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Progesterone treatment enhances the expansion of placental immature myeloid cells in a mouse model of premature labor

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ABSTRACT

Introduction: immature-myeloid cells (IMCs) are proangiogenic bone marrow (BM)-derived cells that normally differentiate into inflammatory cells such as neutrophils, monocytes and dendritic cells (DCs). We characterized placental IMCs comparing their gene expression and subpopulations to tumor IMCs, and tested our hypothesis that progesterone that inhibits preterm labor, may affect their abundance and differentiation.

Methods: differences between IMC-subpopulations in subcutaneous tumors versus placentas in C57BL/6 or ICR (CD-1) mice were analyzed by flow cytometry and gene expression was detected by microarrays. BM- and placental cells were incubated with or without progesterone and IMC subpopulations were analyzed. For preterm labor induction pregnant mice pretreated or not with progesterone were or were not treated with Lipopolysaccharide (LPS).

Results: we detected enrichment of granulocytic-IMCs in placentas compared to tumors, paralleled by a decrease in monocytic-IMCs. mRNA expression of placenta- versus tumor IMCs revealed profound transcriptional alterations. Progesterone treated BM-CD11b^{+} cells ex-vivo induced enrichment of granulocytic-IMCs and a decrease in monocytic-IMCs and DCs. LPS treatment in-vivo led to an increase in BM-IMCs in both progesterone pretreated or non-pretreated mice. In the placenta LPS decreased the IMC population while progesterone led to complete abrogation of this effect.

Discussion: placental IMCs differ from tumor-IMCs in both subpopulations and gene expression. Progesterone enhances the proliferation of placenta-specific granulocytic IMCs ex-vivo and LPS induced labor is accompanied by a decrease in placental IMCs only in progesterone non-pretreated mice. We thus speculate that the protective effect of progesterone in preventing preterm labor may be explained at least in part by this specific anti-inflammatory effect.

1. Introduction

Immature myeloid cells (IMCs) are bone marrow derived cells that normally differentiate into granulocytes, macrophages, and dendritic cells (DCs), but expand under pathological conditions such as malignancy. DCs are antigen-presenting cells that regulate the adaptive immune response. IMCs were shown to be recruited from the bone marrow and to promote tumor angiogenesis as well as tumor growth and metastasis by direct incorporation into tumor endothelium, modulation of the cytokine environment, release of metalloproteinases, and other mechanisms (Yang et al., 2004; Shojaei et al., 2007; Shojaei and Ferrara, 2008; Yang et al., 2008; Kowanetz et al., 2010; Lu et al., 2012).

We have previously shown that proangiogenic IMCs populate the human placenta and their presence correlates with placental- and birthweight (Mei-Dan et al., 2012) and that the proportion of IMCs infiltrating both placenta and malignant tumors in mice is similar

Abbreviations: IMCs, immature-myeloid cells; BM, bone marrow; DCs, dendritic cells; LPS, lipopolysaccharide; PTB, preterm birth

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(Fainaru et al., 2011, 2013). Furthermore, we demonstrated that IMCs that are recruited into the placenta of pregnant mice express a pro-angiogenic transcriptional signature that significantly overlaps with the global expression pattern of tumor derived IMCs (Pencovich et al., 2013).

Progesterone is known to be pivotal for the maintenance of uterine quiescence in pregnancy (Baulieu, 1989; Zakar and Hertelendy, 2007). Preterm birth (PTB) is one of the most common complications of pregnancy leading to major neonatal morbidity and mortality (Blencowe et al., 2012). Progesterone supplementation is now used wildly for the prevention of PTB. (da Fonseca et al., 2003; Meis et al., 2003; O'Brien et al., 2007; Hassan et al., 2011; Choi, 2017; Romero et al., 2018) The mechanism of progesterone action is not fully understood. One potential effect on the immune system was demonstrated in that cultured murine bone marrow (BM) cells, in the presence of progesterone produced increased numbers of immature DCs. These immature DCs may contribute to tolerance in pregnancy as these cells do not activate T cells (Liang et al., 2006). These findings accord with our previous findings indicating that labor and delivery are preceded by myeloid cell alterations, reflected by a decrease in IMCs and an increase in DCs populating the mouse placenta (Fainaru et al., 2014).

