CD19\(^+\)CD5\(^+\) Cells as Indicators of Preeclampsia

Federico Jensen, Gerd Wallukat, Florian Herse, Oliver Budner, Tarek El-Mouslehi, Serban-Dan Costa, Ralf Dechend, Ana Claudia Zenclussen

Abstract—Preeclampsia is a devastating pregnancy-associated disorder affecting 5% to 8% of pregnant women worldwide. It emerges as an autoimmune-driven disease, and, among others, the autoantibodies against angiotensin type 1 receptor II have been proposed to account for preeclampsia symptoms. Despite much attention focused on describing autoantibodies associated with preeclampsia, there is no clue concerning the cell population producing them. CD19\(^+\)CD5\(^+\) B-1a B cells constitute the main source of natural and polyreactive antibodies, which can be directed against own structures. Here, we aimed to identify the B-cell subpopulation responsible for autoantibody production during preeclampsia and to study their regulation, as well as their possible use as markers for the disease. The frequency of CD19\(^+\)CD5\(^+\) cells in peripheral blood of preeclamptic patients is dramatically increased compared with normal pregnant women as analyzed by flow cytometry. This seems to be driven by the high human chorionic gonadotropin levels present in the serum and placenta supernatant of preeclamptic patients versus normal pregnant women. Not only \(\approx 95\%\) of CD19\(^+\)CD5\(^+\) cells express the human chorionic gonadotropin receptor, but these cells also expand on human chorionic gonadotropin stimulation in a lymphocyte culture. Most importantly, isolated CD19\(^+\)CD5\(^+\) cells produce autoantibodies against angiotensin type 1 receptor II, and CD19\(^+\)CD5\(^+\) cells were further detected in the placenta of preeclamptic but not of normal pregnancies where barely B cells are present. Our results identify a B-cell population able to produce pregnancy-pathological autoantibodies as possible markers for preeclampsia, which opens vast diagnostic and therapeutic applications. (Hypertension. 2012;59;00-00.) ● Online Data Supplement

Key Words: preeclampsia ● autoimmunity ● B-1a B cells ● autoantibodies ● AT\(_1\)-AA

Preeclampsia (PE) is a devastating human pregnancy-associated disorder characterized by the onset of hypertension, proteinuria, and edema after the 20th week of pregnancy. It represents one of the most frequent medical complications of pregnancy, affecting \(\approx 5\%\) to 8% of all pregnant women.\(^1\) Limited progress has been achieved in identifying the causes, being the induction of delivery the only available treatment for the clinical symptoms.\(^2\) Recently, the idea that PE may represent an autoimmune disease has emerged.\(^3\) This is based on the fact that many autoantibodies were identified in PE patients and suspected to account for the symptoms of the disease.\(^4\)–\(^6\)

Much attention was focused on autoantibodies against angiotensin type 1 receptor II (AT\(_1\)-AAs), because they have been detected in the serum of PE patients.\(^7\) These autoantibodies are able to bind to and to activate the AT\(_1\) angiotensin receptor, leading to the release of antiangiogenic factors from the placenta, which are reportedly involved in the origin of the disease.\(^8\)–\(^10\)

In spite of the efforts put on identifying autoantibodies related to PE, no attention has been so far focused on the cell population responsible for their production. This is a very important piece of information, because it may provide a novel target of actions in preventing the onset of the disease in patients at risk. B-1a B cells double positive for CD19 and CD5 are a distinctive population of B cells originated during the embryonic life and represent \(\approx 20\%\) of the circulating B cells during adulthood.\(^11\) CD19\(^+\)CD5\(^+\) cells are responsible for the production of so-called natural antibodies, which are characterized by their low-affinity, poly-reactivity and self-reactivity. B-1a B cells possess characteristics that reflect their strong link to autoimmunity.\(^12\) In fact, B-1a B levels have been shown to be elevated in several human autoimmune diseases, such as Sjögren syndrome,\(^13\) arthritis rheumatoid,\(^14\) lupus erythematosus,\(^15\)–\(^17\) and type 1 diabetes mellitus.\(^18\) In murine models, the active participation of these cells in autoantibody production and, thus, in the onset of the disease has been demonstrated (reviewed in Reference 17).
Whether B-1a B cells are linked to PE has not been investigated. The aim of this work was to analyze the dynamic of B-1a B cells in PE versus normal pregnancies and to study whether these cells are responsible for the production of AT1-AA.

