Neutrophils induce pro-angiogenic T cells with a regulatory phenotype in pregnancy.

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Abstract

Whilst fundamental in controlling innate immune responses, the role of neutrophils in regulating adaptive immunity is just being appreciated. We report that human neutrophils exposed to pregnancy hormones progesterone and estriol promote the establishment of maternal tolerance through the induction of a population of $CD4^+$ T-cells displaying a $GARP^+CD127^{lo}FOXP3^+$ phenotype following antigen activation. Neutrophil-induced (ni)T-cells produce IL-10, IL-17 and VEGF, and promote vessel growth *in vitro*. Neutrophil depletion during murine pregnancy leads to abnormal development of the fetal-maternal unit and reduced empbryo development, with placental architecture displaying poor trophoblast invasion and spiral artery development in the maternal decidua, accompanied by significantly attenuated niT-cell numbers in draining lymph nodes. Using CD45 congenic cells, we show that induction of niT-cells and their regulatory function occurs via transfer of apoptotic neutrophil-derived proteins including FOXO1 to T-cells. Unlike in healthy pregnancies, neutrophils from blood and placental samples of pre-eclamptic women fail to induce niT-cells as a direct consequence of their inability to transfer FOXO1 to T-cells. Finally, neutrophil-selective FOXO1 knockdown leads to defective placentation and compromised embryo development similar to that resulting from neutrophil depletion. These data define a novel, non-redundant function of neutrophil-T-cell interactions in the regulation of vascularisation at the maternal-fetal interface.

Significance

Neutrophils are typically known as short-lived cells that act as the first line of defence in response to pathogens. However, data are emerging that neutrophils can have wider implications on the immune system and having direct influence on the ensuing immune response. Establishment of successful pregnancy requires immune tolerance at the maternal-fetal interface. Aberrations in normal placental development can lead to complications, including pre-eclampsia. In this study we describe a novel role for maternal neutrophils in maintaining normal pregnancy, through their interactions with T-cells, resulting in a population of T-cells that are both regulatory and pro-angiogenic and are required for normal placental development. Such interactions are absent in patients with pre-eclampsia, suggesting a potential therapeutic target for pregnancy-related pathologies.

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Introduction

Establishment of immune tolerance at the fetal-maternal interface has long been associated with induction of multiple adaptive immunoregulatory mechanisms. For example, presence of indoleamine 2,3 dioxygenase (IDO) can dampen maternal T-cell responses to paternal alloantigens, leading to fetal protection(1). Moreover, the programmed cell death ligand (PDL1) is crucial for maternal tolerance in allogeneic pregnancies (2). Finally, the presence of regulatory T-cells (Treg) is instrumental to the maintenance of maternal tolerance (3), where fetus-specific Tregs remain after pregnancy(4), and can expand in subsequent pregnancies (5). Immune tolerance of the fetus relies on the concerted actions of hormones and cytokines as well as cross-talk between innate and adaptive immune cells (6). Dysregulation of one or more of these components can lead to pregnancy complications including fetal growth restriction, pre-eclampsia and recurrent miscarriage (7).

Neutrophils - classically considered as short-lived cells –provide a first line of defence during infection ensuring tissue restitution to physiology (8, 9). It is now emerging that neutrophils may also regulate specific processes typical of adaptive immune responses (10), including antibody production from B-cells (11), T-cell suppression (12) and antigen presentation by DC (13). The ability of neutrophils to affect adaptive immunity appears to be dictated by their activation status (14). Recent studies suggest the presence of neutrophils in the human decidua that release pro-angiogenic factors(15, 16). However, their direct influence on pregnancy outcome has not been shown.

In the present study, we describe a novel role for neutrophils in the establishment of adaptive tolerance during pregnancy. We demonstrate that a population of neutrophils, generated by exposure to pregnancy hormones, is able to induce a unique population of T-cells that have regulatory-like and proangiogenic phenotypes. These neutrophil induced (ni)T-cells are necessary for normal placental vascularization and fetal growth during allogeneic pregnancy.

Materials and Methods (Detailed experimental procedures can be found in the Supplemental file)

Healthy volunteer and Patient samples

All volunteers gave informed consent prior to participating in the study.

For *in vitro* experiment blood was collected from healthy male volunteers (aged between 21-35) between 10 and 11am, approved by the East London & The City Local Research Ethics Committee (Rec Ref. QMREC2014.61 London, United Kingdom). For patient samples, blood and placental samples from healthy and pre-eclamptic women were collected at University College, approved by the London and the South-East Research Ethics Committee (Rec/Ref. 13/LO/0287, London, United Kingdom). Supplemental Table 1 (SI Appendix) illustrates the

patient demographics. Both healthy volunteer and patient bloods were collected into 3.2% sodium citrate.

Neutrophil depletion and reconstitution during allogeneic pregnancies

8-12 week old Balb/C males were mated with aged-matched C57 BL/6 females. Following identification of vaginal plug, circulating maternal neutrophils were depleted at days 5 and 8 using a monoclonal neutralizing antibody (Biolegend, clone IA8; 50μg, i.v.). Neutrophils were reconstituted at indicated times during the pregnancy. Neutrophils were made from bone marrow progenitors. Progenitors were isolated via negative selection (Stem Cell Technologies). Following an initial lentiviral transduction (24hrs) to knock down FOXO1, cells were washed and incubated in IMDM medium supplemented with 20% FCS and 100ng/ml recombinant murine GCSF for 5 days. Female CD45.1 (Ly5.1) mice were kindly provided by Professor Andrew Cope, King's College London. Mice were originally obtained from Charles River (Italy). To check for congenic marker following adoptive transfer of donor CD45.1 neutrophils, cells were stained with alexa fluor 488-conjugated anti-mouse CD45.1 (clone A20; Biolegend) along with efluor 450-conjugated CD45.2 (clone 104; eBioscience).