Aiming to shed light on the mechanism by which progesterone may lead to uterine quiescence, we hypothesized that the aforementioned myeloid cell alterations in the placenta may be involved. We first sought to identity placenta specific IMC subpopulations with respect to tumors. This was done by comparing specific phenotypic cell markers defining the granulocytic- versus monocytic IMC subpopulations and by defining specific gene expression patterns. We then tested the effect of progesterone on these myeloid cell populations both ex vivo in bone marrow cell cultures and in vivo in a mouse model of preterm labor.

2. Material and methods

2.1. Experimental models

2.1.1. Mouse model of preterm labor

Experiments were carried out using 6–8 week old ICR (CD-1) female mice, purchased from Harlan Laboratories (Jerusalem, Israel). Pregnant mice were supplied on E8 and allowed to acclimate for 5 days prior to initiating experiments. Animals were maintained in controlled facilities: temperature (25 °C), light (06:00-lights on; 18:00-lights off), with access to food (LabDiet 5001 Rodent Diet, PMI Nutrition International, LLC) and water ad libitum throughout the study. For differences between IMC subpopulations in malignant tumors versus placentas and their differential gene expression patterns mice were sacrificed at E15 and the uterine horns were exposed by midline laparotomy. Placentas were carefully dissected from the decidual tissue and used for further analysis. For evaluation of progesterone effect treatment on myeloid cell populations in the placenta, ICR (CD-1) pregnant mice were or were not pretreated with vaginal progesterone (1 mg/day) (Sigma, St Louis, MO, USA) or carrier (Replens) from day 13 to day 16 of gestation as described in previous studies (Nold et al., 2013; Furcron et al., 2015). In humans, daily administration of 100–200 mg natural micronized progesterone is the most frequently used dosage. 1 mg per/day for a mouse is 10 fold higher dosage and is the dosage commonly used in this model. Because the mechanism by which progesterone prevents PTB is not known we used this high dosage in order to achieve full effect in the mouse. Lipopolysaccharide (LPS) (30 μg) (E. coli serotype 0111; B4, Calbiochem; Merck, Darmstadt, Germany) separated into two doses was administered intrapertioneally at day 15 and at day 16. Four hours after the last dose of LPS, mice were sacrificed, bone marrow was harvested and placentas were dissected as described above, 3 placentas were pooled per sample from each mouse. (Nold et al., 2013; Furcron et al., 2015).

Of note, there are two types of progesterone currently used for prevention of PTB: 1. weekly intramuscular injections of 17α − OHPC and 2. daily administration of natural micronized progesterone. 17α − OHPC has been shown to be effective in preventing PTB in pregnant women with a history of PTB. Micronized progesterone has been shown to be effective for women with short cervical length (Choi, 2017). Natural progesterone was chosen for this study because natural progesterone but not 17α − OHPC has been shown to have anti-inflammatory effects at the murine maternal-fetal interface (Furcron et al., 2015).

2.1.1.1. Culture of myeloid cells. mouse placenta specimens were digested into single cell suspensions as described above. Placenta and BM cells were filtered through a 40 μM cell strainer. Myeloid cells were isolated by magnetic based immunosepartion using positive selection with anti CD11b MACS beads (Miltenyi Biotech, Germany). The BM and placental cells were cultured for five days in the presence or absence progesterone ($1.5*10^{-7}$M). Immunostaining was performed as described above.

2.1.2. Mouse tumor model

Experiments were carried out using 6–8 week old C57BL/6 female mice, purchased from Harlan Laboratories (Jerusalem, Israel). For tumor derived IMCs, Lewis lung carcinoma (LLC) cells (ATCC) were used. Cells were maintained in Dulbecco's modified Eagle medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovineserum (FBS; Invitrogen). Cells were harvested from subconfluent cultures and injected subcutaneously, 1×10^6 cells in 0.2 ml PBS. Mice were sacrificed and tumors were removed when they reached a size of ~ 100 mm3. The entire tumor was minced and processed in order to obtain single cell suspensions for further analysis.

2.2. Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All animal procedures were performed in compliance with the inspection committee on the constitution of the animal experimentation at the Technion (IL-053-04-1.3). Mice were euthanized by controlled release carbon dioxide, and all efforts were made to minimize suffering.