Materials and Methods

**Human Subjects**

All of the experiments involving samples from human subjects were approved previously by the ethics committee of the Otto-von-Guericke University Medical Faculty (EK28/08 to A.C.Z.). All women and patients involved in this work were properly informed about the purpose of our research and gave their written consent before the sampling. The characteristics of the recruited participants are summarized in the Table, and further information is available in the online-only Data Supplement.

**Cell Staining and Flow Cytometry**

Leukocytes were stained for CD19 (PerCP), CD5 (APC; BD Biosciences), or immunoglobulin isotype (PerCP and APC) for 30 minutes at 4°C. After overnight fixation with paraformaldehyde 1%, cells were analyzed by flow cytometry (FACSCalibur). To avoid false-positive results from T cells expressing CD5, we gated on CD19\(^{+}\)/H11001 cells and then analyzed the percentage of CD5\(^{+}\)/H11001 cells within this population, as shown in Figure 1A. Data were analyzed by Cell-quest program (BD Bioscience) or FlowJo (www.flowjo.com).

**Cell Isolation and Culture**

Peripheral blood mononuclear cells were separated by Ficoll-density centrifugation.\(^{19}\) Lymphocytes from third-trimester normal pregnant (NP) or PE (5\(^{+}\)/H11003\(^{10^{5}}\)) were cultured for 24 hours at 37°C and 5% CO2 with RPMI medium added to penicillin/streptomycin 1% enriched with 10% of FBS, 10% of serum from NP women in the third trimester, or 10% of serum from PE patients. Cells were stained for CD19 and CD5 and analyzed as explained above.

### Table. Age and Gestational Age of the Study Participants, as Well as the Clinical Parameters Used to Diagnose PE

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Nonpregnant (n=10)</th>
<th>First Trimester (n=9)</th>
<th>Second Trimester (n=9)</th>
<th>Third Trimester (n=13)</th>
<th>PE (n=16)</th>
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<tr>
<td>Age</td>
<td>27.8±3.47</td>
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<td>30.5±6.92</td>
<td>28±5.43</td>
<td>31±6.34</td>
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<td>GA, wk</td>
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<td>32.2±4.6</td>
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<tr>
<td>GAD, wk</td>
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<td>171.4±16.41</td>
<td>100.57±8.6</td>
<td>32.5±3.51</td>
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<td>&lt;120</td>
<td>32.5</td>
<td>32.5</td>
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<tr>
<td>DBP</td>
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<td>100.57±8.6</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>—</td>
<td>+</td>
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</tbody>
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GA indicates gestational age; GAD, gestational age at delivery; SBP, systolic blood pressure; DBP, diastolic blood pressure; P, proteinuria; PE, preeclampsia.

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**Figure 1.** Peripheral levels of CD19\(^{+}\)CD5\(^{+}\) cells drop toward the third trimester in normotensive normally pregnant women, whereas they are very high in preeclamptic (PE) patients. A, Gate strategies for the analysis of CD19\(^{+}\)CD5\(^{+}\) cells. We first gated on the lymphocyte population and then, to avoid false-positive results because of T cells expressing CD5, a gate in the CD19\(^{+}\) population was defined. The percentage of CD5\(^{+}\) cells within the CD19\(^{+}\) population was analyzed. B, Levels of CD19\(^{+}\)CD5\(^{+}\) in peripheral blood of nonpregnant women; normotensive pregnant (NP) women at their first, second, and third trimesters; and in PE patients. Data are expressed as single dot, whereas means are indicated. \(^{*}P<0.05\), \(^{**}P<0.01\), and \(^{***}P<0.001\) as analyzed by the 1-way ANOVA, followed by a Tukey multiple comparison test.
Human Placenta Explants
Placenta explants from NP or PE patients were performed following a published protocol\textsuperscript{20} and cultured for 24 hours in RPMI medium containing penicillin/streptomycin (1\%) at 37°C and 5\% CO\textsubscript{2}.

Identification of CD19\(^+\) CD5\(^+\) Cells in Human Placentas
CD19\(^+\)CD5\(^+\) cells were detected in frozen placental tissues by immunofluorescence (NP: \(n=8\); PE: \(n=8\)).

