 (514)	1784 (704)	.084
T cells, %, mean (SD)	77 (8.8)	75.5 (6.8)	.837
CD4$^+$ T cells, cells/μL, mean (SD)	871 (343)	917 (238)	.411
CD4$^+$ T cells, %, mean (SD)	44.0 (4.8)	41.5 (6.8)	.360
CD8$^+$ T cells, cells/μL, median (IQR)	548 (206)	672 (237)	.102
CD8$^+$ T cells, %, mean (SD)	29.0 (11.0)	31.5 (8.3)	.569
NKT-like cells, cells/μL, median (IQR)	154 (125)	224 (117)	.025
NKT-like cells, %, median (IQR)	7.5 (4.1)	9.6 (3.3)	.055
B cells, cells/μL, median (IQR)	146 (49)	192 (65)	.046
B cells, %, median (IQR)	7.8 (2.0)	8.7 (3.4)	.525

[a]Normally distributed data expressed as mean (SD, standard deviation): group comparisons with Student *t* test; nonnormally distributed data expressed as median (IQR, interquartile range): group comparisons with Mann-Whitney *U* test.

NKT-like cells. No further significant differences between spontaneous vaginal deliveries and elective cesareans were identified.

Variations in some of the subpopulations in PB may indicate that there is a selective migration of these cells out of the PB (into the sites of inflammation), a change in the survival of these cells, or an obstacle to their differentiation. The determination of these phenotypes in pregnancy and labor may contribute to our understanding of T-cell homeostasis during labor.

In our study, the finding of lower counts of B cell in spontaneous vaginal deliveries compared to elective cesareans could be explained by the migration of these cells into the decidua during labor, which would result in the decrease in the absolute counts of these cells in PB. Indeed, a recent study[29] demonstrated for the first time in humans that specific subtypes of leukocytes infiltrate the decidua during labor, suggesting that the inflammatory mediators produced by leukocytes play a role in decidual activation, a preliminary event in the labor process. Our results are consistent with those of a previous study[30] in an animal model that described immune cell migration from PB to the uterus, as one of the mechanisms responsible for the B lymphopenia that occurs late in pregnancy. We hypothesize that during labor, recruitment events are more intense.

We also found that compared to no labor, spontaneous vaginal deliveries were associated with significantly lower median absolute counts of NKT-like cells. To our knowledge, this finding has not been described previously in labor; however, 1 study found that NKT-like cells may be recruited from the PB immune cell pool after embryo implantation.[31] Similar to B cells, NKT-like cells may also migrate selectively to the decidua during labor, thus decreasing the absolute counts of these cells in the PB. The data pertaining to the function of

Table 3. Comparison of the Maturation Profiles of $CD4^+$ and $CD8^+$ T Cells Between Women Who Gave Birth by Spontaneous Vaginal Delivery and by Elective Cesarean.[a]

	Spontaneous Vaginal Delivery	Elective Cesarean	*P*
Naive $CD4^+$ T cells, cells/μL, median (IQR)	414 (122)	488 (201)	.362
Naive $CD4^+$ T cells, %, mean (SD)	49.9 (12.9)	49.4 (8.9)	.492
CM $CD4^+$ T cells, cells/μL, mean (SD)	335 (185)	332 (112)	.457
CM $CD4^+$ T cells, %, median (IQR)	38.7 (5.5)	34.1 (11.7)	.184
EM $CD4^+$ T cells, cells/μL, median (IQR)	99 (52)	106 (54)	.494
EM $CD4^+$ T cells, %, mean (SD)	9.8 (5.3)	12.3 (4.8)	.621
TEM $CD4^+$ T cells, cells/μL, median (IQR)	12 (11)	24 (50)	.819
TEM $CD4^+$ T cells, %, median (IQR)	1.3 (1.0)	2.3 (4.2)	.909
Naive $CD8^+$ T cells, cells/μL, median (IQR)	303 (118)	321 (125)	.718
Naive $CD8^+$ T cells, %, mean (SD)	52.1 (12.7)	46.8 (14.6)	.056
CM $CD8^+$ T cells, cells/μL), median (IQR)	75 (40)	89 (58)	.482
CM $CD8^+$ T cells, %, mean (SD)	13.4 (5.4)	13.5 (11.8)	.957
EM $CD8^+$ T cells, cells/μL, median (IQR)	73 (42)	114 (97)	.314
EM $CD8^+$ T cells, %, mean (SD)	13.0 (9.3)	14.4 (10.7)	.416
TEM $CD8^+$ T cells, cells/μL, median (IQR)	96 (62)	148 (97)	.160
TEM $CD8^+$ T cells, %, median (IQR)	16.7 (6.6)	21.8 (12.7)	.447