2.3. Flow cytometry

Tumor and placenta specimens, derived from mice as described above, were digested with an enzyme mixture including: 25 μg/ml hyaluronidase (MP biomedical, Solon, OH), 25 μg/ml DNase (Sigma-Aldrich, St. Louis, MO), and 3 μg/ml Liberase (Roche, Nutley, NJ) dissolved in PBS, at 37 °C for 30 min. Digested tissue was then filtered through a 40 μM cell strainer and resuspended in fluorescence-activated cell sorting (FACS) buffer (PBS, 5 mM EDTA, 1% BSA, and 0.05% sodium azide). Immunostaining was performed in the presence of rat anti-mouse Fc receptor III/II (FcgammaRIII/II) (CD16/32; Pharmingen, San Diego, CA, USA), by incubating the cells with monoclonal antibodies for 30 min on ice. Staining reagents included anti-CD11b-FITC (Clone M1/70), anti-CD11c-APC (Clone N418), anti-MHCII-FITC (Clone M5/114), anti-CD45-PE (Clone 30-F11), anti-Gr1-APC (Clone RB6–8C5) anti-Ly6C-PEcy7 (Clone HK 1.4) anti-Ly6G-APC (Clone RB6–8C5) (eBioscience, San Diego, CA). Flow cytometry based cell sorting was performed using FACs Aria (Becton Dickinson, Mountain View, CA, USA).

2.4. RNA extraction and expression analysis

RNA from sorted cells was isolated by EZ-RNA (Biological Industries, Beit Haemek, Israel) and reverse-transcribed, amplified and labeled with Affymetrix GeneChip whole transcript sense target labeling kit. Labeled cDNA was analyzed using Affymetrix mouse ST 1.0

microarrays. Microarrays were scanned using GeneChip scanner 3000 7 G. Microarrays data was normalized using dChip model based expression. Expression data was further analyzed using the Gene Set Enrichment Analysis (GSEA) and DAVID tools.

2.5. Flow cytometry data analysis

Flow cytometry analysis was done using FlowJo 10.1r5 software (Tree Star). Double discrimination of cells was performed prior to every analysis.

2.6. Statistical analysis

Continuous data are presented as mean ± SD. Student *t*-test was performed to compare distributions of samples. $P < .05$ was considered statistically significant.

3. Results

In order to characterize IMC subpopulations in the placenta, we first analyzed the $CD45^+$ hematopoietic cell population in both tumors and placentas. IMC subpopulations were analyzed by flow cytometry in single cell suspensions dreived from placentas (E15) and from LLC tumors grown subcutaneously in mice. We detected a significant enrichment of the $Ly6G^{high}/Ly6C^{med}$ granulocytic IMC subpopulation (over 2-fold, $P < 0.01$) in placenta derived $CD45^+$ hematopoietic cells compared to tumors, paralleled by a concomitant, more than 2-fold decrease ($P < 0.01$) of the monocytic $Ly6G^{med}/Ly6C^{high}$ IMC subpopulation (Fig. 1A).

We next sought to determine whether the observed changes in IMC subpopulations are associated with detectable differences in gene expression. We assessed the global transcriptional signature of tumor derived IMCs (T-IMCs) compared to placental IMCs (P-IMCs) ($CD45^+CD11b^+Gr1^+$ cells). 732 and 420 genes were significantly up- and down-regulated in T-IMCs compared to P-IMCs respectively (Over 2-fold, FDR < 0.05) (Fig. 1B). Analysis of the top overexpressed genes in T-IMCs revealed several key players in tumor angiogenesis including Sema3a (Casazza et al., 2013), Dkk2 (Park et al., 2014), Twist1 (Low-Marchelli et al., 2013), Ddr2 (Zhang et al., 2014), Tm4sf1 (Shih et al., 2009), Pdgfra, Mmp2, Mmp3, Mmp13, Mmp14 and Tgfbr3 (Bandyopadhyay et al., 2002; Yang et al., 2008), as well genes that are involved in cancer progression and cell proliferation including Cep55 (Tao et al., 2014), Nedd4 (Ye et al., 2014), and Prrx1(Reichert et al., 2013) (Fig. 1C). Of note, various genes that were up-regulated in P-IMCs were shown to play a role in reproductive tissue angiogenesis, including Serpine1 (Gomes-Giacoia et al., 2013), Arg1 (Amsalem et al., 2014) Jag1 (Laudanski et al., 2014), Flt1 (Myatt et al., 2013), Gdf15 (Gaafar et al., 2014), Pdgfrn (Cao et al., 2002), and Sfrp4 (Zhang et al., 2013) (Fig. 1C).