ELISAs
Levels of BAFF protein in serum were measured by using a commercial ELISA kit (R&D System). Human chorionic gonadotropin (hCG) quantification was done by using an hCG ELISA kit (DRG Diagnostics). IgG and IgM levels in serum were measured by a homemade ELISA.

Quantification of AT\(_1\)-AA
AT\(_1\)-AA detection was carried out by cardiomyocyte contraction assay as described elsewhere.\textsuperscript{21}

Statistics
In Figure 1, data are presented as dots for every individual showing the mean. The data presented in the rest of the figure are shown as mean±SEM. Data were analyzed for statistical significance using Prism 5 software (GraphPad Software, Inc). Normality was assessed by Kolmogorov-Smirnov tests. Group differences were tested by 1-way ANOVA with Tukey test or Kruskal-Wallis test and Mann-Whitney tests. 2-tailed significance was considered significant and was the threshold to reject the null hypothesis. For more details of materials and methods please see the online Data Supplement.

Results
Frequency of CD19\(^+\)CD5\(^+\) Cells in Normotensive Pregnant Women Is Drastically Diminished at the Third Trimester Compared With the First and Second Trimester, Whereas it Is Very High in PE Patients
We investigated the frequency of CD19\(^+\)CD5\(^+\) cells, the main source of natural polyreactive antibodies,\textsuperscript{17} in NP women and in PE patients. We observed that the frequency of these cells in blood of women at the beginning of pregnancy (first and second trimester) is similar to the one observed in nonpregnant subjects (Figure 1B). However, samples from normotensive women with normally developing pregnancies at their third trimester presented significantly lower levels of CD19\(^+\)CD5\(^+\) cells as compared with the levels observed at any other time point of pregnancy (Figure 1B). Interestingly, in samples from PE patients, the levels of CD19\(^+\)CD5\(^+\) cells were not diminished when compared with NP women in the third trimester. They were even more elevated than in NP subjects in their second trimester (Figure 1B). Of note is the fact that the changes observed in the levels of CD19\(^+\)CD5\(^+\) cells were not a consequence of variations in the levels of total peripheral B cells, because CD19\(^+\) B-cell levels do not change during normotensive pregnancies or in PE patients (Figure S1, available in the Data Supplement). A deeper analysis of the CD19\(^+\)CD5\(^+\) cells revealed that they are further positive for CD27 and CD43 (Figure S2).

Factor(s) Present in the Serum of Pregnant Women Regulates the Levels of CD19\(^+\)CD5\(^+\) Cells In Vitro
After observing that the levels of CD19\(^+\)CD5\(^+\) cells within the lymphocyte population are very high in PE patients compared with NP women in the third trimester, we wondered whether the factor(s) present in the serum is responsible for this drastic difference. To address this, we isolated peripheral blood monocuclear cells from PE patients and cultured them in the absence or in the presence of autologous serum. We next cultured these cells with serum from normotensive NP women in the third trimester. We analyzed the proportion of CD19\(^+\)CD5\(^+\) cells within the lymphocyte population as we did in freshly obtained blood samples. Lymphocytes from PE patients cultured in the presence of FBS or autologous serum presented the same frequency of CD19\(^+\)CD5\(^+\) cells as at time point 0 (data not shown). Interestingly, the addition of serum from NP women provoked a diminution of the frequency of CD19\(^+\)CD5\(^+\) cells within the lymphocyte population after 24 hours of culture (Figure 2A). We next cultured lymphocytes of NP patients (third trimester) in the presence of FBS or autologous serum for 24 hours. Again, no differences could be observed regarding the levels of CD19\(^+\)CD5\(^+\) cells compared with time point 0 (data not shown). The addition of serum from PE patients, however, greatly increased the percentage of CD19\(^+\)CD5\(^+\) cells within the peripheral blood monocuclear cells (Figure 2B). This clearly shows that factors in the serum of PE patients are responsible for the augmented frequency of these cells in the lymphocyte population, which mirrors the observations made in freshly isolated cells (Figure 1B). One of the most prominent molecules released by the placenta and present in serum during pregnancy is hCG. This female hormone is exclusively produced by the trophoblast and shed to the circulation.\textsuperscript{22} Its levels increase rapidly after conception, peaking toward the second trimester and then dropping.\textsuperscript{23} High levels of hCG during pregnancy have been associated with PE,\textsuperscript{24} and we confirmed this (Figure 2C).