Imaging

Neutrophils and autologous T-cells were co-cultured as described above in Nunc™ Lab-Tek™II Chamber Slides (Fisher Scientific). Following co-culture, cells were fixed, then permeabilized and blocked for 3hrs with PBS containing 0.1% fish skin gelatin and 1% fetal calf serum. anti-human MRP8 (clone CF-145 Abcam) at 4°C overnight, followed by staining with secondary antibodies mouse Alexa-555 and mouse Alexa-488 along with nuclear stain DAPI (Life Technologies) Human cells were labeled with mouse anti- Human FOXO1/FKHR MAb (Clone 597554) (R&D Systems).Images were acquired using a LSM 510 META laser scanning confocal microscope

(Zeiss) equipped with a 63x Plan-Apochromat objective.

Mouse placentas were embedded in paraffin and cross-sectional sections taken in order to identify all placental layers. Antigen retrieval was carried out using sodium citrate buffer and stained for haematoxylin and eosin. Some placental sections were also stained for CD31 (AbD Serotec, clone ER-MP12), Cytokeratin-7 (Santa Cruz Biotech; FITC conjugated, clone RCK-105) and DAPI (life Technologies). H&E images were acquired on a Pannoramic 250 High Throughput Scanner at X43 magnification. Immunofluorescence images were acquired on an Ariol System at X20 magnification. Image analysis was performed using Image J (National Institute of Health) and scale bars were added onto the overlaid images

Statistics

Data were analyzed by one- or two-way ANOVA followed by Bonferroni's post hoc test using GraphPad Prism 5. Alternatively, several experiments were analyzed by Student's t-test. Data are presented as mean ± s.e.m., and values of $P < 0.05$ were considered statistically significant.

Results

Human neutrophils exposed to pregnancy hormones induce T-cells with a regulatory-like phenotype and proangiogeneic activity

To study a potential role for neutrophils in maternal tolerance, we tested whether neutrophils exposed to pregnancy hormones could affect T-cell responses. Neutrophils from healthy male donors were used to minimize any effects from endogeneous progesterone and estrogen. Male cells express the same levels of estrogen and progesterone receptors on leukocytes as females (17) and therefore respond to both hormones. Neutrophils (SI Appendix, Fig S1A) were incubated with either progesterone (P, SI Appendix, Fig S1B), or the placental estrogen estriol (E3), or in combination (referred hereafter as E3P) at 100ng/ml - physiological pregnancy levels. Treatment of neutrophils for 30min with E3P led to a distinct $CD16^{lo}CD62L^{lo}CD11b^{lo}AnxA1^{hi}$ phenotype (Figure 1A), typical of an anti-inflammatory/quiescent neutrophil status (14, 18). Addition of E3P-neutrophils to autologous CFSE-labelled T-cells (+anti-CD3, anti-CD28 mAbs), enhanced T-cell proliferation (Figure 1B). These T-cells produced significant levels of IL-10, IL-17, IL-2 and IL-5, but not IFN-ϒ, compared to T-cells activated in the presence of vehicle-treated neutrophils or on their own (Figure 1C, SI Appendi, Fig S1C). Neutrophils cultured alone did produce cytokines, confirming their purity and absence of contaminating monocytes (SI Appendix, Fig S1C).

Given the increased levels of regulatory cytokines detected in E3P-treated neutrophil-T-cell co-cultures, we defined their phenotypic and functional characteristics. A significant increase in the proportion of total CD4+CD45RO+ T-cells expressing high levels of FOXP3 was quantified in the

divided T-cell population, with low levels of CD127 and high levels of the latency associated peptide receptor Glycoprotein A Repetitions Predominant (GARP) (Figure 1D, SI Appendix, Fig S1D and S2A). The induction of Tregs by E3P-treated neutrophils relied upon TCR stimulation, since neutrophils failed to induce FOXP3 expression in unstimulated T-cells (SI Appendix, Fig S2B). Co-culture with neutrophils led to equal expansion of CD4+CD45RO+ T-cells from the starting naïve T-cell population, regardless of neutrophil hormone treatment (Figure 1E, left panel). However, expression of FOXP3 and GARP were induced in CD45RO+ T-cells selectively when co-cultured with E3P neutrophils (Figure 1F right panel), indicating that this pathway of differentiation takes places during T-cell priming. Further analyses revealed that these neutrophil-induced T-cells (referred to hereafter as niT-cells) co-expressed GARP and PD1 (SI Appendix, Fig S2C) – a phenotype typical of functionally suppressive human TCR-activated FOXP3+ Tregs(19). Suppression assays revealed that niT-cells were also functionally suppressive (SI Appendix, Fig S2D), which was cell contact dependent (Figure S2D). This suggested signaling of neutrophil to T-cells during activation either by direct cell-to-cell contact or through the release of large cell material (>0.4μm). The hormone effect on neutrophils was specific to E3P, since treatment of neutrophils with 3rd trimester levels of estrone, 17-β-estradiol or β-human chorionic gonadotrophin (in the presence or absence of 100 ng/ml progesterone) did not induce ni-T-cells following co-culture (SI Appendix, Fig S3A). Neutrophils from healthy female donors (regardless of stage of menstrual cycle) induced niT-cells even without stimulation with E3P (SI Appendix, Fig S3B and C), likely consequent to continued exposure to high level progesterone since this hormone on its own was sufficient induce niT-cells (SI Appendix, Fig S1D)

Further analyses revealed niT-cells produce IL-10 and IL-17 (Figure 2A). IL-17-producing regulatory T-cells have been described (20). IL-17 has been shown to have pro-angiogenic functions by inducing the release of growth factors including VEGF (21). Supernatants from T-cells co-cultured with E3P neutrophils also contained significant levels of VEGF. To confirm the VEGF production in our system was induced by IL-17, an anti-IL-17 neutralizing strategy was

employed, which impaired VEGF production (Figure 2B). More specifically, intracellular staining confirmed VEGF was produced by niT-cells (Figure 2B). The pro-angiogenic function of niT-cells was analysed *in vitro* using Matrigel-based assays. Figure 2D shows supernatants from niT-cells (E3P), but not controls, significantly augmented both vessel length and number of branch points and was inhibited upon IL-17 or VEGF neutralization.