We have previously shown that labor and delivery are preceded by myeloid cell alterations (Fainaru et al., 2014). To test our hypothesis that progesterone induces uterine quiescence by altering myeloid cell populations we first tested its effect on BM myeloid cells in culture. We isolated the $CD11b^+$ myeloid cell population from BM by magnetic based immune-separation (gating depicted in Fig. 2A) and cultured these cells for 5 days in the presence or absence of progesterone ($1.5*10^{-7}$M). IMC subpopulations were then analyzed by flow cytometry (Fig. 2B). We observed a significant decrease in the $Ly6G^{med}/Ly6C^{high}$ monocytic IMC population in the presence of progesterone (9.81 ± 1.06 vs 1.12 ± 0.31% of cultured cells, p = 0.001) and a corresponding increase in the $Ly6G^{high}/Ly6C^{med}$ granulocytic IMC population (45.95 ± 4.45, 85.65 ± 3.04% of cultured cells, p = 0.009) (Fig. 2C). Furthermore, progesterone treatment led to a reduction in the $CD45^+MHCII^+CD11c^+$ DC population (gating depicted in Fig. 2D)

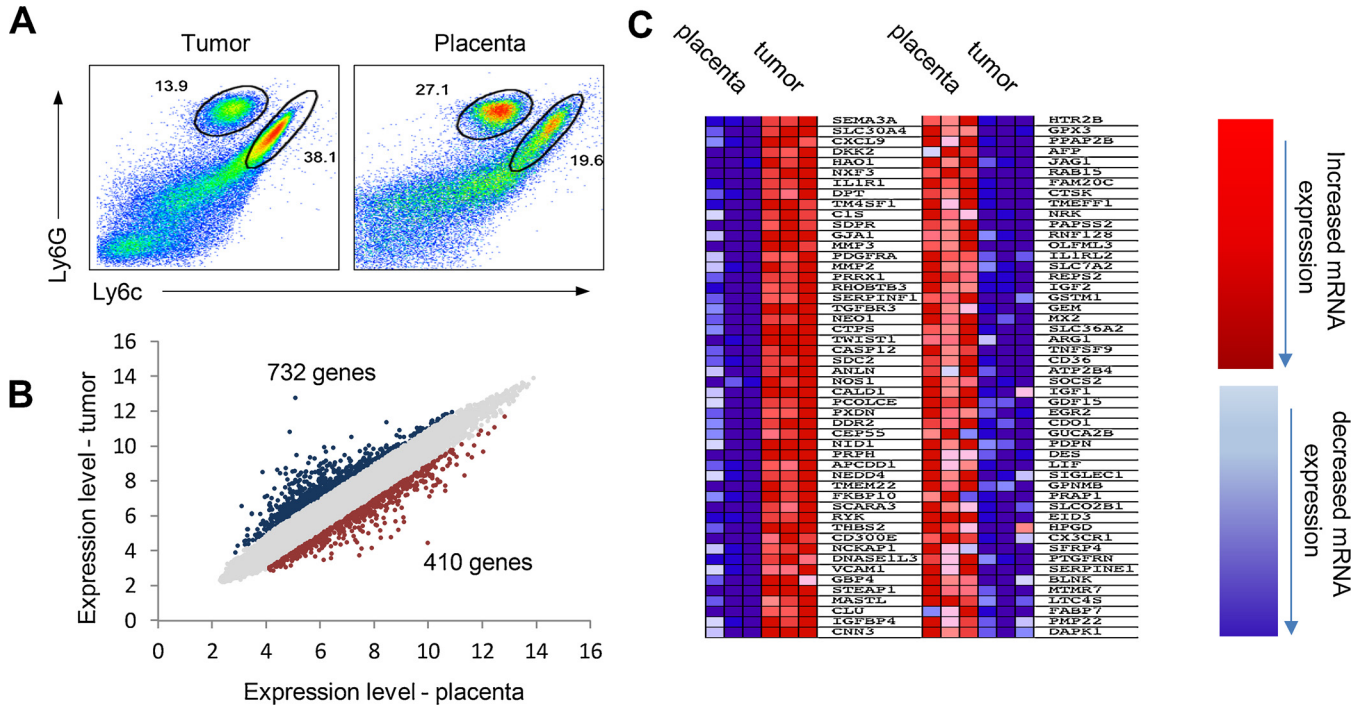

Fig. 1. Differences between IMC subpopulations in malignant tumors versus placentas and their differential gene expression patterns. Single cell suspensions derived from subcutaneous LLC tumors (n = 3) and placentas (pregnancy-day 15) (n = 5), were analyzed by flow cytometry. (A) Representative images of $Ly6G^+Ly6C^+$ IMC percentage out of $CD45^+$ hematopoietic cells in tumor (left) and placenta (right). (B) Genes are plotted based on their average expression level (log2 scale) in $CD11b^+Gr1^+$ IMCs derived from subcutaneous LLC tumors and placentas. Genes showing 2-fold increase or decrease in expression level in tumor compared to placenta derived IMCs are indicated in blue or red respectively. (C) Heat map depicting the top 50 over-expressed genes in tumor versus placenta derived IMCs (left column) and vice versa (right column) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