Abbreviations: CM, central memory; EM, effector memory; TEM, terminally differentiated effector memory.
[a]Normally distributed data expressed as mean (SD, standard deviation): group comparisons with Student *t* test; nonnormally distributed data expressed as median (IQR, interquartile range): group comparisons with Mann-Whitney *U* test.

Table 4. Comparison of Activation Profiles of $CD3^+$, $CD4^+$, and $CD8^+$ T cells Between Women Who Gave Birth by Spontaneous Vaginal Delivery and by Elective Cesarean.[a]

	Spontaneous Vaginal Delivery	Elective Cesarean	*P*
$CD3^+$/HLA DR^+ activated T cells, cells/μL, median (IQR)	253 (185)	283 (144)	.362
$CD3^+$/HLA DR^+ activated T cells, %, median (IQR)	16.4 (7.8)	15.9 (6.2)	.939
$CD3^+$/$CD25^+$ activated T cells, cells/μL, mean (SD)	372 (159)	314 (166)	.714
$CD3^+$/$CD25^+$ activated T cells, %, mean (SD)	22.8 (7.6)	18.8 (12.0)	.172
$CD4^+$/HLA DR^+ activated T cells, cells/μL, median (IQR)	91 (92)	68 (36)	.985
$CD4^+$/HLA DR^+ activated T cells, %, median (IQR)	9.4 (7.0)	7.3 (3.3)	.556
$CD4^+$/$CD25^+$ activated T cells, cells/μL, mean (SD)	329 (157)	302 (165)	.669
$CD4^+$/$CD25^+$ activated T cells, %, mean (SD)	37.3 (9.5)	33.8 (18.3)	.240
$CD8^+$/HLA DR^+ activated T cells, cells/μL, median (IQR)	151 (106)	187 (99)	.239
$CD8^+$/HLA DR^+ activated T cells, %, mean (SD)	24.3 (10.2)	24.4 (15.8)	.948
$CD8^+$/$CD25^+$ activated T cells, cells/μL, median (IQR)	20 (10)	19 (8)	.718
$CD8^+$/$CD25^+$ activated T cells, %, median (IQR)	3.9 (1.8)	3.3 (2.1)	.262

[a]Normally distributed data expressed as mean (SD, standard deviation): group comparisons with Student *t* test; nonnormally distributed data expressed as median (IQR, interquartile range): group comparisons with Mann-Whitney *U* test.

Table 5. Comparison of Regulatory Subsets of T and B Cells Between Women Who Gave Birth by Spontaneous Vaginal Delivery and by Elective Cesarean.[a]

	Spontaneous Vaginal Delivery	Elective Cesarean	*P*
Regulatory T cells $CD4^+CD25^{Hi}CD127^{-/dim}$,cells/μL, median (IQR)	70 (22)	64 (20)	.594
Regulatory T cells $CD4^+CD25^{Hi}CD127^{-/dim}$, %, mean (SD)	8.2 (2.2)	6.8 (3.1)	.072
Regulatory T cells $CD4^+CD25^{Hi}Foxp3^+$, cells/μL, mean (SD)	59 (27)	49 (28)	.403
Regulatory T cells $CD4^+CD25^{Hi}Foxp3^+$, %, mean (SD)	6.4 (2.9)	5.4 (2.2)	.102
Regulatory T cells $CD4^{+(dim)}CD25^{Hi}$, cells/μL, median (IQR)	50 (15)	51 (17)	.894
Regulatory T cells $CD4^{+(dim)}CD25^{Hi}$, %, mean (SD)	6.0 (0.8)	5.6 (1.6)	.516
Regulatory B cells ($CD19^+CD24^{Hi}CD27^+$), cells/μL, median (IQR)	2 (1)	2 (1)	.500
Regulatory B cells ($CD19^+CD24^{Hi}CD27^+$), %, median (IQR)	1.1 (0.8)	1.0 (0.6)	.690
Regulatory B cells ($CD19^+CD24^{Hi}CD38^{Hi}$), cells/μL, median (IQR)	3 (2)	6 (7)	.171
Regulatory B cells ($CD19^+CD24^{Hi}$ $CD38^{Hi}$), %, median (IQR)	2.3 (1.9)	3 (3.2)	.718