Depletion of neutrophils during pregnancy leads to impaired embryo and placenta development.

Although activated neutrophils have been implicated in the pathophysiology of pre-eclampsia (22), very little is known about the physiological role of quiescent neutrophils in pregnancy, despite their increased circulating numbers during gestation (23). Our observations in the human system *in vitro* indicated a potential protective role for quiescent neutrophils in pregnancy. We therefore sought to validate this hypothesis in murine pregnancy. Pregnant C57BL/6 females previously mated with BALB/c males, were treated with anti-Ly6G monoclonal antibody (clone IA8, 50μg i.v.; FigureS3) to deplete maternal neutrophils. Depletions were performed on days 5 and day 8 of pregnancy – a time of active placenta development in the mouse (24).

Analysis of pregnant mice at day 12 revealed systemically fewer $Ly6G^+$ neutrophils in neutrophil depleted mice, compared to isotype control-treated mice. Importantly, neutrophil depletion led to a significant reduction in the numbers of $CD4^+FOXP3^+$ Tregs retrieved in the draining lymph nodes (Figure 3A, SI Appendix, Fig S4). The physiological role of niT-cells in pregnancy was confirmed by the finding that neutrophil-depleted mothers carried significantly smaller fetuses (Figure 3B and C) and placentas (Figure 3C and D) compared to control pregnancies, a phenotype typical of with pre-eclampsia in humans (25).

Histological analyses of placentas in neutrophil-depleted mice revealed a disorganised structure, with a reduced thickness of the maternal decidua, which contains the maternal spiral arteries (decidua basalis; Db (Figure 3E), coupled with poor trophoblast invasion into the maternal layer (Figure 3F). Both fetal and placental size are determined by maternal utero-placental perfusion of

the placenta, which requires co-ordinated trophoblast invasion and recruitment and remodelling of the maternal spiral arteries (26). Given that IL-17 can promote trophoblast invasion *in vitro* (27), it is conceivable that niT-cell-derived IL-17 might play a role in this event. Loss of peripheral (induced) Tregs is known to hinder placental development (28). Collectively, this evidence suggests that maternal neutrophils are required for efficient utero-placental circulation.

Transfer of neutrophil-derived FOXO1 is required for niT-cell induction.

Neutrophils can regulate specific host responses through the release of microstructures, including neutrophil extracellular traps (NETS)(29), apoptotic bodies and microvesicles, which contain a variety of immune-modulatory proteins (30). Exposure to E3P induces a pro-apoptotic neutrophil phenotype with low surface expression of CD16 (31) (Figure 1A). Conversely, the cytokine GM-CSF, which promotes leukocyte survival (32), attenuated the proportion of late apoptotic neutrophils (Annexin-V+, PI+) by 30% and 50% in control and E3P-treated neutrophils, respectively (Figure 4A). Time-course analyses indicated that neutrophil apoptosis following exposure to E3P begins at 24hrs of co-culture (SI Appendix, Fig S5A).

To gain insight into the mechanism of niT-cell induction we investigated the ability of E3P- and/or GM-CSF-treated neutrophils to transfer cytoplasmic material (monitored through myeloid related protein 8 [MRP8] staining) to T-cells. Whereas treatment of neutrophils with E3P significantly promoted transfer of neutrophil proteins to T-cells leading to the generation of niT-cells, this response was reduced and/or inhibited when neutrophils were treated with GM-CSF (Figure 4B, 4C). Finally, induction of FOXP3 expression was specific to neutrophil-derived apoptotic bodies, since co-culture of T-cells with monocytic apoptotic bodies was ineffective (SI Appendix, Fig S5B).

Next, we sought to identify the molecular interactions mediating niT-cell induction. We first interrogated the role of the neutrophil-abundant protein Annexin-A1 (AnxA1) since its upregulation is a feature of an anti-inflammatory neutrophil phenotype as promoted by female sex hormones (14, 18). Pre-treatment of freshly isolated human neutrophils with recombinant AnxA1, at an optimal concentration of 3nM, reproduced E3P exposure and subsequent niT-cell induction. However, direct addition of AnxA1 to T-cells failed to do so (Figure 5A). These observations were further confirmed by experiments with neutrophils and T-cells from AnxA1 KO (33) and WT mice. Neutrophils from AnxA1KO or WT mice were treated with E3P and co-cultured as described above. E3P-treated neutrophils from WT animals induced FOXP3 expression equally in WT T-cells and AnxA1 KO T-cells. In contrast, neutrophils from AnxA1 KO mice failed to induce FOXP3 expression in T-cells irrespective of hormone treatment (Figure 5B, SI Appendix, Fig S6A)**.** Taken together, these data indicate that AnxA1 does not directly induce differentiation of niT-cells, but it may facilitate the transfer of neutrophil-derived signalling proteins necessary for niT-cell formation.