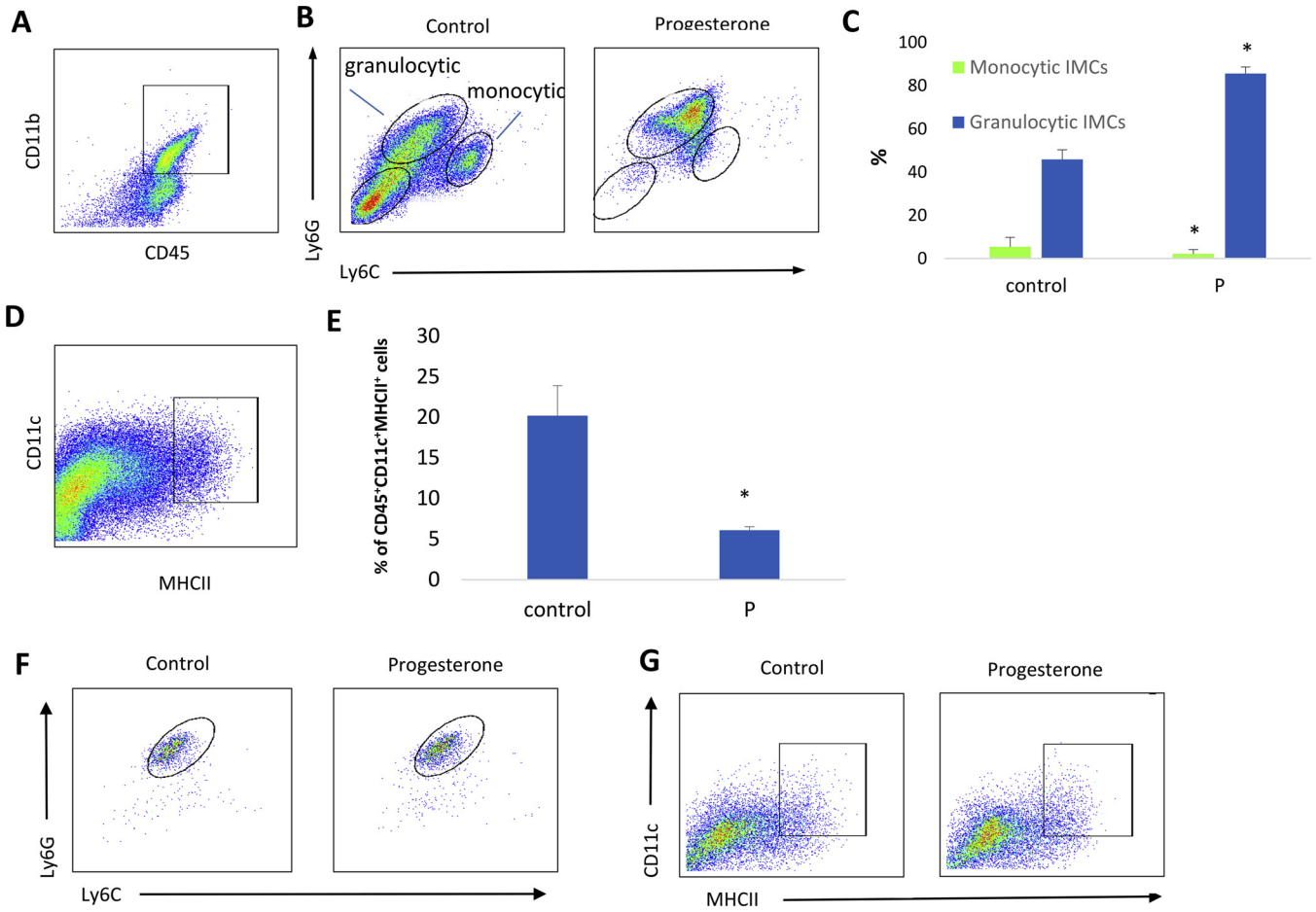

Fig. 2. The effect of progesterone on myeloid cell populations in culture. $CD11b^+$ bone marrow cells or placental cells were isolated by magnetic based immuno separation. These cells were incubated for 5 days in the presence or absence of progesterone ($1.5*10^{-7}$M). IMC subpopuations were gated as $Ly6G^{high}/Ly6C^{med}$ "monocytic" IMCs and $Ly6G^{med}/Ly6C^{high}$ "granulocytic" IMCs in the $CD45^+$ $CD11b^+$ myeloid cell gate (A, B). The effect of progesterone treatment on BM-IMCs (C) The DC population out was gated as $CD45^+CD11c^+MHCII^+$ cells (D). The effect of progesterone treatment on BM-DCs (E), placental IMCs (F) and placental DCs (G) are plotted as mean ± SD; n = 3,3; $p < 0.05$.

(20.20 ± 3.67 vs. 6.09 ± 0.42% of cultured cells, P = 0.03) (Fig. 2E). We next evaluated the effect of progesterone on placenta derived myeloid cells. We cultured mice placental $CD11b^+$ myeloid cells as described above. Intriguingly the alterations observed in BM- derived $CD11b^+$ myeloid cells were not observed in their placental derived counterparts. Specifically we observed no differences in the populations of IMCs (Fig. 2F) or DCs (Fig. 2G).

In light of the observed progesterone effects on cultured bone marrow myeloid cells ex vivo, we next tested the effect of progesterone administration on placental IMCs in a mouse model of preterm labor. ICR pregnant mice were or were not pretreated with vaginal progesterone 1 mg/day or vehicle (Replens) from day 13-16. Mice were then treated by LPS 30 µg or vehicle separated into two doses (day 15 and day 16). 4 h after the last dose of LPS mice were sacrificed and single cells isolated from bone marrow and placentas were immunostained and analyzed by flow cytometry. We analyzed the $CD45^+$ hemaptopoietic placental cell population (Fig. 3A) for the percentage of $CD11b^+Gr1^+$ IMCs (Fig. 3B). LPS treatment led to an increase in IMCs populating the bone marrow in both progesterone pretreated (41.5 ± 9.5 vs. 1.3 ± 0.3%, p = 0.05) or non-pretreated mice (20.8 ± 9.4 vs. 1.3 ± 0.6%, p = 0.05) (Fig. 3C). In the placenta, LPS led to a decrease in the IMC population when compared to untreated controls (Fig. 3D) while strikingly, progesterone pretreatment led to complete abrogation of this effect and maintenance of an intact IMC population (control: 28.2 ± 6.1; progesterone 29.6 ± 4.7 ;LPS: 23.0 ± 2.6; progesterone + LPS: 35.4 ± 2.5% of total $CD45^+$ cells; p = 0.01). Interestingly, the population of DCs (gating as in Fig. 2D) increased upon LPS treatment only in progesterone non pretreated mice, although this effect did not reach statistical significance (Fig. 3E).

4. Discussion

In the present study, we first detected differences in IMC subpopulations infiltrating a physiologic angiogenic tissues (placenta) compared to those infiltrating malignant tumors. Although the overall percentages of $CD11b^+Gr1^+$ cells in placenta and tumor tissues are similar (Fainaru et al., 2011), our current data demonstrates that the placental IMC population is skewed toward a granulocytic subpopulation dominance with over 2-fold increase in $Ly6G^{high}/Ly6C^{med}$ cells compared to the same cell population derived from tumors. It is apparent now that granulocytic and monocytic IMCs are not only phenotypically and morphologically distinct, but also have unique (although partially overlapping) functional characteristics under different physiological and pathological conditions (Bronte et al., 2016; Drabczyk-Pluta et al., 2017).

We have previously shown that tumor and placenta derived IMCs share a common proangiogenic expression pattern when compared to non angiogenic tissue (Fainaru et al., 2013). Development of tumors and placentas represent extreme biological conditions in which a rapid establishment of functional vasculature is required. Nevertheless, placental vascularization is part of an organized and well controlled developmental process as opposed to uncontrolled pathological tumor