hCG Released by the Placenta Regulates the Levels of CD19\(^+\)CD5\(^+\) Cells In Vitro
The placenta is reportedly the organ responsible for the pathogenesis of PE as demonstrated by the fact that mola hidatiforme (placenta without conceptus) can lead to PE.\textsuperscript{25} We discarded that the classical B-cell survival factor BAFF, known to be elevated in autoimmune diseases,\textsuperscript{26} could be responsible for the augmentation in the frequency of CD19\(^+\)CD5\(^+\) cells in PE, because no differences in BAFF levels could be observed between NP women and PE patients (Figure S3). We, therefore, turned our attention to soluble factors released by the placenta. We collected supernatants from placenta explant cultures from term NP women or PE patients and used them for stimulating lymphocytes from PE patients. The addition of supernatant from NP placenta explants provoked a significant reduction in the levels of CD19\(^+\)CD5\(^+\) cells within the lymphocyte population as compared with supernatant from PE placenta explants (Figure 3A).
Similarly, placenta supernatant from PE patients induced an augmentation in the levels of CD19$^+$CD5$^+$ cells within the lymphocyte population as compared with supernatant from NP placenta explants (Figure 3B). This clearly confirms that a soluble factor released by the placenta may be responsible for the kinetics of CD19$^+$CD5$^+$ cells. Having shown that placentas from PE patients released higher levels of hCG than those explants from normotensive pregnant women (Figure

**Figure 2.** Factor(s) present in the serum of pregnant women control the levels of CD19$^+$CD5$^+$ cells in vitro. **A**, Isolated lymphocytes from preeclamptic (PE) patients were cultured for 24 hours in the presence of autologous serum or serum from normal pregnant (NP) women in the third trimester, and control cells were cultured with FBS. Afterward, the levels of CD19$^+$CD5$^+$ cells within the lymphocyte population were analyzed by flow cytometry. Serum from NP women in the third trimester induced a significant diminution in the percentage of CD19$^+$CD5$^+$ cells vs lymphocytes cultured with autologous (PE) serum. **B**, Lymphocytes from normotensive pregnant women in the third trimester were cultured for 24 hours in the presence of autologous serum or serum from PE patients or FBS as control. We observed a significantly augmentation in the levels of CD19$^+$CD5$^+$ cells when lymphocytes were cultured with serum from PE patients vs cells cultured with autologous serum. **C**, Levels of human chorionic gonadotropin (hCG; mIU/mL) in serum of NP women at the third trimester and PE patients. PE patients showed significantly higher levels of hCG vs NP women in the third trimester. Data are representative of 3 experiments and are expressed as mean±SEM. $^*$P<0.05, **P<0.01, and ***P<0.001 as analyzed by the Student t tests.

**Figure 3.** Human chorionic gonadotropin (hCG) released by placental tissue regulate the levels of CD19$^+$CD5$^+$ cells in vitro. Placenta explants from normal pregnant (NP) women and preeclamptic (PE) patients were performed. After 24 hours of culture, the supernatants were harvested and used to stimulate lymphocytes isolated from PE patients or NP women (A). Lymphocytes isolated from PE patients who have been cultured in the presence of supernatants from NP women’s placentas contain much lower CD19$^+$CD5$^+$ cell levels vs lymphocytes cultured with supernatant from PE placenta explants. **B**, Similarly, lymphocytes isolated from NP women in their third trimester and cultured with supernatant from PE’s placenta explants experienced an augmentation in the levels of CD19$^+$CD5$^+$ cells. **C**, hCG concentrations in supernatants of placenta explants from NP women or PE patients after 24 hours of culture. Placenta explants from PE patients released significantly higher levels of hCG than placenta explants from NP women. **D**, hCG induced in vitro an augmentation in the levels of CD19$^+$CD5$^+$ in lymphocytes isolated from nonpregnant women. The levels of CD19$^+$CD5$^+$ cells after 24 hours of culture were normalized with their levels at time 0 and the data expressed as fold increased vs time 0. The data are expressed as mean±SEM. $^*$P<0.05, **P<0.01, and ***P<0.001 as analyzed by the Student t tests.
late with PE symptoms, we wondered whether the levels of AT1-AA autoantibodies in our PE cohort. We observed that AT1-AA on the addition of 10% serum from PE patients to CD19
CD5 cells in placental tissues from normotensive pregnant patients. We performed immune colocalization of CD19 and CD5 cells at the fetal-maternal interface in PE
CD19
CD5 cells strongly express the hCG receptor (Figure S4), we next wondered whether hCG may account for the dynamic of CD19
CD5+ cells. In fact, we found that the addition of recombinant human hCG to peripheral blood mononuclear cells isolated from nonpregnant women provoked an augmentation in the levels of CD19
CD5+ cells (Figure 3D). This is indicative of hCG expanding or even activating CD19
CD5+ cells.

