CD83 is locally regulated and differentially expressed in disturbed murine pregnancy

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Abstract

Alterations in the immunologic balance during pregnancy have been associated with poor pregnancy outcomes. The underlying mechanisms are complex and mouse models delivered valuable information on inflammatory imbalance in disturbed pregnancies and served as model to test potential anti-inflammatory therapies. CD83 is a transmembrane protein (mCD83) with a soluble form (sCD83) which possesses strong anti-inflammatory properties. During murine pregnancy, upregulated mCD83 expression induces sCD83 release after *in vitro* stimulation with LPS, phorbol myristate acetate (PMA) and ionomycin. The release mechanism of sCD83 and its control are yet to be elucidated. In this study, the expression of mCD83 and sCD83 has been extensively studied in the CBA/J × DBA/2J mouse model of pro-inflammatory-mediated pregnancy disturbances. mCD83 was higher expressed on splenic B cells, uterus-draining lymph nodes T cells and dendritic cells from mice with poor pregnancy outcome (PPOM) compared to mice with good pregnancy outcome (GPOM). PPOM, however, was accompanied by lower sCD83 serum levels. *In vitro* treatment of splenic B cells with progesterone led to a reduction of TIMP1 expression, mCD83 expression and sCD83 release, while TIMP1 treatment had a positive effect on sCD83 availability. These results suggest that tissue and matrix components are involved in the regulation of CD83 in murine pro-inflammatory pregnancies.

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Introduction

During pregnancy, the maternal immune system is confronted by the presence of paternal antigens expressed by the semi-allogeneic fetus. In order to avoid fetal rejection, the maternal immune system undergoes several adaptations. Maternal tolerance is orchestrated by a number of different mechanisms regulated by pregnancy hormones and trophoblast to shift decidual immune cells to their regulatory pendants, affecting systemic immune responses as well. Consequently, pregnant women, although not considered immunosuppressed, respond differently for example to viral infections like influenza or auto-antigens compared to non-pregnant women (Piccinni *et al.* 2016, Racicot & Mor 2017).

One major challenge of the immune system during pregnancy is to provide protection against infections despite the tolerogenic shift that immune cells undergo at the fetomaternal interface. This phenomenon is achieved by the interplay of both leukocytes and soluble factors that contribute to finely balance of both immune aspects. An imbalance of pro- and anti-inflammatory components represents a risk that threatens different aspects of pregnancy health. As a result, pregnancy complications, like preterm birth, preeclampsia, intrauterine growth restriction (IUGR) and recurrent

pregnancy loss arise (Raghupathy *et al.* 2012, Kuon *et al.* 2015, Harmon *et al.* 2016).

Mouse models contributed to an improved understanding of the ongoing processes during pregnancy. Thereby, it was shown that a general pro-inflammatory immune reaction, but not necessarily against paternal antigens, contributes to fetal resorption (Robertson *et al.* 2007). Moreover, new therapy approaches were first tested in mouse models, for example the adoptive transfer of leukocytes (DCs, B10 cells, Tregs) and soluble factors (IH-Ig, IL10, TGFB) which contribute to the essential immune balance and decreased fetal resorption (Clark 2016).

Recently, we suggested that the soluble form of the transmembrane molecule CD83 (sCD83) could represent an important factor contributing to pregnancy maintenance (Packhäuser *et al.* 2017). Anti-inflammatory properties of sCD83 have been extensively examined in mouse models of autoimmunity, allograft transplantation and inflammatory disease (Lan *et al.* 2010, Bock *et al.* 2013, Starke *et al.* 2013, Eckhardt *et al.* 2014, Lin *et al.* 2018). However, it remains unclear if sCD83 is generated by alternative splicing or from shedding of the transmembrane form (mCD83) by proteases, as in the case of other immune-related factors (TGFA, TNFA, TACI, etc.) (Murphy 2008, Hoffmann *et al.* 2015).