The transcription factor FOXO1 is the master regulator necessary for FOXP3 expression in inducible Tregs(34-36). Intriguingly, FOXO1 is expressed by neutrophils where it is endowed with pro-apoptotic functions(37). Both estrogen and progesterone can up-regulate FOXO1 in the placenta during pregnancy and this transcription factor is important for normal placental development and decidualization (38, 39). Therefore, we tested whether E3P exposure up-regulates FOXO1 expression in neutrophils, which upon apoptosis could then transfer the transcription factor via apoptotic bodies to induce FOXP3 expression in the targeted T-cells. Confocal microscopy revealed ~30-fold increase in FOXO1 expression in neutrophils treated with E3P compared with untreated cells (Figure 5C). Subsequently, EP3 neutrophils were labelled with anti-FOXO1 antibody by brief osmotic shock and kept overnight in serum-free media to generate neutrophil apoptotic bodies. Feeding these apoptotic bodies to autologous T-cells effectively transferred substantial amounts of neutrophil-derived FOXO1 to T-cells (Figure 5D) and FOXP3

expression, (Figure 5E), while this transfer did not occur with apoptotic bodies from control neutrophils. Consistent with a causal link between FOXO1 transfer via apoptosis and niT-cell induction, pharmacological inhibition of FOXO1 significantly inhibited their ability to undergo apoptosis (Figure S6B) and failed to induce Tregs (Figure 5F). Neutralization of AnxA1 on E3P-derived neutrophil apoptotic bodies significant attenuated transfer of FOXO1 via neutrophil apoptotic bodies on induction of FOXP3 (Figure 5G – compare with Fig 5D).

Activated neutrophils from pre-eclamptic pregnancies fail to induce niT-cells

Many of the pathological features we observed in our in vivo model are compatible with those previously reported in pre-eclamptic pregnancies, including loss of neutrophil quiescence(22) and reduction of circulating Tregs (40). Preeclampsia is a multi-organ syndrome of pregnancy that most commonly affects first time pregnancies in the second trimester of pregnancy (2-7%) and it is a leading cause of maternal and fetal morbidity and mortality(41). Lower levels of estriol have been associated with this pathology (42). Whilst the vascular alterations occurring in pre-eclampsia are well established (43), the impact of immune components in its pathogenesis is less clear (7). Hence, we next interrogated the role of neutrophils in niT-cell induction preeclmpsia.

We observed a significant attenuation in plasma levels of both progesterone and estriol in pre-eclamptic women (SI Appendix, Fig S7), when compared to healthy pregnancies. Next we compared neutrophil phenotypes between healthy and pre-eclamptic patients (Table S1). Elevated CD11b expression in neutrophil from pre-eclamptic patients has previously been reported (22). Our own analysis demonstrated that blood and placental neutrophils from pre-eclamptic patients displayed an activated phenotype with high levels of CD16, CD62L, CD11b, but lower levels of AnxA1 (Figure 6A; SI Appendix, Fig S8A and B). In this context, we note how defective AnxA1 signalling has been associated with pre-eclampsia as it may result from autoantibodies to the protein or by a reduced placental expression of its receptor FPR2/ALX(44, 45). To make a direct

comparison with the observed attenuation in fetal size following our *in vivo* neutrophil depletion model during pregnancy (Figure 3), we could quantify that offspring born to our pre-eclamptic group had a significantly lower birth weight centile compared to offspring born to mothers within our healthy group (pre-eclampsia: 22nd centile, range 11th to 35th centile *vs.* healthy: 52nd centile; range 44-68 centile), suggesting fetal growth restriction in our pre-eclamptic population, akin to our mouse model.

We also quantified that a lower proportion ~10% over 24h neutrophils from pre-eclamptic patients entered apoptosis (Vs 20% of healthy) (Figure 6C), suggesting attenuated apoptotic body formation. In line with this hypothesis and congruent with the data in human neutrophils and T-cells, a significant reduction in the transfer of neutrophil-derived MRP8 and AnxA1 in pre-eclamptic patient-derived T-cells/neutrophil co-cultures compared to co-cultures of cells from healthy pregnancies was observed (Figure 6C). This finding was also associated with attenuated FOXO1 transfer to T-cells from neutrophils from pre-eclamptic patients, compared to their healthy counterparts (Figure 6D).

In line with other reports (40, 46), a significant reduction in circulating and placental CD4+FOXP3+ Tregs was observed in pre-eclamptic patients (SI Appendix, Fig S8C, D). We therefore compared the ability of neutrophils isolated from pre-eclamptic and healthy donors to induce niT-cells. Co-culture of freshly isolated neutrophils and T-cells from healthy samples (blood and placental) led to the induction of functionally suppressive niT-cells (SI Appendix, Fig S9A-C), which produced both IL-10 and IL-17 (SI Appendix, Fig S9 D,E). In contrast, neutrophils from pre-eclamptic patients failed to induce niT-cells, and the minute proportion of these cells found in the co-cultures displayed low levels of IL-10 and IL-17 (SI Appendix, Fig6E and S9F). Importantly, treatment of neutrophils from pre-eclamptic patients with E3P rectified their defect and led to induction of niT-cells and ensuing production of IL-10 and IL-17 (Figure

6E, SI Appendix, Fig S9 D, E). Production of IL-17 by niT-cells may deliver important functions, including microbial defence mechanism and promotor of angiogenesis (21), both of which are vital during pregnancy.

FOXO1-deficient neutrophils fail to induce niT-cells and to restore embryo and placental development.

To validate the mechanistic importance of neutrophil-derived FOXO1 transfer to T-cells, we investigated whether neutrophil-derived protein could be transferred to T-cells *in vivo* and whether loss of neutrophil-derived FOXO1 was responsible for the pregnancy defects observed following neutrophil depletion.

We tested the former by injecting CSFE-labelled neutrophils at day 6 of pregnancy, following an initial neutrophil depletion at day 5. Spleen, uterine-draining paraaortic lymph nodes and non-draining brachial lymph nodes were harvested 48hrs later. As shown in SI Appendix, FigS10A, T-cells from draining lymph nodes expressed high levels CFSE, whilst spleen and non-draining lymph nodes T-cells did not. More specifically, a large proportion of $CD4^+FOXP3^+$ T-cells expressed $Ly6G^+$ $CFSE^+$ (SI Appendix, Fig S10B), suggesting transfer of neutrophil-derived products to T-cells *in vivo.*

To validate the relevance of the FOXO1/FOXP3 axis to niT-cell generation in pregnancy, we selectively knocked-down FOXO1 in murine neutrophils using lentiviral delivery. Two different short-hairpin RNAs (shRNAs) specific for FOXO1 (FOXO1 30 and FOXO1 42) and a non-specific control were transduced into bone marrow progenitor cells (SI Appendix, Fig S11A and B). After 24 h, progenitor cells were stimulated with GCSF for 5 days to develop into $Ly6G^+$ neutrophils (~80% following puromycin treatment; SI Appendix, Fig S11C). *In vitro*, FOXO1-sufficient neutrophils were capable of inducing niT-cells when co-cultured with naïve T-cells,

whilst FOXO1-deficient neutrophils were ineffective (SI Appendix, Fig S12A). This correlated with a reduced ability to induce IL-10, IL-17 and VEGF (SI Appendix, Fig S12B-D).