**CD19
CD5+ Cells Produce AT1-AA Antibodies**

Having observed that the levels of CD19
CD5+ cells correlate with PE symptoms, we wondered whether the levels of CD19
CD5+ cells were also associated with the presence of AT1-AA autoantibodies in our PE cohort. We observed that PE patients showed a very high prevalence of AT1-AA autoantibodies in serum, whereas in their counterpart, NP women (the third trimester), AT1-AA autoantibodies were almost absent (Figure 4A). This is not explained by different levels of total IgG or IgM in the serum of NP or PE patients, as shown in Figure S5. We confirmed that isolated CD19
CD5+ cells from nonpregnant donors produced AT1-AA on the addition of 10% serum from PE patients to the culture, whereas CD19
CD5− cells do not (Figure 4B).

**CD19
CD5+ Cells Were Found in the Placenta From Preeclamptic Patients But Not in NP Placentas**

It has been shown that B-1a B cells migrate from the periphery to the site of inflammation in inflammatory diseases. In this regard and taking into account the inflammatory nature of PE, we wondered whether CD19
CD5+ B-1a B cells can be found at the fetal-maternal interface in PE patients. We performed immune colocalization of CD19 and CD5 cells in placental tissues from normotensive pregnant women and PE patients. We were able to identify CD19
CD5+ B-1a B cells exclusively in placenta samples from PE patients (Figure 5A through 4D), whereas we failed to detect any B-1a B cell in placenta samples from NP women (Figure 5E through 5H). Negative control is shown in Figure S1 through 5L.

**Discussion**

In the last years, the concept of PE as an autoimmune disease has been introduced. This assertion is based on the fact that several autoantibodies were described to be present in PE patients. Among others, the autoantibodies against the AT1-AA have gained attention. Since Wallukat et al described the presence of AT1-AA in PE patients, many features of the disease has been explained by the ability of this autoantibodies to bind to and to activate the angiotensin receptor on a variety of cells. However, despite much information concerning the presence of autoantibodies in PE patients, no attention has so far been focused on the cells producing them. We focused our attention on the B-1a B cells. B1 unlike B2 cells, originate from a progenitor in the fetal liver and are able to self-replenish during adulthood. B-1a B cells are the primary source of natural antibodies. Natural antibodies are produced in the complete absence of antigenic stimulation, are polyreactive, and have been reported to cross-react with multiple antigens, including autoantigens. In fact, B-1a B cells and their produced polyreactive antibodies have been related to autoimmune diseases in humans, whereas their ability to cause autoimmunity has been demonstrated in mouse models of disease.

B-1a B cells were classically defined as CD19
CD5+ cells. Recently, and while this work was in the final stage of elaboration, an elegant article describing a new population (CD20+CD27−CD43+) of B1 cells in humans has arose. To highlight is the fact that 75% of these cells express CD5. On the other hand, more recently, it has been shown that human CD19
CD5+ B-1a B cells may be activated and produce autoantibodies.