[a]Normally distributed data expressed as mean (SD, standard deviation): group comparisons with Student *t* test; nonnormally distributed data expressed as median (IQR, interquartile range): group comparisons with Mann-Whitney *U* test.

NKT-like cells in pregnancy immune modulations are limited.[16,18] Further research is necessary to investigate the exact function of these cells during labor.

Nevertheless, spontaneous vaginal deliveries and elective cesareans did not seem to have significantly different impacts on PB absolute counts or the percentages of the majority of immune cell subsets, and this seems to be supported by the literature. First, it has been shown that in human term pregnancy, a significantly higher percentage of activated T cells are present in the decidua than in the maternal PB as a result of the local expansion of these cells.[32] Additionally, recent research in humans has shown that the absolute counts of Tregs and the estrogen levels in the PB increase progressively from early to late pregnancy[11] and decrease after delivery.[11,33] This suggests that Treg may play a role in the immunological changes that occur before delivery rather than during or after labor. Finally, Bregs were already shown to expand in normal early pregnancy when immunosuppression is necessary.[20] We hypothesize that, like Tregs, they are probably not directly involved in the immunological mechanisms in labor.

In our study, analgesia and/or anesthesia were administered in all cases of vaginal deliveries and elective cesareans. It has been reported that the use of analgesia and/or anesthesia at birth is associated with reduced maternal and fetal endocrine stress responses and that vaginal deliveries are associated with higher endocrine stress responses than elective cesareans are.[34] It is currently unknown whether the use of analgesia and/or anesthesia has any impact on the maternal immune response at delivery.

The main limitation of our study was that there were significantly more nulliparous who delivered their newborns vaginally than by elective cesarean. Nonetheless, our sample was homogenous, as we only included a pregnant woman who had singleton pregnancies between 37 and 41 weeks of gestation, and we used standardized protocols for the management of vaginal deliveries and elective cesareans, with no induction of labor or intravenous analgesia. Studies with larger sample sizes may be required to further investigate the impact of labor on maternal immune system cells. Furthermore, it is important that upcoming studies compare immune cell subsets in the PB and decidua at delivery from the same healthy pregnant women.

In conclusion, this study found that labor does not affect significantly the studied circulating lymphocytes subsets. During labor, these changes may indicate that what is happening in the PB does not reflect what is happening in the placenta. The failure to detect a significant or major difference in maternal PB T-cell subsets, especially in Tregs and Bregs, between the presence or the absence of labor is consistent with the previous studies that reported that these cell subsets have a more important functional role in early pregnancy for tolerating the fetus rather than during labor. In the future, it will be interesting to determine whether the presence or absence of labor has different effects on the immune system of pregnant patients with autoimmune disorders and might eventually affect their clinical outcomes.

Authors' Note

All of the authors designed the study and created the study protocol; J Lima had the original research idea, recruited the patients, collected the data, and drafted the manuscript; C Martins and G Nunes analyzed the blood samples using flow cytometry; LM Borrego supervised all the work and the research protocol; all of the authors contributed to the data analysis and interpretation, revised it, intellectually contributed, and approved the final version of the manuscript.