Although the function of mCD83 still remains under elucidation, different roles have been proposed depending on the cell type expressing it. On dendritic cells (DCs), mCD83 marks mature cells and is necessary for a proper T cell activation and co-stimulation (Zhou & Tedder 1995, 1996, Prechtel & Steinkasserer 2007). mCD83 is also expressed by activated T cells. CD83 silencing in CD4⁺ T cells in mice results in defective functions like antigen presentation, cytokine secretion and proliferation (Su *et al.* 2009). However, the overexpression of mCD83 in naive murine CD4⁺ T cells results in FOXP3 upregulation characteristic for regulatory T cells (Reinwald *et al.* 2008).

mCD83 expression on B cells influences their maturation in the spleen, impacting on the marginal zone (MZ)/follicular zone (FO) B cell relative numbers (Luthje *et al.* 2008, Krzyzak *et al.* 2016). Furthermore, mCD83 expression on B cells is associated to B cell anti-inflammatory responses, including higher IL10 production (Kretschmer *et al.* 2007). Both, MZ/FO ratio and B cell-derived IL10 are increased in murine pregnancies and altered in pathological mouse models of pregnancy (Jensen *et al.* 2013, Muzzio *et al.* 2014a, 2016).

B cells upregulate mCD83 expression after stimulation with stimulatory agents such as lipopolysaccharide (LPS) and PMA. Additionally, direct cell contact plays an important role in the induction of mCD83 expression, as in the case of CD40/CD40L interactions between B cells and CD4⁺ T cells (Kretschmer *et al.* 2011). In our previous work, we showed that splenic B cells from advanced pregnancies were the most reactive to LPS stimulation *in vitro*. *In vivo*, however, the highest mCD83 expression on splenic B cells during gestation was observed at mid-pregnancy. We postulate that the compartmentation of the B cells plays an important role regulating CD83 availability during pregnancy. To test our hypothesis, we used a mouse model of systemic inflammation-induced pregnancy disturbance.

On the one hand, we show that the pregnancy-related hormone progesterone controls matrix components that influence CD83 availability during pregnancy. On the other hand, during inflammatory pregnancies, splenic but not peripheral B cells upregulate CD83 expression. Moreover, despite the higher mCD83 on splenic B cells, lower sCD83 is detected in the sera of affected mice. Our data suggest that tissue-specific components influence sCD83 availability during normal and impaired pregnancy.

Material and methods

Animals

CBA/J (H2^k) female and DBA/J (H2^d) male mice were purchased from Charles River or Janvier Labs (Saint-Berthevin Cedex, France). BALB/c (H2^d) males were bred in our Central Service

and Research Facility for Animals (ZSFV). The animals were kept co-housed in a 12-h light/dark cycle with free access to food and water. Eight- to twelve-week-old CBA/J female mice were either paired with BALB/c males, generating normal pregnancies ('good pregnancy outcome mice' (GPOM)) or with DBA/2J males to obtain immune-induced pathological pregnancies ('poor pregnancy outcome mice' (PPOM)) (Clark *et al.* 1986, Muzzio *et al.* 2014b, 2016). In this mouse model, fetal resorptions are consequence of an immune rejection against paternal antigens (Clark *et al.* 1986), associated to a failure in regulatory T cell (Treg) generation to compensate the pro-inflammatory cytokine boost driven by activated leukocytes (Clark *et al.* 2004). Mice were checked for vaginal plug every morning. Observation of a plug was declared as day 0 of pregnancy, and the female was separated from the male. Mice were killed at day 14 post plug (14 dpp); spleen, thymus, paraaortic and inguinal lymph nodes and blood were collected. Non-pregnant CBA/J female mice were randomly killed and used for *in vitro* experiments. Animal experiments were carried out according to institutional guidelines approved by the Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei Mecklenburg-Vorpommern (LALLF-MV; 7221.3-1-068/13 to DM). The experiments were conducted in conformity with the European Communities Council Directive 86/609/EEC.

Cell preparation

Single-cell suspensions from para-aortic lymph nodes, inguinal lymph nodes, and thymus were obtained as previously described (Packhäuser *et al.* 2017). The tissue was carefully pressed through a 40-µm nylon cell strainer and washed with PBS. In case of spleen tissue, an erythrocyte lysis with 10 mL Lysis Buffer (0.89% NH₄Cl, 0.1% KHCO₃, 0.003% EDTA) was performed and stopped after 5 min with 3 mL FBS. After washing, the cell suspension was filtered a second time with a 40-µm cell strainer. The cell counts of the suspensions were determined using a Neubauer chamber.