We were able to show that both the amount of CD19
CD5+ cells and the levels of AT1-AA autoantibody strongly correlate with the onset of PE. We observed that the levels of CD19
CD5+ cells at the first and second trimester of NP are similar to those observed in nonpregnant women. The levels of CD19
CD5+ cells in normotensive women having normally progressing pregnancies in their third trimester are significantly lower compared with all of the other groups. Notably, PE patients presented elevated frequencies of CD19
CD5+ cells, which were even higher than the levels observed in the second trimester of pregnancy. This is not because of a total augmentation in the levels of CD19
B cells, because they were comparable among all of the groups. In several well-characterized autoimmune diseases, a similar augmentation in the levels of these cells compared with the healthy controls could be observed. To the best of our knowledge, no data are available in the literature regarding B-1a B cells in pregnancy. This interesting dynamic of CD19
CD5+ cells in pregnancy raises the question concerning the physiological relevance of this phenomenon. In this regard it is worth mentioning that immunoglobulins generated during the third trimester of pregnancy cross the placenta.
CD19\(^+\)CD5\(^+\) cells were present in the placenta from preeclamptic (PE) patients but not in placenta from normotensive pregnant (NP) women. Placentas from NP women (n=8) or from PE patients (n=8) were analyzed for the presence of double-positive CD19\(^+\)CD5\(^+\) B-1a B cells by immunofluorescence using specific antibodies. Representative pictures showing CD5\(^+\) cells (green), CD19\(^+\) cells (red), and double-positive cells (yellow) are showed (A through F; PE). We failed to detect CD19\(^+\)CD5\(^+\) double-positive cells in placentas from NP women (E through H). Negative controls are shown (I through L). Nuclei were stained with 4',6-diamidino-2-phenylindole (blue). Bars represent 100 \(\mu\)m.

and enter the fetal circulation. The transfer of immunoglobulin from mother to fetus is a naturally occurring process by which the mother confers immunity to the developing fetus. However, if the immunoglobulins crossing the placenta are potentially autoreactive, as the product of B-1a B cells are, this would have adverse consequences. We hypothesize that the observed diminution in the proportion of CD19\(^+\)CD5\(^+\) cells toward the third trimester in normotensive pregnant women may reflect a protective mechanism to prevent the presence of natural and polyreactive antibodies at the fetal-maternal interface, which would put the growing fetus at risk.

Very interestingly, AT\(_1\)-AA autoantibodies belong to the IgG3 subclass\(^3\) whose production is a hallmark of the humoral response to T-cell–independent type 2 antigens by B-1a B cells.\(^3\) It is tempting to speculate that these autoantibodies that are known to account for the onset of PE are related to CD19\(^+\)CD5\(^+\) cells. PE patients have very high levels of AT\(_1\)-AA, whereas the levels of these antibodies are very low or absent in normotensive pregnant women in their third trimester. Furthermore, we demonstrated that isolated CD19\(^+\)CD5\(^+\) but not CD19\(^+\)CD5\(^+\) cells produce AT\(_1\)-AA when cultured with serum from PE patients. We do not discard the possibility that these cells may also be related to others antibodies. In fact, Torricelli et al\(^4\) have recently reported a similar dynamic for autoantibodies against proteins S and C, in the serum of NP women and PE patients as we observed for CD19\(^+\)CD5\(^+\) cells.

Autoimmune diseases are mainly triggered by the production of autoantibodies by B cells. There are 2 different lineages of B cells, namely, B1 and B2 B cells. B2 cells are originated continuously by precursors present in the bone marrow and then migrate to the spleen, where they complete their maturation.\(^40\) Under nonpathological situations, those B cells that recognize autoantigens are likely to be eliminated. However, in many cases, clones of autoreactive B cells are capable to survive, complete their development, and expand producing autoantibodies that eventually may cause autoimmunity.\(^45\) The tumor necrosis factor family member BAFF plays a crucial role in the developmental progression of transitional B2 cells into the mature B-cell compartments and is required for B2 cell tolerance.\(^35\) In fact, elevated BAFF serum level was found in patients with autoimmune diseases. B2 B cells seems to be not involved in the production of AT\(_1\)-AA in PE, because no differences in the levels of BAFF were observed in the serum of NP women and PE patients. B1 B cell population, unlike B2, does not express BAFF receptor, and, therefore, BAFF is not required for B1-cell development and maintenance.\(^42,43\) This reinforces our hypothesis that autoantibodies found in PE patients are much more likely produced by B1 cells than B2 cells.

CD19\(^+\)CD5\(^+\) cell variations within the lymphocyte population were not related to the levels of total B cells. Our in vitro data indicate that the variations in CD19\(^+\)CD5\(^+\) cells strongly depend on soluble factors present in serum and secreted by the placenta. Pregnancy is strongly associated with changes in the hormonal status, and the placenta is responsible for the secretion of these hormones. The hCG is a glycoprotein produced mainly by the placenta and plays several roles in the maintenance of pregnancy.\(^44\) hCG is being
secreted immediately after pregnancy establishment, whereas its levels diminish toward the third trimester. However, high levels of hCG in PE patients have been described in contrast to NP, which we could confirm in this study. We also showed that placenta explants from PE patients released higher amounts of hCG than placenta explants from NP women. B cells have been shown to express hormone receptors and to respond to hormone treatments in vitro and in vivo. Indeed, we demonstrated here that CD19+CD5+ cells highly express the hCG receptor and expand in vitro on hCG influence. hCG emerges as a pivotal factor dictating the frequency of CD19+CD5+ cells within the lymphocytes as we could observe in vitro.