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CHAPTER VI

General Discussion

Final Remarks

Pregnancy poses a challenge to the maternal immune system because it requires the development of tolerance to a genetically foreign fetus and simultaneously must protect the mother and the fetus against infection. These requirements necessitate tight regulation of the maternal immune system during the different stages of pregnancy. Although several subsets of B and T cells have been shown to have regulatory functions via the production of anti-inflammatory and pro-inflammatory cytokines, their occurrence and function during late pregnancy and the postpartum period remain unclear.[41,42,48,85-95,109-118] Therefore, the main aim of this research was to analyze (i.e., cell quantification and phenotypic identification) B and T cell populations, including their regulatory subsets, in the peripheral blood of healthy pregnant women compared with non-pregnant women from the 3rd trimester to the postpartum period.

Eighty-three patients were selected for observational prospective studies according to the eligibility criteria and allocated into two groups: one group of pregnant women (n = 48) and a non-pregnant women control group (n = 35). During follow-up, only 5 pregnant women dropped out from the study: 3 because they had preterm deliveries (<37 weeks of gestation); 1 that did not come back after collecting blood at the 1st time point (3rd trimester of pregnancy); and 1 woman with a previous caesarean who had an intrapartum uterine rupture during labor with bleeding requiring transfusion prior to the sampling.

We found that the progression of a normal pregnancy from the 3rd trimester to the postpartum period was accompanied by variation in several B and T cell types in the peripheral blood. In chapter III, we showed that the $CD19^+$ B cell population decreased in the late stage of pregnancy, between the 3rd trimester and delivery. These results are consistent with those of several previous studies conducted in humans.[85-95] Moreover, a detailed search of the relevant literature indicated that, to our knowledge, this is the first study to characterize the circulating B cell compartment during human pregnancy while taking into account the maturational stages of the different B cell subsets. We found that the absolute counts of several B

cell subsets (transitional, unswitched memory, resting memory B cells, and plasmablasts) were significantly reduced in the 3rd trimester and on the day of delivery, compared to non-pregnant women, and increased during the postpartum period to normal values. However, the percentages of naïve B cells increased during the 3rd trimester and on the day of delivery, and decreased in the postpartum period to similar levels to those detected in non-pregnant women. This expansion of naïve B cells during pregnancy has been previously described in pregnant mice.[82] Finally, we also showed in chapter III that the absolute counts and percentages of the $CD24^{Hi}CD38^{Hi}$ Breg subset were significantly higher in the postpartum period of pregnant women compared with non-pregnant women, after decreasing during the 3rd trimester and on the day of delivery.

Taken together, the results shown in chapter III support the conclusion that late pregnancy in humans is accompanied by lower levels of B cell subsets and a relative increase in naïve B cells in the peripheral blood. We hypothesized that these observations could result from two conditions: the reduction of the generation or differentiation of B cells, which might be driven by female sex hormones (e.g., progesterone, estrogen)[156,157]; or the increase in the migration of B cells from the peripheral blood to other organs.[158-160] Further studies are required to ascertain the biological meaning of B cell lymphopenia in late pregnancy, but it is probably related to the mechanism that regulates the maternal immune tolerance to the fetus. This hypothesis is supported by the observations in chapter III confirming that most B cell subsets appear to revert to normal levels during the postpartum period, while the percentages of $CD24^{Hi}CD38^{Hi}$ Bregs were significantly higher in the postpartum period compared with non-pregnant women. Because B cell lymphopoiesis could be under endocrine regulation during pregnancy, our results might be explained by the decline in hormonal levels that typically occurs in the postpartum period, which might have lead both to B cell lymphopoiesis recovery and to B cell activation.[82,156]

Although the mechanism that triggers the postpartum increase in the $CD24^{Hi}CD38^{Hi}$ Breg subset remains unclear, these results could have clinical importance for the treatment of patients who might benefit from an increase in B cell lymphopoiesis (e.g., transplant or immunocompromised patients).[156]

We are aware that this study had some limitations. All of the pregnant women in our study received epidural analgesia during labor and/or regional anesthesia. Such anesthetic techniques are unlikely to cause important changes in immune cell counts because regional administration is generally associated with low plasma levels of the administered drugs. Ideally, samples collected before pregnancy would have been compared with samples collected during pregnancy in the same individuals; however, this experimental design would have been very difficult for us to achieve from a practical perspective. Nevertheless, the strength of our research is underscored by its pioneering status as the first investigation conducted in humans to characterize the circulating B cell compartment during pregnancy, while taking into account the maturational stages of the different B cell subsets. In addition, the success of this ambitious and arduous project was only possible because of a team of highly motivated and available people (24 hours a day). Accuracy was maintained because the same research team performed the clinical monitoring (including deliveries) and laboratory tests, using very strict criteria and protocols.