Flow cytometry

Flow cytometry was applied to evaluate the expression of CD83 on B, T, or DCs from spleen, thymus, and lymph nodes as described before (Packhäuser *et al.* 2017). Cell suspensions were first incubated with CD16/32 mAb Fc block (BD Pharmingen, Heidelberg, Germany) for 5 min. Staining with fluorochrome-labeled specific antibodies was performed for 30 min at 4°C in the dark. Samples from spleen were additionally stained with Fixable Viability Dye eFluor 780 (eBioscience) for 30 min and later washed with FACS buffer (1% BSA (Sigma-Aldrich), 0.1% NaN₃ (Carl Roth, Karlsruhe, Germany) in DPBS) before Fc blocking. For intracellular staining, fixation and permeabilization of the cells were accomplished with BD Perm/Wash and BD Cytfix/Cytoperm (BD Biosciences) according to manufacturer's instructions. Subsequently, cells were incubated with fluorochrome-labeled antibodies for 30 min at 4°C for intracellular staining. Following antibodies were purchased from BD Bioscience CD83 (Michel-19); B220 (RA3-6B2); CD4 (RM4-4 and RM4-5); CD23 (B3B4);

CD21 (7G6) and CD19 (1D3). Polyclonal goat IgG biotinylated anti-TIMP was purchased from R&D. DC Marker DCIR2 (33D1) and Streptavidin were purchased from eBioscience. Data were acquired on FACSCanto (BD Bioscience) and analyzed by using FlowJo software (FlowJo, LLC).

Cell stimulation

1×10^6 splenic lymphocytes or magnetic isolated splenic CD19⁺ B cells (CD19 MicroBeads mouse; Miltenyi Biotec GmbH) were cultured 48 h in a total volume of 500 μ L RPMI 1640 culture medium supplemented with 10% FBS and antibiotics on 48-well flat-bottom suspension plates. The stimulation was performed with either 10 μ g/mL LPS O111:B4 for 48 h and 50 ng/mL PMA as well as 500 ng/mL ionomycin (all from Sigma-Aldrich Chemie GmbH) for the last 5 h. In order to examine the influence of metalloproteinases on CD83 expression, cells were additionally stimulated with recombinant Mouse TIMP1 (100 ng/mL; BioLegend, San Diego, USA) or anti-TIMP1 (1 μ g/mL; R&D Systems) from the beginning of the experiment for a total of 48 h. In order to examine the influence of progesterone on CD83 expression, the cells were stimulated 50 ng/mL or 500 ng/mL this pregnancy-related hormone (Sigma-Aldrich). After stimulation, cells and supernatants were separated by 5 min centrifugation at 300 g and the cells were directly used for flow cytometry or by 10 min at 8000 g to store the supernatants at -80°C and lyse cells with peqGOLD TriFast™ (VWR, Radnor PA, USA) for RNA extraction.

ELISA

The levels of sCD83 in sera and supernatants were measured using an ELISA kit for CD83 (Cloud-Clone Corp., Houston, TX, USA). The serum samples were diluted 1:2 in PBS, while the supernatants were examined without dilution. The test was performed following the instruction manual.

Real-time PCR

Magnetic isolated splenic CD19⁺ B cells were treated with TriFast peqGOLD (VWR, Radnor PA, USA). RNA concentration was spectrophotometrically assessed in a the NanoPhotometer PEARL (IMPLEN, Munich, Germany). RNA was reverse-transcribed applying High-Capacity cDNA Archive Kit (Applied Biosystems). For the qPCR, the samples were amplified in duplicate and non-template controls were included. Primer pairs were chosen to span an exon–exon junction to avoid genomic DNA amplification. Real-time PCR was performed using Power SYBR® Green (AB/Life Technologies) in a 7300 Real-time PCR System (Applied Biosystems) with *Actb* as housekeeping gene. Primer sequences were the following: *Cd83* forward: TGAAGGTGACAGGATGCCC; *Cd83* reverse: CTTGGGGAGGTGACTGGAAG; *Actb* forward: TGGAATCCTGTGGCATCCATGAAAC; *Actb* reverse: TAAAACGCAGCTCAGTAACAGTCC. All primers were purchased from Invitrogen.