In a final series of experiments, we wanted to establish the physiological relevance of this mechanism *in vivo*. To this end, we reconstituted neutrophil-depleted pregnant mice with either FOXO1-sufficient (non-specific) or FOXO1-deficient neutrophils (FOXO1 30 or FOXO42) ($3x10^6$;i.v.) at days 6 and 9 of pregnancy. Neutrophil depletion was performed at days 5 and 8 as already described**.** Pregnant mice were analysed at day 12 of gestation. Similar numbers of $Ly6G^+$ neutrophils were present in the uterine-draining lymph nodes following reconstitution with either FOXO1-sufficient or FOXO1 knockdown neutrophils (Figure 7A, left panel). Similarly, there was no difference in the number of $CD4^+$ T-cells (Figure 7A, middle panel). In contrast, the number of $CD4^+FOXP3^+$ T-cells was significantly decreased (~75%) following reconstitution with FOXO1-deficient neutrophils, compared with FOXO1-sufficient neutrophils (Figure 7A, right panel; SI Appendix, Fig 13A). This was accompanied by diminished proportion of cells making IL-10, IL-17 and VEGF following reconstitution of FOXO1 knockdown neutrophils (SI Appendix, Fig 13 B-D).

To conclusively establish the effects of reconstituted donor neutrophils on Treg induction and to confirm results seen in SI Appendix Fig S11), neutrophils from CD45.1 congenic mice were used (see SI Appendix Figure 13E for putiy of inject donor CD45.1+ neutrophils). Bone marrow progenitors from these mice were transduced, cultured into neutrophils, and finally injected into pregnant CD45.2 recipients. We looked at the presence of donor CD45.1+ neutrophils at two distinct time points after injection: 16 hours and 3 days later. We observed two distinct populations of neutrophils within the draining lymph nodes 16 hours post injection: CD45.2+ resident neutrophils and CD45.1+ donor neutrophils. The proportion of neutrophils did not change significantly despite FOXO1 knockdown, suggesting that FOXO1 is not required for migration of neutrophils to the draining lymph nodes. However, we did not see any CD45.1+

donor neutrophils in non-pregnant controls (Figure 7B, contour plots). After 3 days days post-injection (day 12 of pregnancy), we failed to see any live CD45.1+ donor neutrophils (Figure 7B dot plots). This was expected, as neutrophils need to undergo apoptosis in order to induce Treg in this system. Interestingly, despite the low number of CD45.1 Ly6G+ neutrophils identified in the draining lymph nodes, we still observed no difference in total number of Ly6G+ neutrophils in the draining lymph nodes (Figure 7A, left panel), suggesting migration of endogenous neutrophils to the draining is not affected. This could be due to IL-17 be made by the T-cells in the draining LNs (SI Appendix, Figure S13C) following reconstitution of non-specific transduced neutrophils and to a lesser extent FOXO1 knockdown neutrophils, since this cytokine has been shown to promote neutrophil migration into draining LNs(47).

Despite not detecting CD45.1 neutrophils in the draining, a CD45.1+ signal was detected within the CD4+FOXP3+ T cell population (Figure 7C): importantly, this effect was observed only when mice were injected with CD45.1 neutrophils transduced with non-specific virus, but not with FOXO1-depleted CD45.1 neutrophils. The CD45.1+ signal within the Treg cell population was of neutrophil origin, since it was accompanied by Ly6G staining (this was not seen on CD45.2+ cells; Figure 7C histograms and SI appendix 13F for singlet gating). Moreover, this phenomenon was pregnancy-specific, because injection of CD45.1 neutrophils into non-pregnant females did not yield a CD45.1 signal in the Treg population recovered from the draining lymph nodes, and was not seen in the non-draining lymph nodes or spleen of pregnant mice (Figure 7C and SI Appendix, Figure 13G). Taken together, these data conclusively demonstrate that neutrophils are able to transfer their protein content directly to T-cells in vivo in order to induce Tregs during pregnancy and is hindered in the absence of neutrophil-derived FOXO1.

Both embryo and placenta sizes from pregnant mice reconstituted with FOXO1-sufficient neutrophil were analogous to those of mice which did not undergo neutrophil depletion (Figures

7D-F, left panels), coupled with an organized placental structure and normal trophoblast invasion into the maternal layer (Figure 7G, left panel and SI Appendix Fig 13G). Pregnant mice reconstituted with FOXO1-knockdown neutrophils yielded significantly smaller embryos and placentas (Figures 7D-F, middle and right panels). The placental layers of these mice displayed a disorganized morphology with poor trophoblast invasion and were comparable to placentas following neutrophil depletion (Figure 7G, middle and right panels). This difference in placental phenotype between total loss of neutrophil (depletion) and selective loss of neutrophil FOXO1 suggests that, in addition to niT-cell induction by FOXO1 transfer, maternal neutrophils might exert other direct effects (i.e. niT-cell-independent) on placental development.

