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## Peripheral CD300a+CD8+ T Lymphocytes with a Distinct Cytotoxic Molecular Signature Increase in Pregnant Women with Chronic Chorioamnionitis

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### Abstract

**Problem**—CD300a is an immunomodulatory molecule of the immunoglobulin receptor superfamily expressed in the leukocytes of myeloid and lymphoid lineages. However, its biological function on CD8+ T lymphocytes remains largely unknown. This study was conducted to assess the biological significance of CD300a expression in T lymphocytes and to determine whether its expression in peripheral T lymphocytes changes in pregnant women presenting with anti-fetal rejection.

**Methods of Study**—Microarray analysis was performed using total RNA isolated from peripheral CD300a+ and CD300a– T lymphocytes. Flow cytometric analysis of the peripheral blood samples of pregnant women and pathologic examination of the placentas were conducted.

**Results**—A large number of genes ( $N = 1,245$ ) were differentially expressed between CD300a– and CD300a+ subsets of CD8+ T lymphocytes, which included CCR7, CD244, CX3CR1, GLNY, GZMB, GZMK, IL15, ITGB1, KLRG1, PRF1, and SLAMF7. Gene Ontology analysis of differentially expressed genes demonstrated enrichment of biological processes such as immune response, cell death, and signal transduction. CD300a expression in CD8+ T lymphocytes was coupled to a more cytotoxic molecular signature. Of note, the proportion of CD300a+CD8+ T lymphocytes increased in pregnant women with chronic chorioamnionitis (anti-fetal rejection of the chorioamniotic membranes;  $P < 0.05$ ).

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### Disclosure of Conflict of Interest

The authors declare no conflict of interest.

**Conclusion**—The findings of this study strongly suggest an increase of systemic T lymphocyte-mediated cytotoxicity in pregnant women with chronic chorioamnionitis as a manifestation of maternal anti-fetal rejection.

### Keywords

placenta; maternal anti-fetal rejection; prematurity; preterm birth; transcriptome

### Introduction

Tolerance of the fetus by the mother during pregnancy is essential for viviparity.<sup>1, 2</sup> Biological characteristics such as separation of maternal circulation from fetal circulation<sup>3, 4</sup> and expression of non-polymorphic HLA-G on trophoblasts<sup>5</sup> are well-established mechanisms protective of the fetus. In addition, a large body of evidence indicates that the maternal immune system also undergoes alterations to avoid rejection of the semi-allogeneic fetus, and a role for regulatory T lymphocytes has been emphasized recently.<sup>6–8</sup> As T lymphocytes play a key role in immune response, many investigators have studied changes of peripheral T lymphocyte subsets in the context of maternal tolerance of the fetus.<sup>9–14</sup> Several studies have noted that the proportion of peripheral CD8+ T lymphocytes either increases or does not change during pregnancy,<sup>9–11, 13</sup> while the absolute number of CD3+, CD4+, and CD8+ T lymphocytes decreases during pregnancy.<sup>12–14</sup> There are also other reports showing that the expression of activation markers such as HLA-DR and CD25 in T lymphocytes changes during pregnancy.<sup>9, 12</sup> These observations overall strongly suggest that pregnancy is associated with changes involving peripheral T lymphocyte numbers and activation status.

CD300a is a leukocyte surface molecule of the immunoglobulin receptor (IgR) superfamily. It has extracellular V-type Ig-like domains and a cytoplasmic domain with immunoreceptor tyrosine-based inhibitory motifs (ITIM). CD300a can be detected on the surface of various leukocyte populations such as monocytes, macrophages, granulocytes, dendritic cells, NK cells, and T and B lymphocytes.<sup>15, 16</sup> Among CD4+ T lymphocytes, CD300a expression is mainly restricted to Th1 memory and effector cells.<sup>17</sup> IFN $\gamma$ -producing CD4+ T lymphocytes are enriched in CD300a+ expressing cells, and stimulated CD4+ T lymphocytes producing TNF $\alpha$  and IL-2 are also largely positive for CD300a. Marked up-regulation of T-box transcription factor Eomesodermin (Eomes) is another characteristic of stimulated CD300a+CD4+ T lymphocytes.<sup>18</sup> However, the biological significance of CD300a expression on CD8+ T lymphocytes remains largely unknown.

Villitis of unknown etiology is a destructive inflammation of the placenta and a consequence of maternal anti-fetal rejection.<sup>19</sup> Infiltration of maternal CD8+ T lymphocytes into the fetal chorionic villi is its characteristic feature, and we have found increased expression of CD300a in villitis of unknown etiology. Increased expression of CD300a in villitis of unknown etiology led us to think that CD300a-expressing CD8+ cells represent a functionally distinct subset of T lymphocytes involved in maternal immune response against the fetus during pregnancy.

This study was performed to assess the biological meaning of CD300a expression in CD8+ T lymphocytes using gene expression profiling. A further flow cytometric analysis of peripheral blood samples from pregnant women was conducted to determine whether the expression of CD300a in T lymphocytes changes in women presenting with anti-fetal rejection.

## Materials and methods

### Patient Samples

Whole peripheral blood was collected by venipuncture from healthy non-pregnant women ( $N=11$ ) and normal pregnant women ( $N=40$ ). All normal pregnant women eventually delivered babies at term ( $37^{+0} \sim 41^{+6}$  weeks) without any maternal and neonatal complications such as intra-amniotic infection, hypertensive disorders, diabetes mellitus, and small-for-gestational-age neonates. Cord blood ( $N=17$ ) was collected from the umbilical vein during delivery; both maternal and cord blood samples were obtained in two cases. All patients provided written informed consent, and the collection and use of the samples were approved by the Institutional Review Boards of the participating institutions.

### Cell Isolation/Sorting

Peripheral blood mononuclear cells (PBMCs) were isolated from samples of the peripheral blood of healthy non-pregnant women by discontinuous density gradient centrifugation using Histopaque 1077 and Histopaque 1119 (Sigma-Aldrich, St. Louis, MO, USA), and labeled with CD300a-PE (Beckman Coulter, Miami, FL, USA), CD3-FITC (BD Biosciences, San Jose, CA, USA), CD4-APC (BD Biosciences), and CD8-APC-CY7 (BD Biosciences). CD4+CD300a-, CD4+CD300a+, CD8+CD300a-, and CD8+CD300a+ T lymphocyte subsets were sorted using a FACSaria III cell sorter (BD Biosciences). The purity of sorted cells ranged from 80.3 to 98.4%.