Statistical analysis

In vivo and *ex vivo* data comparing GPOM vs PPOM pregnancies were analyzed by unpaired Student's *t*-test. *In vitro* data comparing treatments and control were analyzed by paired Student's *t*-test or repeated-measures ANOVA with Dunnett's posttest where applicable.

Results

mCD83 is upregulated in PPOM in a cell- and compartment-dependent manner

We observed higher mCD83 expression on splenic B cells in PPOM as compared to GPOM (2.26 $\pm$ 0.27-fold increase; $P < 0.001$; Fig. 1B). This was accompanied by higher percentages of mCD83⁺ B cells (1.74 $\pm$ 0.19 in GPOM vs 2.54 $\pm$ 0.22 in PPOM; $P < 0.05$; Fig. 1C). Although an increment in the percentage of CD83⁺ cells could be observed within splenic DCs (8.31 $\pm$ 1.06 in GPOM vs 20.7 $\pm$ 2.62% in PPOM; $P < 0.001$; Fig. 1C) and T cells (0.11 $\pm$ 0.01 in GPOM vs 0.19 $\pm$ 0.03% in PPOM; $P < 0.05$; Fig. 1C), the overall mCD83 expression level was not significantly different. We also analyzed the expression of the main mature splenic B cell subsets, CD21/35^{hi}CD23^{low} marginal zone (MZ) and CD21/35^{int}CD23^{hi} follicular zone (FO) B cells. Both, FO and MZ B cells expressed significantly higher levels of mCD83 in PPOM than in GPOM mice (2.05 $\pm$ 0.24-fold increase in FO and 3.52 $\pm$ 0.75-fold increase in MZ B cells respectively; $P < 0.001$; Fig. 1B). Within B cell subpopulations, mCD83⁺ percentages were significantly increased only in MZ B cells (1.60 $\pm$ 0.15 in GPOM vs 2.57 $\pm$ 0.21% in PPOM; $P < 0.01$).

In contrast, no significant differences were found in the mCD83 expression on peripheral B cells of thymus and uterus-draining lymph nodes (Fig. 1B). In thymus, however, we observed that mCD83 was elevated in DCs as well in CD4⁺ T cells of PPOM mice as compared to GPOM (2.32 $\pm$ 0.18-fold increase in DC and 1.18 $\pm$ 0.02-fold increase in T cells; $P < 0.001$ and $P < 0.01$ respectively; Fig. 1B). The percentage of mCD83⁺ cells was, however, reduced in thymic CD4⁺ T cells (0.66 $\pm$ 0.08 in GPOM vs 0.44 $\pm$ 0.04% in PPOM; $P < 0.05$; Fig. 1C). Both uterine-draining lymph nodes (inguinal (ILN) and paraaortic (PLN)) showed higher mCD83 expression on DCs in PPOM as compared to GPOM (2.07 $\pm$ 0.17 and 2.29 $\pm$ 0.23-fold increase respectively; $P < 0.001$; Fig. 1B).

In summary, mCD83 was upregulated on different lymphocyte subpopulations of PPOM. There was a significant difference in the expression of mCD83 molecule in splenic B cells and dendritic cells from thymus and uterus-draining lymph nodes.

Figure 1 PPOM express higher mCD83 than GPOM in splenic but not in peripheral B cells *in vivo*. B cells (B220⁺), T cells (CD4⁺) and dendritic cells (DCIR2⁺) from GPOM ($n=16$) were compared to cells from PPOM ($n=22$). (A) Representative plots show gating strategies for determination of mCD83 expression. In overlapping histograms (right), the gray area represents the unstained control and the black line the positive staining. Bars show the relative median fluorescence intensity (MFI) of mCD83 (B) or the percentage of mCD83⁺ cells (C) in live B cells (B220⁺), T cells (CD4⁺) and dendritic cells (DCIR2⁺) from spleen (SPL), thymus (THY), paraaortic (PLN) and inguinal (ILN) lymph nodes. In spleen, B220⁺ B cells were further subdivided in CD21/35^{hi}CD23^{low} marginal zone B cells (MZ) and CD21/35^{int}CD23^{hi} follicular zone B cells (FO). All data were analyzed using Student's *t*-test ($n \geq 16$). Significant differences are indicated (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$).