Discussion

The ability of neutrophils to impact adaptive immunity has recently emerged (10) though never considered in the context of a regulatory/tolerant phenotype. Here, we describe a novel role for a specific neutrophil functional phenotype – consequent to exposure to maternal and placental hormones - in the induction of a population of pro-angiogenic T-cells with regulatory properties in pregnancy. The differentiation of niT-cells is reliant on AnxA1-facilitated transfer of FOXO1 contained in neutrophil apoptotic bodies to T-cells during activation: niT-cells are required to maintain normal pregnancy outcomes as depletion of neutrophils during pregnancy leads to smaller embryo sizes and abnormal placentation in mice. While the presence of activated neutrophils in the maternal circulation has been previously found to be detrimental in pregnancy complications including pre-eclampsia (22, 48), our observations suggest that a regulatory non-activated pro-apoptotic neutrophil phenotype promoted by pregnancy hormones might be essential for normal placentation, including its vascular development. The cytokine milieu that results from niT-cell generation could favour the establishment of a pro-angiogeneic environment, with both IL-10 and IL-17 promoting vessel development (21, 49) and IL-17 promoting trophoblast invasion (27, 50), both key processes in spiral artery remodelling (26). Additionally, and more specifically,

we have shown that niT-cells make IL-17-dependent VEGF, adding further support to their pro-angiogenic function within the placenta.

Analyses carried out in pre-eclampsia samples provide pathological relevance to this mechanistic work, identifying defects in this novel physiological circuit that may be contributory to poor pregnancy outcomes of pre-eclampsia. During healthy pregnancy, the maternal immune system adapts to allow survival of a partially histo-incompatible fetus, and failure of this adaptation contributes to poor placental invasion that pre-dates the clinical onset of pre-eclampsia. Studies have suggested that impaired expansion of inducible Treg cells (40, 51) particularly in the decidua might represent a pathogenic defect in pre-eclampsia. A key feature of the niT-cell population we describe is its ability to produce IL-17. While there is general agreement that in healthy pregnancy, there is a preferential differentiation of iTreg cells over Th17 cells systemically, the functional significance of Th17 cells in the decidua is controversial. Increased decidual Th17 cell percentage in women with unexplained recurrent miscarriages compared to healthy pregnancy has been described (52). However, another study reported higher percentages of Th17 cells in the decidua in healthy pregnancy (27). Our observations suggest that IL-17-producing niT-cells rather than 'conventional' Th17 effectors - in the placenta might in fact be required to support vascular development in healthy pregnancy, as IL-17 is known to promote angiogenesis and tissue growth (21).

Neutrophil-derived FOXO1 plays an important role in niT-cell induction and the maintenance of embryo size and normal placentation. It is known that FOXO1 is an important transcription factor, which promotes FOXP3 expression in T-cells. However, FOXO1 has broader implications in placentation, and is necessary for normal placental development as indicated by the abnormalities observed in FOXO1 null mice (38). Similarly, the role of Tregs in pregnancy might not be confined to regulating immune responses: broader effects of Tregs on the maternal vasculature

have been reported, with downstream relevance in controlling hypertension and other cardiovascular events (53). Additionally, Tregs can modulate cell-surface proteins on endothelial cells, leading to an anti-inflammatory endothelial cell phenotype (54). Hence, it is tempting to speculate that failure to promote appropriate niT-cell generation, might prevent the establishment of a pro-angiogenic environment within the placenta. Further studies are warranted to confirm this hypothesis.

The induction of niT-cells by neutrophils is likely to occur physiologically in the uterine-draining LNs during the presentation of paternal antigens to circulating allospecific T-cells, as previously reported (55). In this context, we show that neutrophils can access LNs where they exchange cellular material with T-cells. In our system, neutrophil protein transfer occurs selectively in the uterine-draining LNs, the only likely site of active immunity during pregnancy in otherwise healthy mice, suggesting T-cell activation is also required for the transfer of neutrophil material – as we show in our in vitro studies – and that TCR triggering likely induces a permissive status for the 'acceptance' of neutrophilic proteins into T-cells. The molecular features of this permissive status remain to be established.

Importantly, as the induction of a this neutrophil phenotype occurs in the absence of other signals (e.g. inflammatory) as a consequence of exposure circulating hormones, it is conceivable that this functional status might arise in neutrophils systemically during pregnancy and might affect ongoing T-cell activation in sites other than the fetal maternal unit – hence the well described remission of antigenically-unrelated autoimmune responses during pregnancy(56).

In conclusion we report a novel cell-to-cell cross-talk whereby E3P programmed neutrophils to induce proangiogenic T-cells thus enabling sustainable placenta/fetus interface development.

We predict that neutrophil regulatory properties on several facets of the adaptive immune response, and tolerance in particular, could be further identified to decipher the way innate and adaptive immune systems co-operate, providing, on one hand, opportunities for defining new pathogenic mechanisms and, on the other, novel modes for therapeutic interventions.

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Author contributions

SN designed, performed and analyzed experiments, wrote the manuscript. JS performed and analyzed in vivo experiments. MK performed and analyzed experiments. SM performed experiments. ASP analysed experiments. RH performed experiments. CM performed experiments and contributed to manuscript writing. DW provided clinical samples and contributed to manuscript writing. SF, provided funds, designed and analyzed in vivo experiments. FMB designed and analyzed experiments, wrote the manuscript. MP designed and analyzed experiments, wrote the manuscript.