### Microarray Analysis

Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. DNase-treated total RNA (500 ng) was amplified and biotin-labeled with the Illumina TotalPrep RNA Amplification Kit (Ambion, Austin, TX, USA). Labeled cRNAs were hybridized to Illumina's HumanHT-12 Expression BeadChip (Illumina, San Diego, CA, USA). BeadChips were imaged using a BeadArray Reader, and raw data were obtained with BeadStudio Software V.3.4.0 (Illumina). Gene expression data was quantile normalized,<sup>20</sup> probes non-detected in at least three samples were removed, and differential expression was tested using a paired moderated t-test.<sup>21</sup> A threshold of 5% on the False Discovery Rate<sup>22</sup> adjusted  $P$  values (called  $q$ -values) together with a minimum of 1.5-fold change was used to determine significance. Gene ontology analysis was performed using enrichment methods previously described<sup>23, 24</sup> while pathway analysis was conducted with the Signaling Pathway Impact Analysis (SPIA) method.<sup>25, 26</sup> The complete data set is available in a MIAME (Minimal Information about a Microarray Experiment) compliant format in ArrayExpress (<http://www.ebi.ac.uk/arrayexpress/>) database (entry ID: E-TABM-902).

### Real-time Quantitative RT-PCR (qRT-PCR)

Reverse transcription of total RNA was done using SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) and oligo(dT) primers. qRT-PCR analyses were performed with TaqMan gene expression assays (CCL5, Hs00174575\_m1; CCR7, Hs99999080\_m1; CD244, Hs00175569\_m1; CD300a, Hs00381974\_m1; CX3CR1, Hs00365842\_m1; GNLY, Hs00246266\_m1; GZMB, Hs00188051\_m1; GZMK, Hs00157878\_m1; INFG, Hs99999041\_m1; IL15, Hs99999039\_m1; ITGB1, Hs01127543\_m1; KLRG1, 00929964\_m1; PRF1, Hs00169473\_m1; SLAMF7, Hs00221793\_m1; Applied Biosystems, Austin, TX, USA) using a Biomark System (Fluidigm, South San Francisco, CA, USA). The human ribosomal protein, large, P0 (RPLP0; Applied Biosystems) was used for normalization.

## Flow Cytometry

Peripheral blood in EDTA (100  $\mu$ l) was incubated with a combination of CD300a-PE, CD3-FITC, CD45RA-FITC (BD Biosciences), CCR7-PE-CY7 (BD Biosciences), CD4-APC, and CD8-APC-CY7. Analyses were performed with the BD FACS LSR II flow cytometer and BD FACSDiva software (BD Biosciences). For intracellular staining of granzyme A, granzyme B, and perforin, 100  $\mu$ l of whole blood were first labeled with antibodies against surface antigens [CX3CR1-FITC (Biolegend, San Diego, CA, USA), CD300a-PE, CD3-PE-CY7 (BD Biosciences), and CD4-APC-CY7 (Biolegend) or CD8-APC-CY7]. After lysis with 1X FACS Lysing Solution (BD Biosciences) and fixation/permeabilization with Fixation/Permeabilization solution (BD Biosciences), the cells were stained with Granzyme A-Alexa Fluor 700 (Biolegend), Granzyme B-Alexa Fluor 647 (Biolegend), and Perforin-PerCp-Cy5.5 (Biolegend).

## Placental Pathology

Four hematoxylin-and-eosin stained sections (two full-thickness sections of the placental disc, one umbilical cord section, and one roll of chorioamniotic membranes) were reviewed for the presence of placental pathologic findings according to the criteria proposed by the Perinatal Section of the Society for Pediatric Pathology. Placental histological changes were categorized as those associated with amniotic fluid infection (acute chorioamnionitis), maternal vascular underperfusion, and fetal vascular obstruction.<sup>27–30</sup> Villitis of unknown etiology, chronic deciduitis, and chronic chorioamnionitis were diagnosed according to previously described criteria.<sup>19, 31, 32</sup>

## Statistical Analysis

For continuous variables, distributions were examined for normality using Kolmogorov-Smirnov tests. When data were far from normality, the Mann-Whitney U test was performed for independent variables, and the Wilcoxon signed rank test was used for related variables. Statistical analyses were performed using SPSS Version 19.0 (SPSS, Inc., Chicago, IL, USA). All *P* values were two-sided, and a value of *P* < 0.05 was considered to be statistically significant.

## Results

### Transcriptome of CD300a+ and CD300a– T Lymphocytes

Microarray analyses of CD300a+CD8+ T lymphocytes and CD300a–CD8+ T lymphocytes from peripheral blood of five healthy adult women revealed differential regulation of 1,245 genes out of more than 25,000 annotated genes on the array (adjusted *P* value < 0.05, fold change > 1.5) (Figure 1a, 1c). Gene Ontology (GO) analysis showed enrichment of 141 biological processes, largely related to immune process, cell death, and signal transduction (Table I). One hundred thirty-six genes were differentially expressed among 613 genes associated with the immune system process (Table II). The impact evidence captured by SPIA from signaling perturbations and enrichment revealed eight pathways such as cytotoxicity and cytokine-cytokine receptor interaction (Table I).

On the contrary, a relatively smaller number of genes (123 genes) was found to be differentially expressed between CD300a+ and CD300a–CD4+ T lymphocytes than in CD8+ T lymphocytes (Figure 1b, 1c). On GO analysis, 63 biological processes were enriched including signal transduction, immune response, and cell communication, which were similar to those for CD300a+CD8+ T lymphocytes (Table III). Among the genes related to the immune process, 16 genes were differentially expressed in CD300a+CD4+ T lymphocytes (Table IV). Cytokine-cytokine receptor interaction was found to be enriched by the SPIA pathway analysis.

Among the differentially expressed genes between CD300a+CD4+ and CD300a-CD4+ T lymphocytes, 87 genes were regulated in the same direction as in the comparison between CD300a+CD8+ and CD300a-CD8+ T lymphocytes. The gene FCRL3, down-regulated in CD300a+CD4+ T lymphocytes compared to CD300a-CD4+ T lymphocytes, was found to be up-regulated in CD300a+CD8+ compared to CD300a-CD8+ T lymphocytes (Figure 1d).