CD19+CD5+ cells able to produce autoantibodies are potentially dangerous for pregnancy, especially in the third trimester, because these immunoglobulins may have the ability to reach the placenta as we already discussed above. In PE patients, not only the production of AT1-AA by CD19+CD5+ cells was found, but also CD19+CD5+ cells were detected in placental tissue. In line with this, it has been shown that B-1a B cells in autoimmune diseases not only contribute to the onset of the pathology by the antibody production but also migrate to the sites of inflammation and are able to produce and secrete inflammatory, hazardous molecules, for example, cytokines, which will exacerbate the symptoms.

In summary, CD19+CD5+ cells emerge as a novel PE marker and their levels correlate with the disease, as well as with the levels of AT1-AA. Their frequency seems to be regulated by increased hCG levels secreted by the placenta during PE and present in the serum. Our data enormously lead to the onset of the pathology by the antibody production but also migrate to the sites of inflammation and are able to produce and secrete inflammatory, hazardous molecules, for example, cytokines, which will exacerbate the symptoms.

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Disclosures
None.

References


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Expanded Material and Methods

**Human subjects**
Peripheral blood samples were obtained from healthy non-pregnant female volunteers (n=10) as well as from first (n=9), second (n=9) or third trimester (n=13) healthy pregnant women (normal pregnancy=NP). It is worth to point that samples were not taken from the same donors. Blood from PE patients (n=16) was sampled as well. PE was clinically defined as hypertension (i.e., diastolic blood pressure of at least 90 mm Hg on at least two occasions 4 to 168 hours apart) and proteinuria, characterized as one of the following: urine dipstick results of at least 1+ (30 mg per deciliter) on at least two occasions 4 to 168 hours apart; a protein:creatinine ratio of at least 0.35; urine dipstick results of at least 2+ (100 mg per deciliter); or a 24-hour urine specimen containing at least 300 mg of protein.

**Cell staining and flow cytometry**
Peripheral blood samples were collected on 6 ml BD Vacutainer tubes containing EDTA and incubated with 50 ml of lysis buffer (1,5M NH4Cl, 100mM KHCO3, 0,9M EDTA) for 10 min at room temperature (RT). After washing the staining was performed.

**Cell characterization and culture**
CD19^+CD5^- and CD19^+CD5^+ cells were isolated by magnetic cell isolation of CD19^+ cells (MACS technology, untouched B cell isolation kit, Miltenyi Biotech) followed by CD5^+ isolation using an APC-CD5 antibody and anti-APC microbeads (Miltenyi Biotech). These cells were characterized in detail by flow cytometry using antibodies against CD27 (PerCP), CD43 (PE) (BD Biosciences, Germany).

**Expression of Lh/hCG receptor on CD19^+CD5^- cells**
The expression of Lh/hCG receptor on CD19+CD5+ isolated cells was analyzed by using a mouse anti human primary antibody (Luteinizing Hormone Receptor (C-term) ACRIS, Germany)) followed by a rabbit anti mouse FITC secondary antibody.

**Quantification of AT1-AA**

Single cardiomyocyte cells were dissociated from pieces of the heart ventricle with a 0.2% solution of trypsin and were cultured in SM20-1 medium equilibrated with humidified air and containing 10% heat inactivated neonatal calf serum (NCS) and 2µM flordeoxyuridine (Serva, Heidelberg, Germany). On the day of experiment the cells were incubated in 2ml fresh medium for 2 h. The experiments were performed on a heated desk (37°) of an inverted microscope. Six selected spontaneously beating cardiac cells or cell cluster per culture flask were counted for 15 s to measure the basal beating rate of the cells. The basal beating rate was 142 ± 11 beats /min. After the estimation of the basal beating rate the immunoglobulins isolated from serum of NP or PE patients were added in a dilution of 1:40. Similarly, supernatant of CD19+CD5+ or CD19+CD5- cell culture were analysed by this assay and used undiluted. After 1 to 2 hours the beating rate was measured again. The effects of the antagonist of the angiotensin II AT1 receptor irbesartan (1µM) was measured 5 min after addition.