Disclosures

None

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Figure Legends

Figure 1. Human neutrophils treated with estriol and progesterone display a distinct phenotype and induce regulatory T cells from a naïve T cell population

(A) Neutrophils from healthy male donors were treated with progesterone (P; 100ng/ml) or estriol (E3; 100ng/ml) or in combination (E3P) for 30 min, washed. Neutrophil phenotype was analysed by flow cytometry for surface markers CD16 (FcγRIII), CD62L, CD11b and the neutrophil anti-inflammatory protein, Annexin-A1 (AnxA1) **P*<0.05 ***P*<0.01 compared to control treated neutrophils.(B) Neutrophils were treated with hormones as described in (A) and co-cultured with autologous lymphocytes (labelled with 3μM CFSE) at a 1:1 ratio for 5 days in the presence of 2μg/ml soluble anti-CD3 and anti-CD28 antibodies. The scheme of cell treatment is reported in Figure S1b.* P<0.05 compared to control. (C) Supernatants were collected from co-cultures, from lymphocyte or neutrophil cultured on their own, in the presence or absence of P and E3 (100 ng/ml each). Cytokines levels were quantified using a multiplex assay. *P<0.05 compared to control treatments. (D) Following culture, lymphocytes or lymphocytes that had been co-cultured with neutrophils (with or without estriol and progesterone) were stained for CD4, CD45RO and FOXP3, as well as GARP and CD127. *P<0.05, compared to medium. (E) CD45RA naïve T cells were isolated by negative selection from healthy male donors and co-cultured at 2:1 ratio with control- or E3P-treated neutrophils for 5 days (plus 2μg/ml of soluble anti-CD3 and anti-CD28 antibodies).

Figure 2. Human neutrophil-induced T-cells secrete regulatory cytokines and are pro-angiogeneic.

(A) Control (white bars) or E3P (green-lined bars) CD4+ lymphocytes co-cultured with neutrophils were stained for intracellular FOXP3 and IL10 (Gated on CD4+FOXP3+; left panel; **P<0.01 compared to medium) or IL17 (right panel; *P<0.01). ((**P<0.01). (B) ELISA for VEGF carried out from supernatnats of co-cultures decribed in 1(A). In some cases, IL-17 was blocked during co-culture at a concentration of 630ng/ml, which was the optimal dose to inhibit the level of IL-17 released in these co-cultures (950pg/ml). As a positive control, recombinant human IL-17 was added to media at the same concentration found in our co-cultures. (C) Intracellular staining for VEGF in $CD4^+$ T cells following co-cultures described above. (D) Vessel growth assay using growth factor-reduced Matrigel. *P< 0.05 compared to control;♯P<0.05 compared to E3P. In all cases data are mean ± SEM of 3-5 experiments with 3-5 donors per experiment

Figure 3. Neutrophil depletion during allogeneic pregnancy leads to abnormal placentation
Balb/C males were mated with C57 BL/6 females. Following identification of vaginal plugs, circulating maternal neutrophils were depleted using a monoclonal neutralising antibody or some pregnant females were treated with isotype control antibody (both antibodies at 50µg, i.v.) at day 5 and day 8 and pregnant animals sacrificed at day 12. (A) Absolute numbers of $Ly6G^+$ neutrophils, $CD4^+$ T-cells and $CD4^+FOXP3^+$ Tregs in the draining paraaortic lymph nodes. *P<0.05 **P<0.01 compared to isotype control-treated animals. (B) Crown to rump measurements were taken to determine embryo lengths (C) differences between placenta diameter from isotype control and neutrophils depleted mice *P<0.05 **P<0.01 compared to isotype control-treated animals. (D) Representative images of embryos and placentas from isotype control and neutrophil-depleted animals. Scale bar = 2.5mm. (E) H&E staining of placentas from two groups of mice to look at organisation of placental layers (dotted lines). Gi giant cell trophoblasts; Db decidua basalis; Jz junctional zone; Lz labyrinthine zone. Scale bar = 1000µm. (F) Immunofluorescent staining of placentas for CD31 (red), trophoblasts (cytokeratin-7; green) and cell nuclei with DAPI (blue). Dotted lines indicate placental layers as described in (E). Yellow dotted square indicates zoomed in section of the Db layer to look at trophoblast invasion more closely. Invasion of trophoblasts into the maternal layer was quantified using Image J software. **P<0.01 compared to isotype control. In all cases data mean ± SEM of 2-3 experiments with 3-4 animals per group.

Figure 4. Human neutrophils induce Tregs via apoptotic bodies.

(A) Control and E3P-treated (see Figure 1) neutrophils were incubated with or without GMCSF (50ng/ml) in co-culture with autologous T cells. Following 24hr co-culture, cells were stained with Annexin-V and propidium iodide (PI). Based on forward/side scatter, neutrophils were gated and the degree of apoptosis (**P<0.01) compared to control. (B) T cells were stained for CD4 (yellow) MRP8 (red) and DAPI (blue) and analyzed by confocal microscopy (***P<0.001 compared to without GMCSF) (scale bar, 7-μm). (C) Flow cytometric analyses of niT-cells induction in the presence or absence of GMCSF with respect to CD45RO, FOXP3, CD127 and GARP; ***P<0.001. In all cases data mean ± SEM of 4-5 experiments with >three distinct donors per experiment.

Figure 5. Neutrophils induce FOXP3 to T cells via the transfer of FOXO1.
(A) The effect of recombinant human AnxA1 on niT-cells induction was assessed by treating either T cells alone (white bars) or co-culture following direct treatment of neutrophils (green bar). ***P*<0.01 compared to neutrophil co-culture in the absence of AnxA1 treatment. (B) Neutrophils and splenic T cells were isolated from male WT and AnxA1 KO mice. Neutrophils were treated E3P (green lined bars) or left untreated (white bars) and co-cultured (at 1:1 ratio) for 3 days with autologous or counterpart (WT or KO) T cells, stimulated with 2μg/ml anti-CD3 and anti-CD28 antibodies. T cells were stained for CD4 and FOXP3 (***P<0.001 compared to T cells in absence of neutrophils). (C) Neutrophils from male donors were treated with progesterone and estriol (E3P; 100ng/ml each) for 30 min, washed and stained with DAPI and mouse anti-human FOXO1 (10μg/ml) in 0.1% Triton-X to gently permeablize the cells, prior to adding secondary Alexa-555 antibody. FOXO1 expression by neutrophils was quantified by confocal microscopy. Scale bar 7μM. **P<0.001 compared to control. (D) FOXO1 protein contained by E3P neutrophils were labelled with a fluorochrome-conjugated antibody as in (B). After 18hr incubation, FOXO1-labelled apoptotic bodies were collected and co-cultured with autologous T cells for 3 days in the presence of 2μg/ml soluble anti-CD3 and anti-CD28 antibodies. FOXO1 transfer to T cells was quantified by confocal microscopy and measured as corrected total cell fluorescence (CTCF). Scale bar 3.5μM. ***P<0.001 compared to control. (E) T cells - cultured as described in (D) - were stained for CD4 (pink), FOXP3 (green), and FOXO1 (red) expression and counter-stained with DAPI (purple). The intensity of FOXP3 and FOXO1 expression in the same cell was determined by ImageStreamX analysis by comparing bright detail similarity (median fluorescence) of the two. ***P*<0.01 compared to control. Scale bar = 7μm. (F) The ability of E3P-treated neutrophils to induce Tregs was determined following neutrophil-specific inhibition of FOXO1. Log scale shown. (G) T cells were co-cultured with neutrophil apoptotic bodies as described in (D) Additionally, AnxA1 was neutralised on neutrophils prior to apoptotic body