### Differential mRNA Expression of Immune-related Genes between CD300a+ and CD300a-CD8+ T Lymphocytes

**Chemokines/Cytokines**—Among chemokine genes, CCL3, CCL5, and CCL20 showed an increased mRNA expression in CD300a+CD8+ T lymphocytes, whereas mRNA expression of CXCL16 was decreased in CD300a+CD8+ T lymphocytes compared to CD300a-CD8+ T lymphocytes. mRNA expression of some cytokines of the hematopoietin family, including interleukins, were up-regulated in CD300a+CD8+ T lymphocytes. These included IL15 sharing certain biological activities with IL-2 such as T and NK cell activation and proliferation; IL29, a member of the IL-10 family; and CLCF1, a member of the IL-6 family. mRNA expression of several members of the TNF family (TNF, TNFSF9, TNFSF10, TNFSF12, TNFSF14, FASLG, and CD40LG) was up-regulated, while mRNA expression of another member of the TNF family, LTB, was down-regulated in CD300a+CD8+ T lymphocytes compared to CD300a-CD8+ T lymphocytes. Among the members of the interferon family, mRNA expression of interferon-gamma (IFNG), interferon gamma-inducible protein 16 (IFI16), and interferon-induced protein with tetratricopeptide repeat 2 (IFIT2), was up-regulated in CD300a+CD8+ T lymphocytes, while mRNA expression of interferon-induced transmembrane protein 3 (IFITM3) was down-regulated.

**Chemokine/Cytokine Receptors**—Among chemokine receptor genes, mRNA expression of CXCR3 (a receptor for CXCL9, -10, and -11), CXCR6 (a receptor for CXCL16), CX3CR1 (a receptor for CX3CL1), and CCR6 (a receptor for CCL20) was up-regulated in CD300a+CD8+ T lymphocytes compared to CD300a- cells, while mRNA expression of CCR7 (a receptor for CCL19 and -21) was down-regulated. Regarding interleukin receptor genes, mRNA expression of IL10RA, IL12RB1, IL18R1, IL18RAP, and IL28RA was up-regulated in CD300a+CD8+ T lymphocytes, while mRNA expression of IL6R and IL11RA were down-regulated. Among TNF receptor genes, mRNA up-regulation of TNFRSF1B, TNFRSF4, FAS, and TNFRSF9 was observed in CD300+CD8+ T lymphocytes, whereas mRNA expression of CD27 (TNFRSF7), TNFRSF25, and ectodysplasin A receptor (EDAR) was down-regulated. mRNA expression of interferon gamma receptor 2 (IFNGR2) and TGFBR2 was down-regulated in the CD300+ subset.

**Effector Molecules for Cytotoxicity**—One of the major effector functions of cytotoxic T lymphocytes is secretion of cytotoxic granules to induce apoptosis of target cells. mRNA expression of the major components of the granzyme genes (GZMA, GZMB, and GZMK) was up-regulated in CD300+CD8+ T lymphocytes. mRNAs of granulysin (GNLY) and perforin (PRF1) were also highly expressed in CD300+CD8+ T lymphocytes.

**Killer Cell Receptors**—CD300+CD8+ T lymphocytes showed the enriched mRNA expression of killer cell receptors. A subset of killer cell immunoglobulin-like receptors (KIR2DL1, -2, -3, -4, -5A, KIR2DS5, KIR3DL1, -2, and -3) and killer cell lectin-like receptors (KLRA1, -B1, -C1, -C3, -D1, -F1, and -G1) showed increased mRNA expression in CD300a+CD8+ T lymphocytes. Among inhibitory killer cell receptors, mRNA expression of KIR3DL1, KIR3DL2, KIR2DL, and KLRC1/KLRD1 (CD94) were up-regulated. Among activating killer cell receptors, mRNA expression of KIR2DS and KLRC3/KLRD1 (CD94) were up-regulated. mRNA expression of NCR3 (NKp30) and CD244, among other membrane receptors, was also upregulated.

### Differential mRNA Expression of Immune-related Genes between CD300a+CD4+ and CD300a-CD4+ T Lymphocytes

Among genes related to the immune process, 16 genes were differentially expressed in CD300a+CD4+ T lymphocytes (Table IV). Among the differentially expressed immune-related genes between CD300a+CD4+ and CD300a-CD4+ T lymphocytes, 12 genes were regulated in the same direction as those expressed differentially between CD300a+CD8+ and CD300a-CD8+ T lymphocytes: CD300a, IL15, CCL5, TIMP1, TNFRSF4, CD226, GZMA, IFNG, TNFSF14, APOBEC3G, CFH, and CTSW. Four genes differentially expressed only in CD300a+CD4+ T lymphocytes, but not in CD300a+CD8+ T lymphocytes, were CTLA4, OSM, GPR44, and TNFSF13B. CTLA4 was down-regulated but the others were up-regulated in CD300a+CD4+ T lymphocytes compared to CD300a-CD4+ T lymphocytes.

### Validation of Differential Expression of Immune-related Genes in CD300a+ T Lymphocytes

For validation of the microarray results, the differential mRNA expressions of the selected genes (CCL5, CCR7, CD244, CD300a, CX3CR1, GLNY, GZMB, GZMK, IFNG, IL15, ITGB, KLRG1, PRF1, and SLAMF7), related to cytotoxicity and cytokine-cytokine receptor interaction, between peripheral CD300a+ and CD300a-CD8+ T lymphocytes ( $N = 9$ ), were analyzed by qRT-PCR. All genes other than CCR7 were expressed significantly higher in CD300a+CD8+ T lymphocytes than in CD300a-CD8+ T lymphocytes, while the expression of CCR7 was lower in CD300a+CD8+ T lymphocytes than in CD300a-CD8+ T lymphocytes, which confirmed the microarray analysis (Figure 2).

Granzyme A, granzyme B, perforin, and CX3CR1 protein expressions in peripheral CD300a+ and CD300a-CD8+ T lymphocytes ( $N = 30$ ) were also assessed by flow cytometry, all of which were expressed higher in the CD300a+ sub-population than in the CD300a- sub-population (Figure 3).

### CD300a+ T Lymphocytes in Peripheral Blood of Pregnant Women

The demographic characteristics of the study population are shown in Table V. On flow cytometric analysis of peripheral blood from pregnant women ( $N = 19$ ), CD300a was detected in 26.6% (5.9–65.2%) of CD8+ lymphocytes and in 18.3% (8.4–67.3%) of CD4+ T lymphocytes. When evaluated in subsets of Tc and Th lymphocytes by co-labeling for CD45RA and CCR7,<sup>33</sup> the proportion of CD300a+ cells was greater in the order of effector, effector memory, central memory, and naïve T lymphocytes ( $N = 40$ , Figure 4a, 4b). The proportion of CD300a+ T lymphocytes was not different between preterm and term pregnant women [preterm ( $N = 7$ ) vs. term ( $N = 12$ ): CD8+, 30.4% (11.7–41.6%) vs. 19.7% (5.9–65.2%); and CD4+, 21.9% (14.0–67.3%) vs. 18.3% (8.4–48.1%)]. The proportions of CD300a+ T lymphocytes in cord blood of neonates ( $N = 17$ ) were quite small [CD8+, 7.3% (1.4–17.9%); CD4+, 6.1% (1.3–18.1%)], compared to those in adults ( $P < 0.001$ ).