Ex vivo stimulation reveals no difference between PPOM and GPOM expression of mCD83 and sCD83

Taking into account the discrepancy in terms of mCD83 expression between splenic and peripheral B cells, we moved forward to compare splenic B cell expression of mCD83 after *ex vivo* stimulation with inflammatory signals. Here, neither differences in the mCD83 expression nor in the sCD83 release could be depicted between the two groups of mice after 48-h stimulation (Fig. 2A and B).

A pregnancy-specific hormone influences sCD83 expression

In our previous work, we found splenic B cells to be a major source of sCD83 during pregnancy and we suggested that it may contribute to anti-inflammatory responses that support pregnancy maintenance (Packhäuser *et al.* 2017). In this experiment, we found that GPOM had higher serum levels of sCD83 than pro-inflammatory PPOM at day 14 of pregnancy (2308.65 ± 176.92 vs 1638.89 ± 76.3048 pg/mL respectively, Fig. 3A).

During pregnancy a variety of immune and non-immune adaptations take place regulated that is by the sexual hormones estradiol and progesterone. In our previous work, we found progesterone but not estradiol regulating mCD83 on splenic B cells, T cells and DCs.

Here, splenic lymphocytes or magnetically isolated CD19⁺ splenic B cells from non-pregnant CBA/J female mice were stimulated with 500 ng/mL progesterone (the same order of magnitude as during murine pregnancies; Hashimoto *et al.* 2010). The treatment led to a significant reduction of sCD83 in the supernatants of the cell culture of splenocytes (374.40 ± 20.60 vs 303.69 ± 16.07 pg/mL) and B cells (590.63 ± 26.55 vs 508.95 ± 10.16 pg/mL) compared to the respective controls; $P < 0.05$; Fig. 3B).

To determine if the effect of progesterone was linked to a reduced production of CD83, we stimulated splenic lymphocytes with progesterone and/or LPS. We did not observe any significant changes in the levels of intracellular staining for CD83 (iCD83), although a significant reduction at the mRNA levels could be seen (from 1.472 ± 0.59 to 1.303 ± 0.52 after progesterone treatment; Fig. 3C).

The effect of progesterone on CD83 expression on resting lymphocytes was also assessed performing intra and extracellular staining of CD83. Progesterone treatment led to a reduction on both, mCD83 (216.75 ± 6.09 MFI for untreated controls, 199.67 ± 4.20 for 50 ng/mL progesterone and 183.84 ± 5.00 with 500 ng/mL, Fig. 3D) and iCD83 (241.8 ± 32.86 MFI for untreated controls, 229.6 ± 34.94 with 500 ng/mL progesterone, $P < 0.05$, $n = 7$, data not shown). The effect of progesterone on mCD83 was stronger than on iCD83 (0.849 ± 0.016 vs 0.937 ± 0.025). At the mRNA levels, progesterone did not have any significant effect on *Cd83* expression (Fig. 3D).

TIMP1 increases sCD83 availability ex vivo

Since the reduction of sCD83 in the supernatants of progesterone-treated B lymphocytes correlated with a reduction of mCD83 levels, we looked for mechanisms that would affect CD83 membrane turnover, decreasing mCD83 and sCD83 availability. Many membrane proteins are target of degradation by proteinases which are regulated by proteases inhibitors. The metalloproteinase inhibitor 1 (TIMP1) is expressed on B cells (Alter *et al.* 2003) and a known target of progesterone as well (Imada *et al.* 1994, Hampton *et al.* 1999). Using isolated lymphocytes, we were able to show that progesterone led to significantly decreased TIMP1 expression on splenic B cells (378.17 ± 12.97 after progesterone treatment vs

Figure 2 *Ex vivo* stimulation of splenic B cells reveals no difference in mCD83 expression between GPOM and PPOM. (A) Splenic lymphocytes were stimulated with LPS for 48 h with PMA and ionomycin for the last 5 h (LPI). Plots show a representative gating strategy for determination of mCD83 expression on CD19⁺ B cells (left). The overlapping histograms display mCD83 expression of untreated (filled line) and LPI-stimulated B cells (dashed line). Gray area represents the FMO for CD83. Bars show the corresponding median fluorescence intensities (MFI) of mCD83 (bottom-right) on LPI-stimulated B cells. (B) Supernatants of the LPI-stimulated lymphocytes were analyzed by ELISA to determine sCD83 levels. All data were analyzed using Student's *t*-test (A: $n = 10$, B: $n = 9$).