In chapter IV, we found that Treg subset counts, namely $CD4^{Dim}CD25^{Hi}$, $CD4^{+}CD25^{Hi}Foxp3^{+}$ and $CD4^{+}CD25^{Hi}CD127^{-/dim}$, increased in the postpartum period compared with the 3rd trimester and the day of delivery. These results are in accordance with the conclusions of Wegienka et al.,[118] but are inconsistent with earlier studies.[42,112,115] Despite the limitations of our study, namely the hemodilution effect and heterogeneity of the Treg subsets, additional data regarding the analysis of Foxp3,[161,162] a transcription factor that is essential for Treg induction and stability, support the conclusions shown in this study. Accordingly, the Foxp3 expression ratio in the $CD4^{Dim}CD25^{Hi}$ Treg subset increased significantly in the postpartum period, after decreasing during pregnancy and on the day of delivery compared with the levels measured in non-pregnant women. Furthermore, these fluctuations in Foxp3 expression and in Treg subset levels showed a similar pattern to those previously found in chapter III for the $CD24^{Hi}CD38^{Hi}$ Breg subset (i.e., increased levels during the postpartum period). Hence, the results shown in chapters III and IV provide further evidence that Breg play an important role in Treg differentiation, probably by promoting Foxp3 expression in these cells. This assumption is also supported by recent studies conducted in human cell lines.[163,164]

The increase in circulating Treg subsets and Foxp3 expression in the postpartum period compared with the 3rd trimester and the day of delivery, also could be a consequence of the action of known Treg modulators, such as hormones and cytokines,[107] driven by a yet unclear mechanism. It has been reported that the female sex hormones progesterone and estradiol reduce Foxp3 expression in Tregs during mid pregnancy.[116] Our data are consistent with these findings, which probably extend through the 3rd trimester and delivery. During the postpartum period, Foxp3 expression increases significantly compared with the 3rd trimester and the day of delivery, reaching levels similar to those measured in the control group of non-pregnant women. This phenomenon confirms that pregnancy results in a modulation of Foxp3 expression within the $CD4^{Dim}CD25^{Hi}$ Treg subset. However, the increase in Treg suggests the presence of an immunosuppressed environment, permitting maternal-fetal tolerance during pregnancy, as well as the postpartum period. Strikingly, labor has been linked to the progression of maternal-fetal immune cell trafficking with gestational age, which might trigger the activation of maternal immune cells.[165] According to the findings presented in chapter IV, we hypothesize that Treg expansion in the postpartum period is a potentially physiological mechanism that leads to the down regulation of maternal immunological events that are activated during delivery (i.e., reducing susceptibility to systemic inflammatory responses).

The results presented both in chapters IV and III included only women with a normal singleton pregnancy between 37 and 41 weeks of gestation to obtain a relatively homogenous sample and to reduce potential bias. In addition, all enrolled pregnant women contributed samples at all of the study time points (3rd trimester, delivery day and postpartum). We also accounted for the phase of the menstrual cycle during which the samples were collected from the non-pregnant women. Peripheral blood samples were collected from this control group during the follicular phase of the menstrual cycle because hormonal status during the luteal phase is comparable to that during pregnancy.[166] However, a previous study[167] reported that Treg subsets expand during the follicular phase, peaking immediately before ovulation (to induce immune tolerance to facilitate implantation), and then decline in the subsequent luteal phase. This phenomenon may have contributed to the absence of significant differences in Treg numbers between the non-pregnant and pregnant

groups. Preferably, samples from additional time points, such as during the first and second trimesters of pregnancy, would be included, as well as samples obtained during the monitoring of women before becoming pregnant through the postpartum period. However, these additional time points are beyond the scope of the current analyses.