We analyzed the proportion of the CD300a+ subset of T lymphocytes in samples of the peripheral blood of pregnant women according to placental pathology findings: intra-amniotic infection, maternal vascular underperfusion, fetal vascular thrombo-occlusive diseases, and chronic placental inflammation (villitis of unknown etiology, chronic chorioamnionitis, and chronic deciduitis). The median sampling to delivery interval was 1 day (range: 0–194 days). The proportion of CD300a+CD8+ T lymphocytes was higher in cases with chronic chorioamnionitis than in those without chorioamnionitis (Figure 4c). When evaluated in subsets of Tc and Th lymphocytes by co-labeling for CD45RA and CCR7,<sup>33</sup> the proportion of CD300a+ cells was greater in the effector memory and the central memory subsets of CD8+ T lymphocytes but not in either effector or naïve subsets

(Figure 4e). The other placental findings were not associated with changes in CD300a+ peripheral T lymphocytes.

## Discussion

This study reports the transcriptome of CD300a+CD8+ and CD300a+CD4+ T lymphocytes for the first time. The principal findings of this study are: 1) CD300a expression defines a distinct sub-population of CD8+ T lymphocytes; 2) the gene expression profile of CD300a+CD8+ T lymphocytes is consistent with a more cytotoxic phenotype than that of CD300a-CD8+ T lymphocytes; and 3) the proportion of CD300a+ peripheral Tc lymphocytes is significantly increased in pregnant women with chronic chorioamnionitis (anti-fetal cellular rejection of the chorioamniotic membranes) than in women without chronic chorioamnionitis.

### Significance of CD300a Expression in CD8+T Lymphocytes

CD300a is constitutively expressed in granulocytes, NK cells, and dendritic cells, in which it functions as an inhibitory receptor.<sup>34–39</sup> Co-ligation of CD300a exerts an inhibitory effect on CD32a-mediated signaling in neutrophils.<sup>34</sup> In mast cells, CD300a inhibits kit-mediated SCF-induced signaling to cause impairment of differentiation, survival, and activation.<sup>35</sup> CD300a also suppresses eotaxin-dependent transmigration of eosinophils and the anti-apoptotic effects of IL-5 and GM-CSF.<sup>39</sup> CD300a cross-linking inhibits the cytotoxicity of NK cells.<sup>36, 38</sup> In dendritic cells, CD300a inhibits TNF- $\alpha$  production from activated plasmacytoid dendritic cells and up-regulates type I IFN production.<sup>37</sup> In contrast to non-lymphocytic leukocytes, only a subset of B and T lymphocytes was found to express CD300a.<sup>16–18</sup>

In this study, the majority of CD300a+CD8+ T lymphocytes were memory and effector cells as was the case with CD4+ T lymphocytes.<sup>17</sup> Fetal T lymphocytes, most of which are of a naïve subset, were almost negative for CD300a. Interestingly, far more genes were differentially expressed between CD300a+CD8+ and CD300a-CD8+ T lymphocytes than between CD300a+CD4+ and CD300a-CD4+ T lymphocytes. This suggests that CD300a is biologically more significant in CD8+ T lymphocytes than in CD4+ T lymphocytes. This is in contrast to the comparison of the transcriptional profiles between activated CD4+ T lymphocytes and CD8+ T lymphocytes, each group of which showed similar numbers of differentially expressed genes.<sup>40</sup>

CD300a+CD8+ T lymphocytes had differential expressions of cytokine/chemokine and related genes. CCL3 and CCL20 were up-regulated upon T lymphocyte activation and responsible for the pro-inflammatory response of T lymphocytes, while CCL5 was highly expressed in resting T lymphocytes, suggesting its importance in the homeostasis of resting T lymphocytes.<sup>40</sup> CXCL16 and CXCR6 are examples of co-expression of ligand and its receptor in the same cell, and activation of T lymphocytes is known to lead to marked down-regulation of CXCL16.<sup>41</sup> In addition, IFNG, a major effector cytokine of T lymphocytes, is up-regulated in CD300a+CD8+ T lymphocytes. Several key effector molecules for cytotoxicity were also up-regulated in CD300a+CD8+ T lymphocytes. CD300a+CD8+ T lymphocytes expressed GZMA, GZMB, and GZMK. GZMB is specific for effector-type cells, while GZMK is found mainly in a subset of memory-type cells. GZMA is highly expressed on primed CD8+ T lymphocytes regardless of differentiation status. High expression of CX3CR1 is also compatible with the features of peripheral cytotoxic effector CD8+ T lymphocytes with intracellular perforin and granzyme B.<sup>42</sup>

## Differential Expression of Immune-related Genes in CD300a+CD4 T Lymphocytes

A certain number of the genes ( $N=87$ ) shared by CD300a+CD4+ and CD300a+CD8+ T lymphocytes reflect common cellular events shared by CD4+ and CD8+ T lymphocytes in the CD300a+ sub-population. Four immune process-related genes differentially expressed only in CD300a+CD4+ T lymphocytes, but not in CD300a+CD8+ T lymphocytes, were CTLA4, OSM, GPR44, and TNFSF13B. CTLA4 is a co-stimulatory molecule expressed by activated T lymphocytes and binds to B7-1 and B7-2 on antigen-presenting cells. CTLA4 transmits an inhibitory signal to T lymphocytes.<sup>43</sup> Oncostatin M encoded by OSM is a member of the IL-6 family acting as a growth regulator<sup>44</sup> and as an inflammatory mediator.<sup>45</sup> Th1 lymphocytes regulate hematopoietic progenitor cell homeostasis by production of oncostatin M.<sup>46</sup> GPR44 encoding G-protein-coupled receptor 44 is selectively expressed in Th2 cells.<sup>47</sup> TNFSF13B encodes a cytokine of the tumor necrosis factor (TNF) ligand family, which is a ligand for receptors TNFRSF13B/TACI, TNFRSF17/BCMA, and TNFRSF13C/BAFFR.