Figure 3 Progesterone reduces mCD83 and sCD83 availability *in vitro*. (A) Sera of pregnant mice were analyzed by ELISA to determine sCD83 levels. Data were analyzed using Student's *t*-test (GPOM $n=19$, PPOM $n=8$). Significant differences are indicated ($*P\leq 0.05$). (B) Splenic lymphocytes or magnetically isolated CD19⁺ B cells from the spleen of non-pregnant CBA/J mice were cultured for 48 h with progesterone or with medium alone as control. Graph shows levels of sCD83 of supernatants determined by ELISA. Data were analyzed using paired *t*-test ($n=5$). Significant differences are indicated ($*P\leq 0.05$). (C) Splenic lymphocytes from non-pregnant CBA/J mice were cultured for 48 h with LPS alone or with LPS and 500 ng/mL progesterone. PMA and ionomycin were added for the last 5 h. The plots show a representative gating strategy (left). Overlapping histograms (right) display differences in iCD83 between LPS (filled line) and LPS with progesterone (dashed line) stimulated CD19⁺ B cells. Gray area represents the FMO for CD83. Data represent the fold increase of iCD83 MFI (middle) or *Cd83* mRNA (right) in splenic CD19⁺ B cells over the corresponding untreated controls. Data were analyzed using paired *t*-test (flow cytometry: $n=7$; qPCR: $n=6$). (D) Splenic lymphocytes from non-pregnant CBA/J mice were cultured for 48 h with or without the presence of 50 or 500 ng/mL progesterone. The plots show a representative gating strategy (left). Overlapping histogram displays differences between with progesterone-stimulated B cells (dashed line) and untreated control (filled line). Gray area represents the FMO for CD83. Bars (top-middle) show the corresponding median fluorescence intensity (MFI) of mCD83 within CD19⁺ B cells (right-bottom). Bars (right) display the fold change of either mCD83 ($n=4$) or iCD83 ($n=7$) MFI after progesterone treatment. Graph (bottom) shows *Cd83* mRNA levels of control vs progesterone-treated B cells ($n=6$). Data from flow cytometry were analyzed by ANOVA and Dunnett's Multiple Comparison test to compare treatments against control (top-middle) or unpaired *t*-test (right). Data from qPCR were analyzed by paired *t*-test ($n=6$). Significant differences are indicated ($**P\leq 0.01$, $***P\leq 0.001$).

Figure 4 The metalloproteinase inhibitor TIMP1 increases mCD83 and sCD83 availability *in vitro*. (A) Splenic lymphocytes from non-pregnant CBA/J mice were cultured for 48 h with 50 or 500 ng/mL progesterone. Cells cultured in medium alone served as control. Overlapping histograms (top) display differences between progesterone-stimulated B cells (dashed line) and untreated control (filled line). Gray area represents the FMO for TIMP1. Bars show the median fluorescence intensity (MFI) of TIMP1 within CD19⁺ B cells (bottom). Data were analyzed by ANOVA and Dunnett's multiple comparison test to compare treatments with controls ($n=4$). Significant differences are indicated (** $P\leq 0.01$). (B) Splenic lymphocytes were cultured for 48 h with LPS and for the last 5 h with PMA and ionomycin (LPI). Recombinant TIMP1 or anti-TIMP1 blocking antibody were added from the beginning of the experiment. Scatter dot plots show a representative gating strategy to CD19⁺ B cells and CD4⁺ T cells (top). Overlapping histograms display differences between TIMP1 (middle-left) or anti-TIMP1 (middle-right) expression on CD19⁺ B cells (dashed lines) and the respective controls (filled line). Gray curve represents the FMO for mCD83. The bottom-left graph shows the fold increase of the corresponding MFI of mCD83 on CD19⁺ B cells after stimulation with LPI and TIMP1 ($n=4$). The bottom-right graph represents the normalized MFI of mCD83 on CD19⁺ B cells after stimulation with LPI and anti-TIMP1 ($n=5$). Data were analyzed using paired *t*-test. Significant differences are indicated (* $P\leq 0.05$). (C) Graph shows the amount of sCD83 in supernatants of LPI or LPI and TIMP1-treated splenocytes determined by ELISA. Data were analyzed using paired *t*-test ($n=5$). Significant differences are indicated (* $P\leq 0.05$).