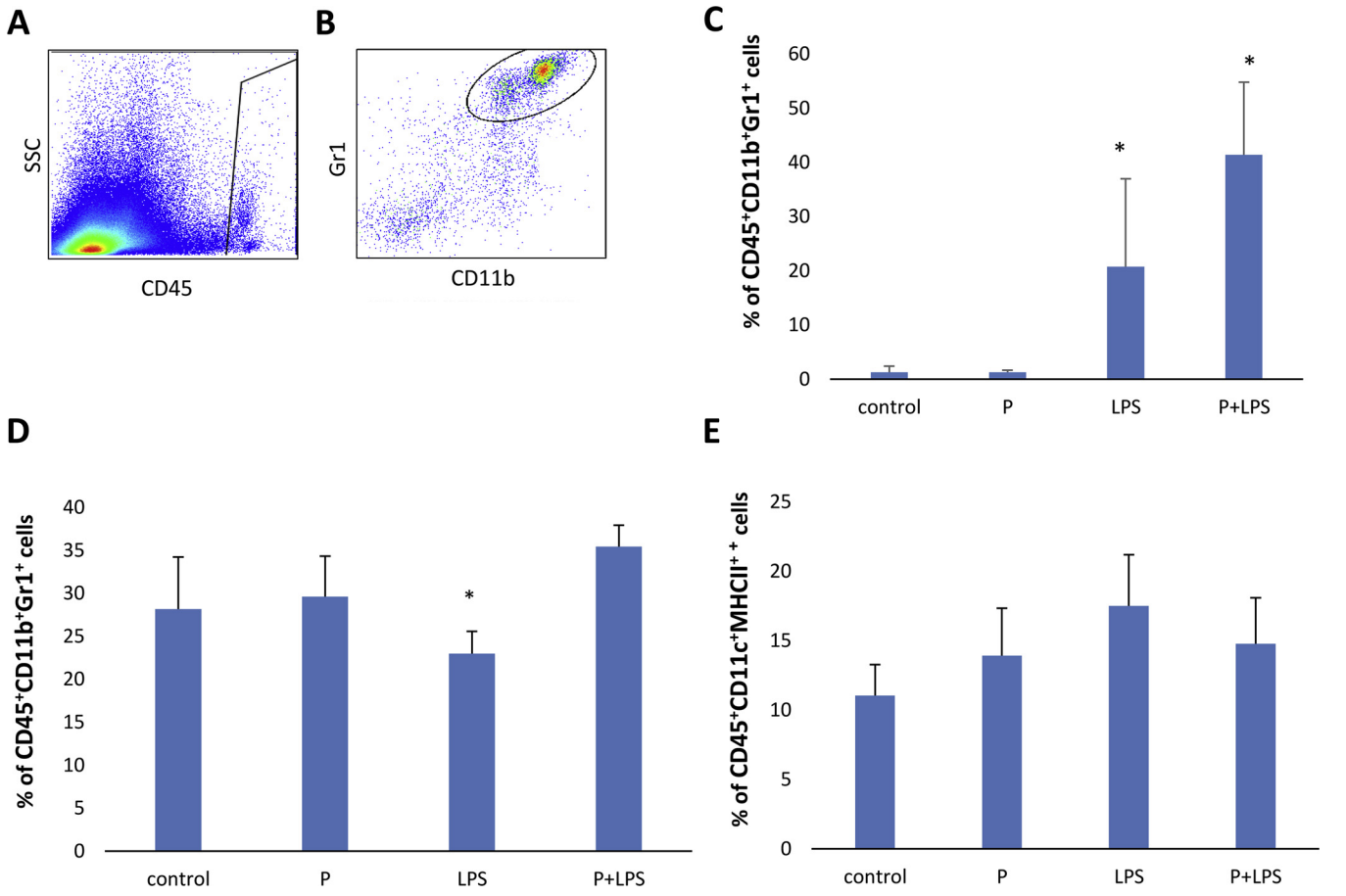

Fig. 3. The effect of progesterone treatment on myeloid cell populations in a mouse model of preterm labor. ICR pregnant mice were or were not pretreated with vaginal progesterone 1 mg/day or Replens from day 13-16. Mice were or were not treated by LPS 30 μg separated into two doses (day 15 and day 16). 4 h after last dose of LPS mice were sacrificed and single cell suspensions derived from bone marrow (n = 3) and placentas (n = 5) were analyzed by flow cytometry. IMCs are gated as $CD45^+CD11b^+Gr1^+$ cells (A,B). The percentage of $CD11b^+Gr1^+$IMCs in BM (C) and placentas (D) out of total $CD45^+$ hematopoietic cells are plotted as mean ± SD. The percentage of $CD45^+CD11c^+MHCII^+$ DCs in placentas out of total $CD45^+$ hematopoietic cells are plotted as mean ± SD (E); n = 7,5,9,8; *$p < 0.05$.