## Changes in T Lymphocyte Immune Responses during Pregnancy

Most studies about T lymphocyte tolerance of the fetal allograft have been done mainly about T-helper cells.<sup>48, 49</sup> In earlier studies, maternal tolerance toward the fetal allograft was explained by the predominant Th2 immunity over Th1 immunity.<sup>50</sup> The Th1/Th2 theory is now blending into the Th1/Th2/Th17/Treg paradigm, and the role of Treg cells is increasingly emphasized.<sup>6, 51, 52</sup>

In contrast, studies about the changes of cytotoxic T lymphocytes during normal pregnancy are limited.<sup>9-14</sup> Alteration of cytotoxic T lymphocytes in pregnancy-related complications such as implantation failures and preeclampsia supports the significance of cytotoxic T lymphocytes for the successful maintenance of normal pregnancy.<sup>53-55</sup> Yang et al. showed increased peripheral CD8+ T lymphocyte activation in women with multiple implantation failures and recurrent pregnancy losses. The proportion of activated T lymphocytes was inversely correlated with IFN $\gamma$ /IL-10 producing CD3+/CD4+ T lymphocyte ratios.<sup>54</sup> De Groot et al. reported that women with preeclampsia had a higher partner-specific cytotoxic T-lymphocyte precursor frequency when compared to women with uncomplicated pregnancies, suggesting an increased cytotoxic T-cell response to paternal antigens in women with preeclampsia.<sup>53</sup>

Chronic chorioamnionitis is a common placental lesion found in cases of late preterm birth, and represents maternal anti-fetal cellular rejection involving the chorioamniotic membranes.<sup>32, 56, 57</sup> We demonstrated in this study that CD300a+CD8+ T lymphocytes are mainly cytotoxic effector cells and memory cells, and the proportion of CD300a+CD8+ T lymphocytes in peripheral blood was higher in pregnant women who subsequently delivered placentas showing chronic chorioamnionitis than in those who did not. It suggests that peripheral CD8+ T lymphocytes of pregnant women with chronic chorioamnionitis have increased systemic cytotoxic capacity as a manifestation of maternal anti-fetal rejection. It is intriguing that the pregnant women with villitis of unknown etiology (anti-fetal cellular rejection involving the chorionic villi) did not show a significant increase of CD300a+ T lymphocytes in peripheral blood.<sup>19</sup> Further analysis of more cases would be necessary to address this issue.

## Conclusion

We report the transcriptome of CD300a+ peripheral T cells for the first time. The findings herein strongly suggest a role for CD300a+CD8+ T cells in the maternal anti-fetal immune response. There is a significant increase in the proportion of CD300a+CD8+ T cells in women presenting with anti-fetal cellular rejection in the chorioamniotic membranes.