465.92 ± 26.59 MFI in controls, respectively; $P < 0.01$; Fig. 4A), supporting the idea of a protease involvement regulating CD83 availability.

To confirm that TIMP1 was involved in the changes of m- and sCD83 expression, we cultured splenic

lymphocytes with LPS and either recombinant TIMP1 or a blocking antibody against TIMP1. TIMP1 treatment was able to elevate mCD83 expression (2.02 ± 0.08 in LPI vs 2.22 ± 0.08 -fold increase with LPI and TIMP1, respectively; $P < 0.05$, Fig. 4B), while anti-TIMP1 reduced

it (2.197 ± 0.07 in LPI vs 1.926 ± 0.09 -fold increase with LPI and Anti-TIMP1, respectively; $P < 0.05$; Fig. 4B). Furthermore, TIMP1 treatment of splenocytes led to significantly increased sCD83 release (1917.66 ± 78.81 with LPS and TIMP1 vs 1758.61 ± 75.40 pg/mL for LPS-treated controls; $P < 0.05$; Fig. 4C).

In summary, TIMP1 effects on mCD83 inversely correlate with those observed with progesterone, favoring sCD83 availability.

Discussion

CBA/J $\times$ DBA/2J pregnancies (here referred as PPOM) are characterized by a failed maternal tolerance (Clark *et al.* 1980). The pro-inflammatory manifestations can be observed systemically in the mother and locally at the resorptive fetoplacental unit (Ahmed *et al.* 2010). Innate cells, including neutrophils, NK cells, macrophages and DCs have been found to be the main effectors of the resorptive phenotype (Bonney & Brown 2014).

mCD83 was first described as a maturation marker for DCs and its expression on DCs is linked to a higher pro-inflammatory potential (Aerts-Toegaert *et al.* 2007, Wilson *et al.* 2009, Pinho *et al.* 2014, Seldon *et al.* 2016). mCD83⁺ DCs are present in human decidua and in high numbers associated with miscarriage (Askelund *et al.* 2004, Qian *et al.* 2015). Our data show that DCs from the uterus-draining lymph nodes in PPOM expressed higher levels of mCD83 than in GPOM, which supports the observations in human studies.

CD4⁺ T cells can as well express mCD83, for which both anti- and pro-inflammatory roles were suggested, depending on the subpopulation of T cell that was studied (Wolenski *et al.* 2003, Reinwald *et al.* 2008). Although we did not analyze different CD4⁺ T cell subsets in detail, we observed the significant differences between both groups of mating only in thymic T cells. As PPOM expressed higher levels of mCD83 than GPOM, we assume that mCD83 expression on thymic CD4⁺ T cells is accompanying the pro-inflammatory T cell repertoire that was already described in those mice.

Similarly, B cells in spleen depicted significant differences in mCD83 expression. In our previous work, we showed that healthy murine pregnancy is accompanied by an upregulation of mCD83 expression on splenic B cells *in vivo*. Moreover, splenic B cells from pregnant mice acquire a higher capacity to upregulate mCD83 expression after stimulation with pro-inflammatory signals as LPS (Packhäuser *et al.* 2017). PPOM pregnancies are characterized by higher levels of pro-inflammatory cytokines, including TNFA and IFNG, which contribute to the disturbed pregnancy phenotype (Clark *et al.* 1998). These cytokines are as well inducers of mCD83 expression (Li *et al.* 2012, Lin *et al.* 2018). As expected for pro-inflammatory pregnancies, splenic B cells from PPOM displayed higher levels of mCD83 than in GPOM.