**Production of AT1-AA by CD19+CD5+ isolated cells**

Isolated CD19+CD5+ or CD19+CD5- cells (5 x 10^5/ml) were cultured in the presence of 10% of PE serum. After 72 h of culture, supernatant was harvested and AT1-AA levels were measured as described below.

**In vitro hCG treatment**

For understanding how hCG modifies the CD19+CD5+ population within the PBMCs, we cultured them with human recombinant hCG (100 IU/min; Pregnyl, Schering-Plough) for 24h afterward the percentage of CD19+CD5+ population within the PBMCs was analyzed by flow cytometry.

**Identification of CD19+ CD5+ cells in human placentas**

Placental tissues were obtained from NP women having vaginal delivery, gestational age at delivery (38± 1,41 weeks) or PE patients having c-section, gestational age at delivery (31± 3,8 weeks). Samples were fixed in iced cold acetone for 10 min. and the endogenous Fc receptors were blocked with a solution of 10% FBS + 10% BSA in PBS for 2 h at RT. Primary antibodies (mouse anti-human CD19 and rabbit anti-human CD5; Dako Cytomation, Germany) were used at a 1:50 dilution. Secondary antibodies (goat anti rabbit IgG FITC (Green) and goat anti mouse IgG Alexa Fluor 555 (Red) (Invitrogen, Germany) were applied at a 1:1000 dilution. Nuclei were counterstained with 4’,6-diamidino-2-phenylindole (DAPI) for their visualization. Slides were mounted with 4’, 6-diamidino-2-phenylindole “Vectashield” mounting medium (Vector laboratories, Germany) and analyzed under the Axio observer A1 fluorescent microscope (Zeizz, Germany). Pictures were taken by using the Axiocam MRe camera (Zeizz, Germany) and analyzed with Axiovision Rel.4.6 program (Zeizz, Germany) program. Green (CD5), red (CD19) or co-localization (yellow, overlapping, CD5+CD19+) were documented and analyzed. Negative controls were performed by replacing the first antibody with diluted mouse and rabbit serum.

**IgG and IgM home made ELISA**
IgG and IgM levels in serum were measured by a home-made ELISA by using a rabbit anti human IgG and IgM (Dako, Germany) capture antibodies (1:600) and rabbit anti human IgG-HRP, rabbit anti human IgM-HRP (Dako, Germany) as detecting antibodies (1:2000).
Figure S1. CD19+ cells (total B cell population) in peripheral blood of normal pregnant women and pre-eclamptic patients. Comparable numbers of total B cells (CD19+ cells) in NP and PE. Data are expressed as single dots, whereas means are indicated. No statistical significant differences were found after analyzing the data with the one-way analysis of variance.
Figure S2. Characterization of isolated CD19^+CD5^+ B cells. CD19^+CD5^+ and CD19^+CD5^- B cells were isolated from peripheral blood of pregnant and non-pregnant women as described in M&M. Cells were further stained for CD27 and CD43 B1 markers. Both, CD19^+CD5^+ and CD19^+CD5^- cells express similar levels of CD27. However, CD19^+CD5^+ CD27 gated cells express higher levels of CD43 (~30%) compared to CD19^+CD5^- CD27 gated cells (~4%).
Figure S3. Levels of B cell activating factor (BAFF) in the serum of normal pregnant women (3rd trimester) and pre-eclamptic patients. BAFF levels in serum of patients suffering from pre-eclampsia do not differ from the levels observed in normal pregnant women. No statistical significant differences were found after analyzing the data with the Mann-Whitney-U-Test.
Figure S4. HCG receptor expression in CD19+CD5+ Cells. Isolated CD19+CD5+ B cells express high levels of hCG receptor. The expression levels of HCG receptor was analyzed by flow cytometry and confirmed by immunofluorescence.
Figure S5. Levels of IgG and IgM in serum from NP and PE patients. The levels of total IgM (A) and IgG (B) in serum from pre-eclamptic patients do not differ from those observed in normotensive pregnant women at their third trimester. No statistical significant differences were found after analyzing the data with Mann-Whitney-U-Test.