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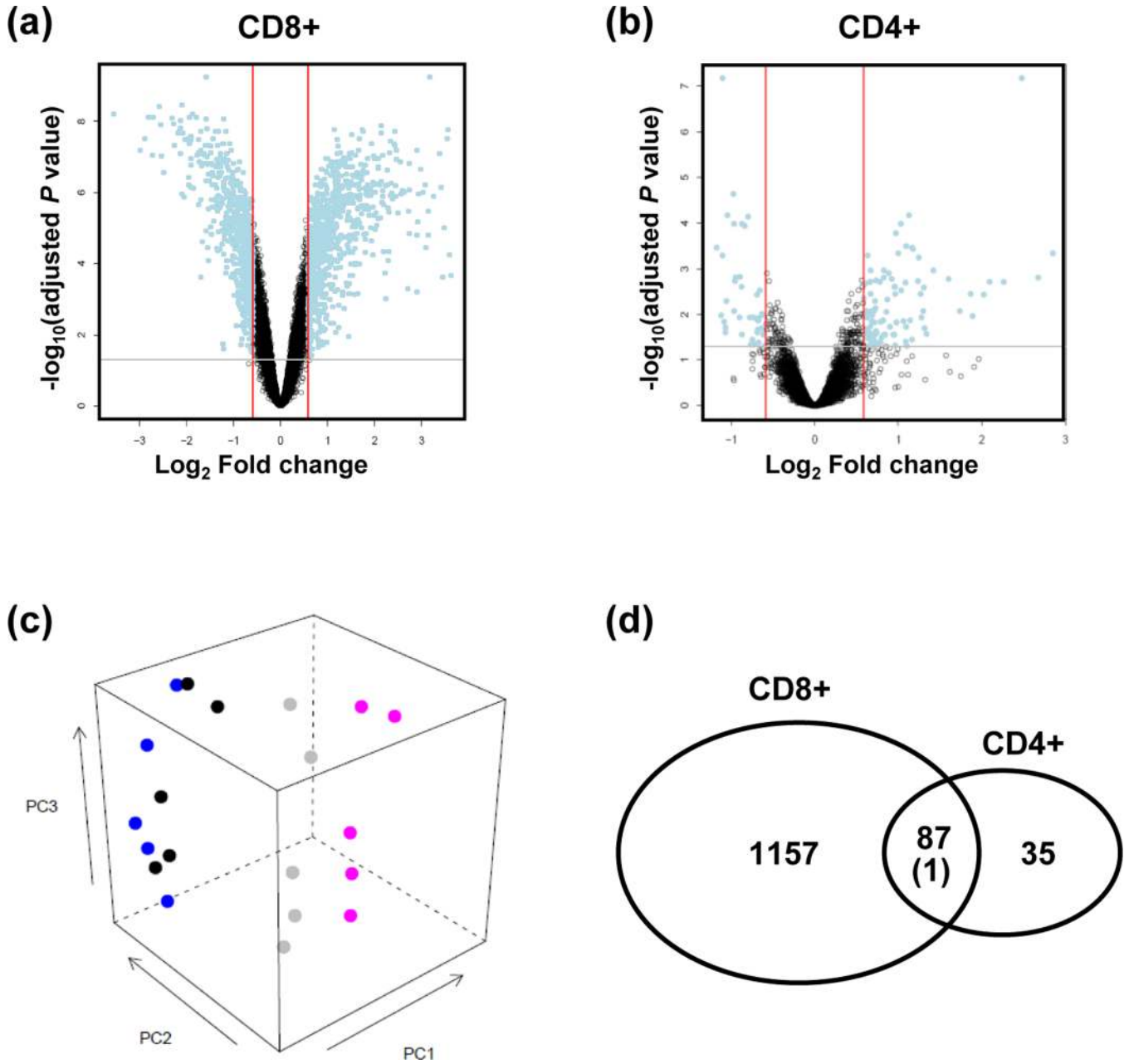
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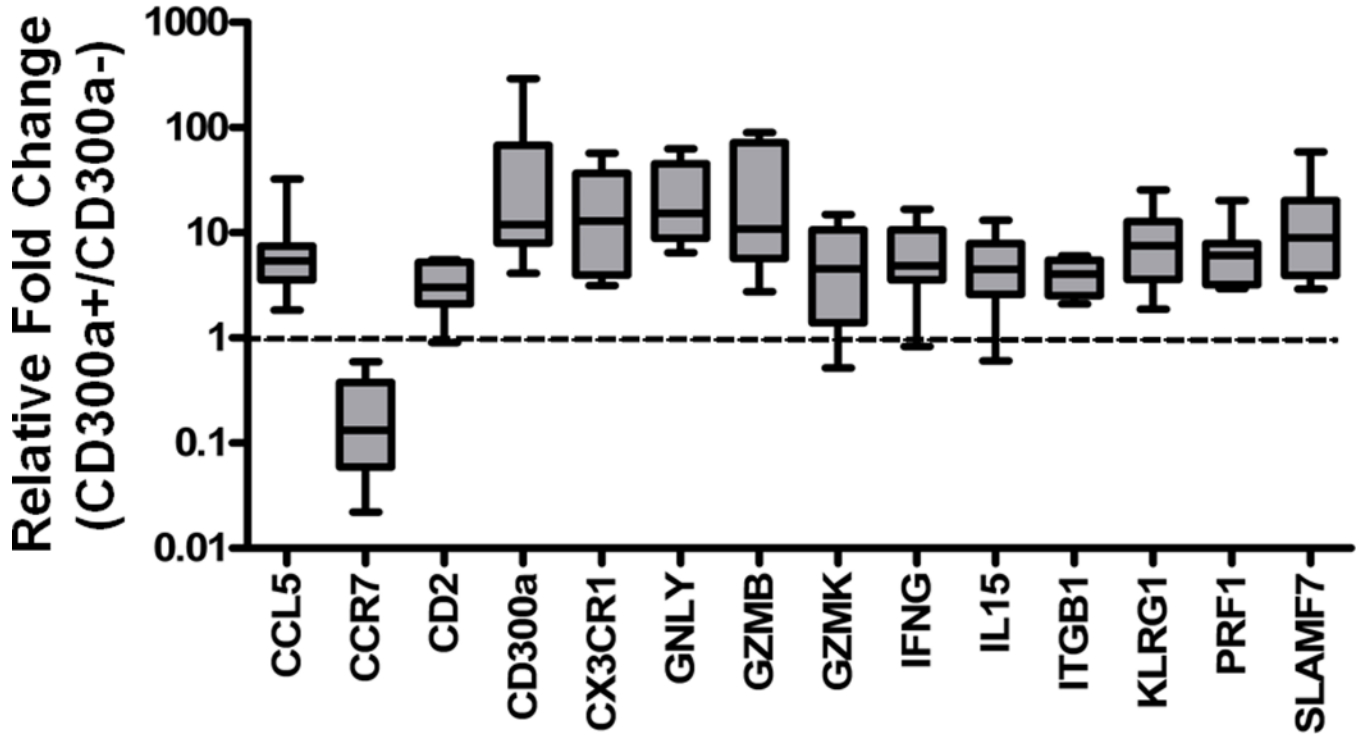
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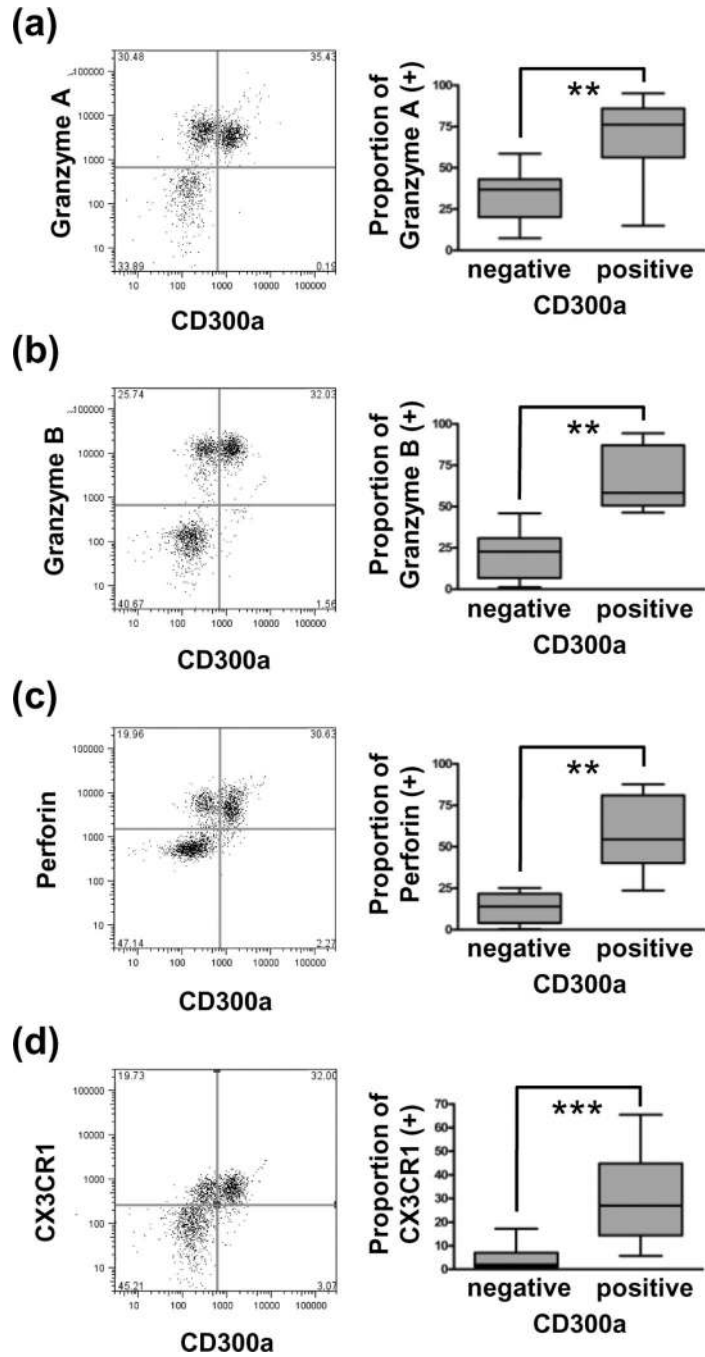