In chapter V, we addressed the question of whether normal labor has an effect on maternal circulating T cell subsets, Treg, and Breg. Specifically, differences between spontaneous vaginal deliveries (after completion of labor) and elective cesareans (no labor) were analyzed in the peripheral blood of healthy women. We found that labor had no major effects on the levels of the majority of B and T cell subsets, although it was associated with lower $CD19^+$ B cell counts. This decrease in $CD19^+$ B cells may indicate the presence of obstacle to their differentiation, a change in their survival, or selective migration of these cells out of the peripheral blood. Indeed, previous studies support the hypothesis that the reduced counts of maternal immune cells result from their migration into the decidua during labor.[158,168] We hypothesize that during labor, recruitment events are more intense. In chapter V, we showed that spontaneous vaginal deliveries were associated with lower counts of NKT-like cells. To our knowledge, this finding has not been previously described during labor. In fact, data concerning the function of NKT-like cells in the immunomodulatory events associated with pregnancy are limited.[103,105] Further research is necessary to investigate the precise functions of these cells during labor. However, similarly to B cells, NKT-like cells might also migrate selectively to the decidua during labor, resulting in a decrease in these cells in the peripheral blood.

Nevertheless, spontaneous vaginal deliveries and elective cesareans did not seem to have significantly different impacts on the absolute counts or percentages of the majority of B and T cell subsets in the peripheral blood. In fact, these results appear to be inconsistent with those of previous studies in humans, suggesting that Treg play a role in immunological changes that occur before labor and that human labor may be initiated by the decrease in Treg.[115,117] Several factors could explain our observations. First, it has been shown that a significantly higher percentage of activated T cells are present in the decidua rather than in peripheral blood as a result of the local expansion of these cells.[108] Thus, any significant variation in immune

cells might not be detected in maternal peripheral blood on the day of delivery. In addition, the study could be limited by the sample size of pregnant women who gave birth by vaginal delivery (n = 18) or elective cesarean (n = 14). Furthermore, recent research in humans has shown that absolute counts of Treg and estrogen levels increase progressively in the peripheral blood from early to late pregnancy[42] and decrease after delivery.[42,117] This phenomenon suggests that Tregs might play a role in the immunological changes that occur before delivery rather than during or immediately after labor. Accordingly, Breg were shown to expand in early pregnancy when immunosuppression is probably most necessary.[15] Therefore, we hypothesize that, like Treg, Breg are probably not directly involved in immunological mechanisms related to labor.

The main limitation of our study presented in chapter V was that there were significantly more nulliparous patients who delivered their newborns vaginally than by elective cesarean. Nonetheless, our sample was homogenous because we only included pregnant woman who had singleton pregnancies between 37 and 41 weeks of gestation. Furthermore, we used standardized protocols for the management of vaginal deliveries and elective cesareans, with no induction of labor or intravenous analgesia. Studies with larger sample sizes may be required to further investigate the impact of labor on the cells of the maternal immune system.

The results shown in chapter V together with those described in chapters III and IV provide important answers to our research questions. First, we found that the levels of the $CD24^{Hi}CD38^{Hi}$ Breg and Treg subsets also varied during the postpartum period, despite their best known involvement in the establishment of early pregnancy. Second, this variation was not due to labor, as evidenced by the absence of differences in circulating Breg and Treg subset levels between women with spontaneous vaginal labor and elective cesareans on the day of delivery. This finding indicates that labor alone does not influence the expansion of B cells and T cells, nor is a consequence of their suppression, bearing in mind that phenomena in the peripheral blood do not necessarily reflect those in the placenta. Nevertheless, labor has been likened to an immunological reaction that results in the recruitment of immune cells not only to the maternal-fetal interface but also to the peripheral blood.[98] Finally, the increase in the Breg and Treg subsets during the postpartum

period suggest a regulatory mechanism for the suppression of immune cell activation events after the day of delivery that is not triggered by labor "per se". These results might also explain the increased susceptibility to infections that occur during the postpartum period in healthy women.