angiogenesis. In this study we compared mRNA expression patterns of placenta- versus tumor derived IMCs and revealed profound transcriptional alterations consisting of hundreds of differentially expressed genes. Analysis of the top overexpressed genes in T-IMCs revealed several key players in tumor angiogenesis as well as genes that are involved in cancer progression and cell proliferation (Fainaru et al., 2013). Of note, various genes that were up-regulated in P-IMCs were shown to play a role in reproductive tissue angiogenesis (Tayade et al., 2005; Jones et al., 2008; Song et al., 2012). The data presented here provide a proof of concept that IMCs differ substantially between normal and malignant proangiogenic tissues not only in their mode of differentiation (granulocytic vs. monocytic) but also in distinct gene expression patterns corresponding to their different functions in these tissues.

Progesterone is known to be pivotal for the maintenance of uterine quiescence in pregnancy (Baulieu, 1989)(Zakar and Hertelendy, 2007) and progesterone supplementation is now used wildly for the prevention of PTB. The anti-inflammatory effects of progesterone have been studied. In one study no effect of vaginal progesterone treatment was demonstrated on macrophage accumulation at the cervices after LPS injection (Nold et al., 2013). Nevertheless, it has been shown to increase the proportion of decidual CD4+ T-regulatory cells and to decrease the proportion of decidual $CD8^+CD25^+Foxp3^+$ T cells and macrophages (Furcron et al., 2015). To test the role of myeloid cells in uterine quiescence and labor we analyzed the effect of progesterone on cultured myeloid cells. When analyzing bone marrow derived myeloid cells ex vivo, we observed a significant increase in the IMC population in the presence of progesterone and a corresponding reduction in the bone marrow DC population. This effect was only seen in placenta specific granulocytic IMCs and not in monocytic IMCs, that are more abundant in tumors and in which an opposite effect was observed. This effect suggests that progesterone contributes to the survival of anti-inflammatory proangiogenic IMCs and limits their differentitaion into inflammatory mature cells such as DCs. Interestingly, this effect was not observed when analyzing placental derived myeloids cells exposed to progesterone ex vivo. It is possible that these cells are already saturated with local high levels of progesterone and therfore do not respond to addition of additional progesterone in vitro.

In a mouse model of preterm labor we observed a similar response when pretreating mice with progesterone. LPS induced inflammation led to an increase in bone marrow IMCs. In the placenta, LPS led to a decrease in IMCs, probably by inducing their differentiation into mature cells such as the observed increase in DCs. This effect was completely abrogated in progesterone pretreated mice. Previously we showed that labor and delivery are preceded by a decrease in IMCs and an increase in DCs populating the mouse placenta (Fainaru et al., 2014). Our results suggest that progesterone may help keeping IMCs in an undifferentiated state. We thus speculate that the protective effect of progesterone in preventing preterm labor may be explained at least in part by this specific anti-inflammatory effect.

There are chemical, biological, and pharmacologic differences between the two types of progesterone that are used to prevent PTB (as

mentioned above). Natural progesterone decreases myometrial activity, prevents cervical ripening and does not increase the risk of gestational diabetes. However, 17α − OHPC does not decrease myometrial activity, has no effect on cervical ripening and increases the risk of gestational diabetes. (Romero and Stanczyk, 2013) Because of the significantly different nature of the two types of progesterone it is very hard to speculate what will be the effect of 17α − OHPC in our model, however it is an interesting question for future studies. This study unfortunately cannot answer the important question as to what is the right timing to start progesterone treatment to prevent PTB. This is mainly because of the difficulty comparing human and mice gestational ages and the inherent biological differences in their pregnancies. Nevertheless, it is an important question for future studies comparing the efficiency of progesterone supplementation at different stages of pregnancy in order to prevent PTB.

5. Conclusions

We hereby demonstrate that IMC subpopulations infiltrating the placenta differ from those that infiltrate tumors in both cellular phenotype and in gene expression. Progesterone that has been shown to inhibit premature labor, enhances the proliferation of bone marrow derived granulocytic IMCs ex vivo. In mice, inflammation induced labor leads to a decrease in the placental IMC population. This effect was completely abrogated in progesterone pretreated mice. We thus speculate that the protective effect of progesterone in preventing preterm labor may be explained at least in part by this specific anti-inflammatory effect.

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Conflict of interest

None

Acknowledgements

NA

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