**Figure 1.** The transcriptome of CD300a+ and CD300a- T lymphocytes. (a, b) Volcano plots showing the relationship between the FDR-corrected *P* values of the genes ( $-\log_{10}$  of) and their fold changes ( $\log_2$  of). Blue dots outside the two red lines and above the gray line have fold changes  $>1.5$  and FDR-corrected *P* values  $< 0.05$ . Positive values on the *x*-axis indicate genes whose expression increased in CD300+ T lymphocytes, while negative values indicate genes down-regulated in CD300+ T lymphocytes. (a: CD8+ T lymphocytes; b: CD4+ T lymphocytes) (c) A principal component analysis plot generated with data from five pairs of samples (CD300+CD8+ (pink), CD300a-CD8+ (gray), CD300a+CD4+ (black), CD300a-CD4+ (blue) T lymphocytes), showing a more clear segregation between CD300a+CD8+ and CD300a-CD8+ than between CD300a+CD4+ and CD300a-CD4+ T lymphocytes. (d) Among the differentially expressed genes between CD300a+CD8+ and CD300a-CD8+ T

lymphocytes, 87 genes were regulated in the same direction as those expressed differentially between CD300a+CD4+ and CD300a-CD4+ T lymphocytes. Far more significantly enriched genes ( $N= 1,157$ ) were identified in the CD8+ T lymphocytes with CD300a expression, while only 35 more genes were differentially regulated in the CD4+ T lymphocytes with CD300a expression.

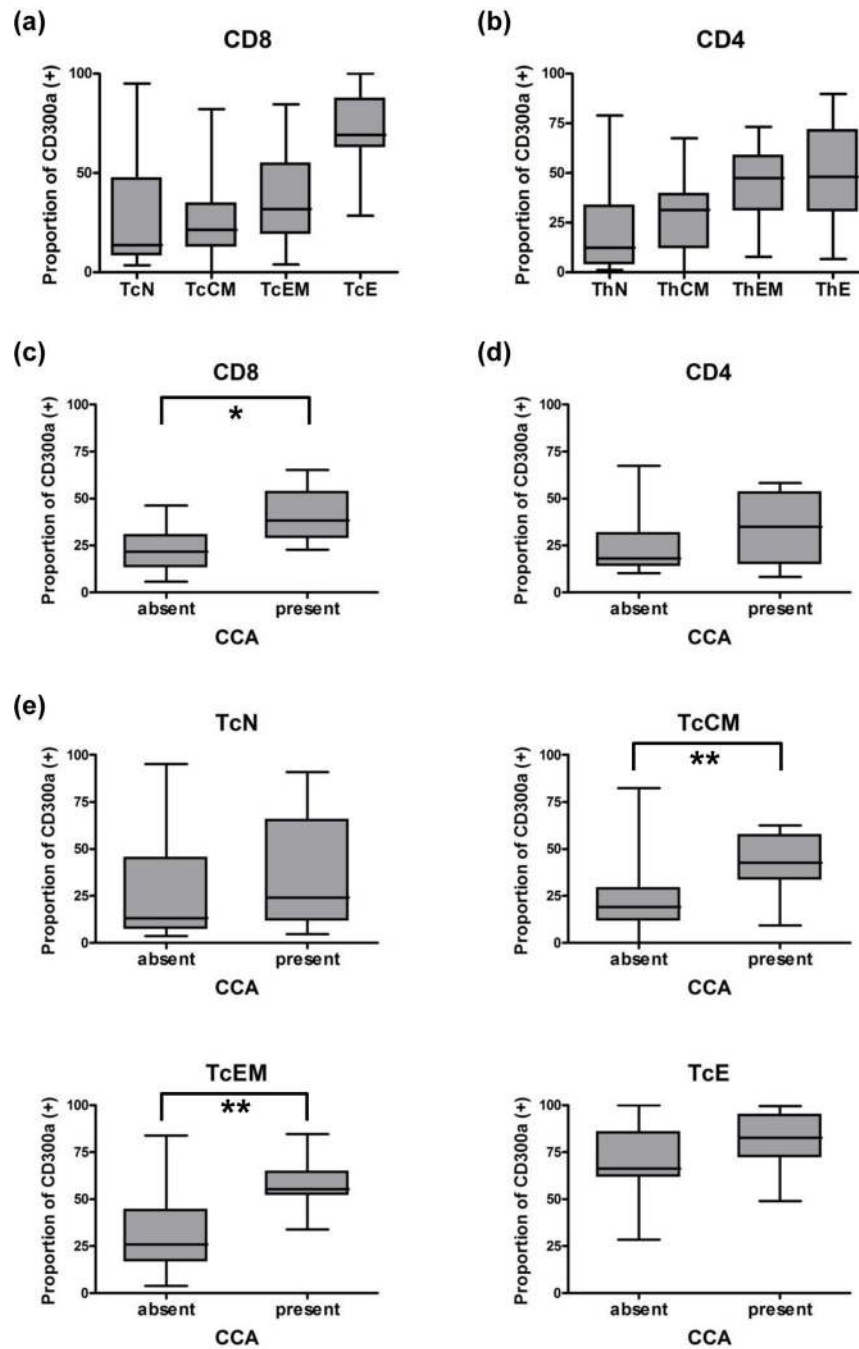


**Figure 2.** Validation of microarray analysis to compare gene expression between CD300a+CD8+ and CD300a-CD8+ T lymphocytes. Differential mRNA expression of the genes related to cytotoxicity and cytokines-cytokine receptor interaction between CD300a+ and CD300a-CD8+ T lymphocytes was confirmed by quantitative real-time-RT-PCR. CCR7 mRNA expression was lower, whereas the others were higher in CD300a+ than in CD300a- CD8+ T lymphocytes.



**Figure 3.** Expression of cytotoxicity effector proteins in CD300a+CD8+ peripheral T lymphocytes. Granzyme A (a), granzyme B (b), perforin (c), and CX3CR1 (d) were expressed higher in CD300a+ than CD300a- CD8+ T lymphocytes. \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .





**Figure 4.** The proportion of CD300a+ T lymphocytes in peripheral blood by flow cytometry. (a,b) The naïve, central memory, effector memory, and effector subsets of CD8+ T lymphocytes (a) and CD4+ T lymphocytes (b) showed the gradual increase of CD300a expression. (c,d) The increase of CD300a expression in pregnant women with chronic chorioamnionitis compared to the expression in those without was detected in CD8+ T lymphocytes. (c: CD8+ T lymphocytes; d: CD4+ T lymphocytes) (e) Central memory and effector memory CD8+ T lymphocytes showed higher expression of CD300a in cases with chronic chorioamnionitis than in those without. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; TcN, naïve cytotoxic T lymphocytes,

TcCM, central memory cytotoxic T lymphocytes; TcEM, effector memory cytotoxic T lymphocytes; TcE, effector cytotoxic T lymphocytes.

**Table I**

Top 10 biological processes and 8 pathways enriched in CD300a+CD8+ T lymphocytes compared to CD300a-CD8+ T lymphocytes

		No. Enriched Genes / No. Total Genes	Adjusted P value
Biologic process	Immune system process	136/613	6.43E-16
	Immune response	103/410	1.31E-15
	Defense response	83/353	1.52E-10
	Death	123/690	1.71E-07
	Cell death	122/687	2.10E-07
	Response to stimulus	244/1678	2.83E-07
	Programmed cell death	114/650	1.31E-06
	Apoptosis	113/646	1.59E-06
	Signal transduction	293/2154	3.00E-06
	Cell communication	312/2340	6.13E-06
Pathway	Natural killer cell mediated cytotoxicity	35/104	7.19E-10
	Graft-versus-host disease	21/37	8.47E-10
	Cytokine-cytokine receptor interaction	40/146	1.51E-09
	Antigen processing and presentation	24/68	3.54E-06
	Allograft rejection	14/30	3.62E-05
	Asthma	10/20	0.00070
	Type I diabetes mellitus	13/33	0.00172
	Autoimmune thyroid disease	12/35	0.00604

**Table II**

Top 20 differentially expressed genes related to immune system process in CD300a+CD8+ lymphocytes

Gene Symbol	Gene Name	Fold Change	Adjusted P value
CD300A	CD300a molecule	9.00	5.86E-10
CD79A *	CD79a molecule, immunoglobulin-associated alpha	2.98	5.86E-10
KIR2DL3	killer cell immunoglobulin-like receptor, two domains, long cytoplasmic tail, 3	11.82	1.71E-08
PLCG2	phospholipase C, gamma 2 (phosphatidylinositol-specific)	4.40	2.16E-08
HLA-DOA *	major histocompatibility complex, class II, DO alpha	3.82	4.26E-08
CCL3	chemokine (C-C motif) ligand 3	3.81	4.26E-08
TNFSF14	tumor necrosis factor (ligand) superfamily, member 14	3.17	6.53E-08
PRKCA *	protein kinase C, alpha	3.35	7.78E-08
CCL3L3	chemokine (C-C motif) ligand 3-like 3	3.95	9.28E-08
SLAMF7	SLAM family member 7	1.99	1.10E-07
IL15	interleukin 15	2.53	1.16E-07
FYB *	FYN binding protein (FYB-120/130)	1.84	1.37E-07
CD55 *	CD55 molecule, decay accelerating factor for complement (Cromer blood group)	2.99	1.53E-07
VIPR1 *	vasoactive intestinal peptide receptor 1	3.20	1.84E-07
TBK1	TANK-binding kinase 1	2.60	1.92E-07
APOBEC3F	apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3F	2.88	1.94E-07
CLCF1	cardiotrophin-like cytokine factor 1	2.90	3.25E-07
DLL1 *	delta-like 1 (Drosophila)	2.70	3.46E-07
TGFBR2 *	transforming growth factor, beta receptor II (70/80kDa)	2.02	3.79E-07
MAPK1	mitogen-activated protein kinase 1	2.26	4.09E-07

\* down-regulated

**Table III**

Top 10 biological processes and the pathway enriched in CD300a+CD4+ T lymphocytes compared to CD300a-CD4+ T lymphocytes

		No. Enriched Genes / No. Total Genes	Adjusted P value
Biologic process	Signal transduction	41/2154	0.00449
	Immune response	15/410	0.00449
	Negative regulation of smooth muscle cell proliferation	3/6	0.00449
	Smooth muscle cell proliferation	4/17	0.00449
	Regulation of smooth muscle cell proliferation	4/17	0.00449
	Cell communication	42/2340	0.00455
	Response to external stimulus	15/438	0.00455
	Response to stimulus	33/1678	0.00731
	Response to wounding	11/267	0.00888
	Muscle cell proliferation	4/24	0.00950
Pathway	Cytokine-cytokine receptor interaction	9/146	4.76E-05

**Table IV**

Differentially expressed genes related to immune system process in CD300a+CD4+ lymphocytes

Gene Symbol	Gene Name	Fold Change	Adjusted P value
CD300A	CD300a molecule	5.57	6.53E-08
CTLA4*	cytotoxic T-lymphocyte-associated protein 4	1.79	1.17E-04
IL15	interleukin 15	1.96	1.68E-04
CCL5	chemokine (C-C motif) ligand 5	7.21	4.58E-04
TIMP1	TIMP metalloproteinase inhibitor 1	2.00	4.58E-04
TNFRSF4	tumor necrosis factor receptor superfamily, member 4	2.05	2.97E-03
OSM	oncostatin M	1.95	3.53E-03
CD226	CD226 molecule	1.64	8.41E-03
GZMA	granzyme A (granzyme 1, cytotoxic T-lymphocyte-associated serine esterase 3)	3.70	1.11E-02
GPR44	G protein-coupled receptor 44	1.64	1.51E-02
IFNG	interferon, gamma	2.13	1.86E-02
TNFSF14	tumor necrosis factor (ligand) superfamily, member 14	1.55	2.17E-02
APOBEC3G	apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G	1.76	2.95E-02
TNFSF13B	tumor necrosis factor (ligand) superfamily, member 13b	1.54	3.33E-02
CFH	complement factor H	1.73	4.08E-02
CTSW	cathepsin W	1.61	4.98E-02

\*  
down-regulated

**Table V**

## Demographics and clinical characteristics of pregnant women

Maternal age (years) (median, range)	25 (15–40)
Gestational age at sampling (weeks) (median, range)	39.0 (13.3 – 41.7)
Gestational age at delivery (weeks) (median, range)	39.7 (37.0 – 41.9)
Labor at sampling (present/absent/unknown)	14/25/1
Race (African American /others/unknown)	35/4/1
Para (primiparity/multiparity)	13/27
Baby gender (male/female)	17/23
Smoking (yes/no)	3/37
Interval between sampling and delivery (days) (median, range)	1 (0–194)