In conclusion, the present results demonstrate that maternal immunomodulation also occurs during late pregnancy and postpartum period and is associated with an interplay between different B and T cell types, especially Breg and Treg. These findings may facilitate the future identification of new strategies for the diagnosis and treatment of pregnancy-associated disturbances, as well as mechanisms underlying maternal responses to infection and vaccination.

The use of maternal immunization to protect the health of the pregnant woman, her fetus, and the infant, has increased over the past decade. The efficacy of maternal vaccines relies on the secretion of antibodies at the maternal–fetal interface. The recent resurgence of pertussis and diphtheria has been reported in several industrialized countries with a high associated morbidity and mortality, occurring primarily in young infants.[169] These events have resulted in policy changes for vaccines aimed at protecting both mothers and their infants during the first months of life, and many health authorities are now recommending certain immunizations during pregnancy.[170] These new strategies have been reinforced by a recent study demonstrating that tetanus, diphtheria and pertussis vaccination during pregnancy increases antibody titers against diphtheria and pertussis, which may prevent neonatal pertussis infection.[171] In Portugal, it is actually recommended that pregnant women be vaccinated with a combined vaccine against pertussis, tetanus and diphtheria, ideally at 32 weeks. This vaccination scheme for pregnant women is planned for inclusion in the National Immunization Program for 2017.[172] According to health entities, the recommended optimal timing for this specific vaccination is between 27 and 36 weeks of gestation. In our study with normal pregnant women during their 3rd trimester, we identified quantitative changes in the peripheral B cell compartment while considering the different maturational stages. Thus, it is likely that the maternal response to vaccination during this critical window may differ between pregnant and non-pregnant women. Therefore, the impact of pregnancy-associated changes in the B cell compartment on humoral immune function should

be carefully clarified. These basic immunological insights can be useful for the improvement of maternal vaccination programs to increase their safety and efficiency.

Future perspectives

Further studies in the near future should include a larger sample size to ensure a more homogenous distribution between pregnant and non-pregnant women with respect to age and parity. In addition, it would be interesting to investigate samples from additional time points in the same individuals, such as during the first and second trimesters of pregnancy, as well as before becoming pregnant.

Because crucial immunological events are more likely to occur at the maternal-fetal interface,[2,49] it is important that future studies compare B and T cells in both peripheral blood and at the decidua in the same healthy pregnant women. The combination of these analyses would discriminate whether the variations in some immune cell populations result from their selective cellular migration out of the peripheral blood or from differences in differentiation events.

In future research, it will also be important to fully characterize the regulatory functions of the B and T cell subsets. Specifically, it will be essential to examine T cells expressing Foxp3 in relation to their regulatory activity because activated T cells without regulatory functions can also present a Foxp3-positive phenotype.[173]

Moreover, it will be interesting to determine whether the presence or absence of labor has different effects on the immune system of pregnant patients with autoimmune disorders and might eventually affect their clinical outcomes. In addition, it will be important to investigate whether the characterization of B cell subsets can facilitate the identification of risk markers for the development of obstetric complications in pregnant women with or without autoimmune diseases. In this context, it will also be important to clarify the role of B-cell activating factor (BAFF), an essential regulator of B cell maturation and function. Nevertheless, our group is currently undertaking a preliminary analysis of the BAFF levels in all of the samples collected during our study.

Several findings have supported the importance of female sex hormones, namely estrogen and progesterone, in the modulation of B cells and T cells during human pregnancy. Specifically, Xiong et al.[42] has shown that Treg subsets in the peripheral blood are positively correlated with estrogen levels, a relationship that might play an important immunomodulatory role during pregnancy. Another study recently demonstrated that membrane progesterone receptor alpha is present on Treg, suggesting an association between the immune system and the initiation of human labor.[119] Future research addressing the mechanism underlying the activation and modulation of Breg and Treg, mediated by hormones, could facilitate the design of new approaches for the treatment of disturbances related to pregnancy or autoimmune diseases.

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Note: **additional references for each study were placed in their respective chapter**

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