The fact that differences in mCD83 expression on B cells are absent in uterus-draining lymph nodes suggests that there may be mechanisms to limit CD83 expression in the periphery even during pro-inflammatory pregnancies. Our results suggest that compartmentation and matrix components seem to play a role regulating the CD83 biology. In fact, B cells in spleen are compartmentalized and they migrate upon activation (Pereira *et al.* 2010). In the splenic stroma, B cells interact with several cell types that exert a considerable influence on the B cell fate (Mueller & Germain 2009). We observed that PPOM, despite their higher mCD83 expression on different lymphocyte population, displayed lower serum levels of sCD83. It has been suggested that proteolytic shedding may lead to the release of sCD83 from its membrane-bound form (Hock *et al.* 2001). Then, a higher splenic mCD83 expression accompanied with lower sCD83 release suggests that if a protease is involved, its function diminished in PPOM (Fig. 5).

Consistent with this, we observed that in *ex vivo* stimulated splenic B cells, progesterone limits CD83 availability in a process which seems to be mediated by TIMP1. TIMP1 influenced CD83 availability, increasing mCD83 expression and sCD83 release after *ex vivo* stimulation of splenic B cells. Progesterone treatment, on the other hand, induced TIMP1 downregulation, reducing CD83 availability (Fig. 6).

Figure 5 Schematic summary of CD83 shedding in GPOM and PPOM on splenic B cells. In GPOM (A), a balanced shedding of mCD83 provides the source of sCD83 in healthy pregnancies. In contrast, an imbalanced shedding of mCD83 in PPOM (B) results in reduced sCD83 release.

Figure 6 Schematic summary of the proposed progesterone-dependent regulation of CD83 availability by TIMP1. (A) TIMP1 inhibition of a proteinase that degrades mCD83 results in a higher mCD83 expression and therefore improved availability for sCD83 release. (B) Progesterone, acting through inhibition of B cell TIMP1 expression, facilitates the degradation of mCD83, reducing mCD83 expression and sCD83 release.

Progesterone may display contradictory roles depending on the target cell. In the case of TIMP1 production, for example, progesterone showed a negative effect on B lymphocytes but induces TIMP1 expression in uterine fibroblasts (Imada *et al.* 1994) and human endometrial stromal cells (Hampton *et al.* 1999). Then, if the influence of stromal-derived TIMP1 or metalloproteinases prevails over the local effect on B cell expressed TIMP1, we may observe the contrary phenotype in splenic B cells *in vivo* than in *ex vivo*-treated cells. So the effect that we observed on isolated lymphocytes may correspond to a peripheral/activated phenotype, as in the case of a local inflammatory process. In this case, activated B cells could leave their niches and act to contribute to a pro-inflammatory response during pregnancy minimizing the anti-inflammatory effect of sCD83 release.

In this scenario, progesterone would exert a regulatory role on sCD83 availability in B cells that had left the splenic compartment, which find no impediment in acting in a pro-inflammatory manner. Splenic B cell residents, on the other hand, remain as source of systemic sCD83 that may contribute to pregnancy maintenance.

As proposed for sCD83, certain soluble factors as TNFA, TGFA and TACI are derived by cleavage from their latent or the extracellular domains of their respective membrane proteins in a process that is mediated by metalloproteinases from the ADAM family (Murphy 2008, Hoffmann *et al.* 2015). These metalloproteinases can be produced by the same lymphocytes that express such factors or they can be released by cells of the stromal cells into the extracellular matrix. Metalloproteinases and their inhibitory molecules have critical roles during pregnancy, and their synthesis and release are under hormonal control. We found that while progesterone treatment reduces sCD83 release, ADAM10 expression is reduced and ADAM17 is unchanged in B cells (data not shown). Because this is associated to a reduced mCD83 expression and TIMP1 expression, it seems unlikely that these ADAM proteases are involved in the sCD83 release. The mechanisms of sCD83 release need further evaluation.

Overall, our data indicate that matrix components regulate mCD83 expression during pregnancy impacting on sCD83 availability. These components may be compromised in pro-inflammatory pregnancies, leading to altered sCD83 release.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

R E and K P performed experiments, analyzed data and contributed to the elaboration of the manuscript. J E and A T performed experiments and analyzed data. M Z contributed with reagents, the design of experiments and the writing of the manuscript. D M conceived and designed the experiments, analyzed data, contributed with reagents, wrote the paper and supervised the work.

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