formation and the ability of neutrophil-derived FOXO1 transfer to T cells was measured by confocal microscopy. Data are mean ± SEM of two experiments conducted with three donors per experiment.

Figure 6. Neutrophils from pre-eclamptic pregnancies fail to induce niT-cells.
(A) Peripheral blood neutrophils from pre-eclamptic patients were stained for CD15, CD16, CD62L, CD11b and AnxA1 to establish their phenotype as compared to cells from age and gestation-matched healthy pregnant women (demographic data in Supplementary Table 1). Data are mean ± SEM of 15 healthy and 10 pre-eclamptic samples; ***P<0.001, **P<0.01 using Student's T test for each marker. (B) Comparison of gestation-adjusted birth weight centiles for babies born from normotensive mothers (n= 15; mean 52nd centile; range 44-68 centile) and pre-eclamptic mothers (n=10; 22nd centile, range 11th to 35th centile)
(C) Extent of apoptosis of peripheral blood neutrophil from healthy and pre-eclamptic samples measured by AnxV and propidium iodide (PI) labeling. Data are mean ± SEM of 15 healthy and 10 pre-eclamptic samples; **P<0.01 compared to healthy pregnancy. (D) T cells from healthy and pre-eclamptic pregnancies were co-cultured for 5 days with autologous neutrophils (no additional hormones added) and were subsequently stained for DAPI (blue), AnxA1 (red), and MRP8 (green). The amount of neutrophil protein transferred to T cells was analyzed by confocal microscopy and co-localisation of AnxA1 and MRP8 was analyzed using Mander's overlap coefficient indices. Data are mean ± SEM of 15 healthy and 10 pre-eclamptic samples; *** P<0.001. Scale bar, 7μm.
(D) Neutrophils from healthy and pre-eclamptic donors were co-cultured with autologous T cells as described in (B). T cells were then stained for DAPI (blue) and FOXO1 (red) and analyzed by confocal microscopy to establish the efficiency of FOXO1 transfer to the T cells, as measured by corrected total cell fluorescence (CTCF). Data are mean ± SEM of 15 healthy and 10 pre-eclamptic samples; *** P<0.001. Scale bar, 7μm. (E) Neutrophils from healthy or pre-eclamptic patients were co-cultured with autologous T cells at a 1:1 ratio for 5 days in the presence of 2μg/ml soluble anti-CD3 and anti-CD28 antibodies. T cells were stained for niTreg markers as in Figure 2. Additionally, neutrophils from pre-eclamptic patients were treated with exogenous E3P and then co-cultured with T cells (blue lined bar). Data are mean ± SEM of 15 healthy and 10 pre-eclamptic samples; ** P<0.01. Data are mean ± SEM of 3 experiments with n= 15 and 10 pre-eclamptic donors**.**

Figure 7. Knock down of FOXO1 in neutrophils leads to abnormal pregnancy

(A) Balb/C males were mated with C57 BL/6 females and neutrophil depleted as described in Figure 3. Bone marrow progenitors were transduced in order to knock-down FOXO1, followed by culture with 100ng/ml GCSF for neutrophil differentiation. $3X10^6$ transduced neutrophils were injected i.v (tail vein) at days 6 and 9 of pregnancy and mothers were sacrificed at day 12. Absolute numbers of $Ly6G^+$ neutrophils, $CD4^+$ T cells and $CD4^+FOXP3^+$ Tregs in the uterine draining lymph nodes were counted. n= 3 mice per group from 3 distinct timed matings $P<0.01$. (B) Bone marrow from $CD45.1^+$ donor mice were transduced with non-specific reconstituted as described above and the presence of donor CD45.1 $Ly6G^+$ (clone Gr-1)$F4/80^-$ neutrophils was analysed by flow cytometry after 16hrs (contour plots) and after 72 hours (day 12) post injection. (C) The presence of transferred CD45.1 donor neutrophil material to the $CD4^+FOXP3^+$ population was also analysed by flow cytometry, their origin confirmed by Ly6G staining, compared to $CD45.2^+$ cells. n= 3-4 mice per group from 3 distinct timed matings.

Embryo size (D) and placenta diameter (E) were measured as described in Figure 3. n =22 embryos and placentas from non-specific, 16 from FOXO30 and 12 from FOXO1 42 from 3 distinct pregnancies per group $^{**}P<0.01$ compared to non-specific. (F) Representative embryo and placenta images of all three groups shown. Scale bar = 2.5mm. (G) Representative immunofluorescence images of placentas for CD31 (red), trophoblasts (cytokeratin-7; green) and cell nuclei with DAPI (blue). Dotted lines indicate placental layers as described in (E). Yellow dotted square indicates zoomed in section of the Db layer to look at trophoblst invasion more closely. Invasion of trophoblasts into the maternal layer was quantified using Image J software. n= 3 placentas from each group $^{*}P<0.05$ compared to non-specific. Data are mean ± SEM (A and F) line indicates mean (B and C).