Epithelial and stromal-specific immune pathway activation in the murine endometrium *post-coitum*

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Abstract

The endometrium is a dynamic tissue, demonstrating cyclical growth/remodelling in preparation for implantation. In mice, seminal constituents trigger mechanisms to prepare the endometrium, a process dubbed 'seminal priming' that modifies immune system components and mediates endometrial remodelling in preparation for pregnancy. An array of cytokines has been reported to mediate this interaction, although much of the literature relates to *in vitro* studies on isolated endometrial epithelial cells. This study measured changes in immune-related gene expression in endometrial epithelial and stromal cells *in vivo* following natural mating. CD1 mice were naturally mated and sacrificed over the first 4 days *post-coitum* ($n=3$ each day). Endometrial epithelial and stromal compartments were isolated by laser capture microdissection. Labelled cRNA was generated and hybridised to genome-wide expression microarrays. Pathway analysis identified several immune-related pathways active within epithelial and stromal compartments, in particular relating to cytokine networks, matrix metalloproteinases and prostaglandin synthesis. Cluster analysis demonstrated that the expression of factors involved in immunomodulation/endometrial remodelling differed between the epithelial and stromal compartments in a temporal fashion. This study is the first to examine the disparate responses of the endometrial epithelial and stromal compartments to seminal plasma *in vivo* in mice, and demonstrates the complexity of the interactions between these two compartments needed to create a permissive environment for implantation.

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Introduction

The endometrium is a dynamic tissue that undergoes cyclical growth and remodelling to varying degrees in different species in preparation for blastocyst implantation. In mice, seminal constituents are thought to trigger the mechanisms preparing the endometrium for implantation in a process dubbed 'seminal priming' (Fig. 1), which can modify immune system components and influence subsequent events in pregnancy (Robertson *et al.* 2013). The endometrium responds rapidly to seminal fluid exposure by triggering an inflammatory cascade which is soon followed by an influx of leukocytes (Sharkey *et al.* 2012). Seminal plasma proteins interact with oestrogen (E_2)-primed uterine epithelial cells, resulting in the synthesis of a large array of cytokines and other immunoregulatory factors, such as granulocyte macrophage colony stimulating factor (CSF2), interleukin (IL)6, IL8, monocyte chemoattractant protein (CCL2), matrix metalloproteinases (MMPs) and prostaglandins (PGs) (Sanford *et al.* 1992, Robertson *et al.* 1996a). Within hours of this rise in cytokine production, inflammatory cells invade the cervix as the primary site of semen deposition in

humans, pigs, rabbits and mice, although in rodents this inflammatory response can be seen throughout the endometrium (Lovell & Getty 1968, Taylor 1982, Pandya & Cohen 1985, Claus 1990, McMaster *et al.* 1992, Rozeboom *et al.* 1998, Robertson 2005). Myeloid lineage cells such as monocytes/macrophages, dendritic cells and granulocytes of circulatory origin extravasate to accumulate in uterine stromal tissue, with macrophages, neutrophils and other granulocytes in particular further traversing the epithelium to accumulate in the uterine cavity (De *et al.* 1991, McMaster *et al.* 1992, Bischof *et al.* 1994). This initial neutrophil response occurs prior to implantation, with a purported concurrent drop in cytokine profiles and a rise in progesterone (P_4) (Robertson *et al.* 1996a). Endometrial leukocytes, however, persist for several days (O'Leary *et al.* 2004).

Cytokines such as CSF2 (Robertson & Seamark 1990, Robertson *et al.* 1996b), leukaemia inhibitory factor (LIF), CCL2 (Wood *et al.* 1997, 1999, Meter *et al.* 2005, Boomsma *et al.* 2009), regulated upon activation normal T cell expressed and secreted (CCL5) (Wood *et al.* 1997, Altman *et al.* 1999, Arima *et al.* 2000) and TGFB have been shown to be expressed in endometrial cell compartments *post-coitum*, although much of these

Figure 1 Schematic diagram representing the complex interplay between murine endometrial epithelial and stromal cells following exposure to semen/seminal plasma. Cytokines, matrix metalloproteinases (MMPs) and prostaglandins (PGs), mediate the development of immunotolerance and tissue remodelling in the peri-implantation period to prepare for implantation and subsequent pregnancy.

data relate to *in vitro* models. However, most of the peri-implantation tissue remodelling (both prior to and during decidualisation) appears to be mediated by MMPs (Aplin 1997, Tang *et al.* 2005). Several MMPs have been implicated, with macrophages being the main source of these proteases, which they produce in response to the prevailing cytokine and PG milieu (Goetzl *et al.* 1996). However, MMP function is reasonably species-specific: in women, these enzymes are the main mediators of endometrial shedding, whereas this process is largely absent in rodents, where endometrial remodelling merely involves functional regression (Marbaix *et al.* 1996, Tang *et al.* 2005).

Alongside endometrial remodelling, a concurrent modification of the maternal immune system occurs in order to promote maternal immunotolerance of the paternal antigens displayed by the implanting blastocyst. Following initial semen deposition in the female reproductive tract, paternal antigens are phagocytosed, processed and presented to maternal CD4⁺ and CD8⁺ T cells by antigen presenting cells in the para-aortic lymph nodes (Johansson *et al.* 2004, Moldenhauer *et al.* 2009). This induces a functional anergy/hyporesponsiveness in these cells, which promotes conceptus allograft tolerance (Tafari *et al.* 1995), in line with a concurrent expansion of the CD4⁺CD25⁺Foxp3⁺ regulatory T cell (Treg) pool (Aluvihare *et al.* 2004, Shima *et al.* 2010). The strength of this maternal response depends on seminal plasma composition, particularly paternal antigen content and TGFB concentrations (Robertson *et al.* 2009). Seminal plasma TGFB in humans and swine is largely accounted for by the TGFB1 isoform (although TGFB2 and 3 are also present), which is secreted in an inactive

form requiring activation in the acidic environment of the vagina (Politch *et al.* 2007). The activation of TGFB1 induces naïve CD4⁺CD25[−] T cells to differentiate into Foxp3⁺ suppressor/anergic T cells (Chen *et al.* 2003). The PG component of seminal plasma may also synergise with TGFB in this regard, since PGE₂ enhances this inhibitory effect and has been shown to induce a regulatory phenotype in human CD4⁺CD25[−] T cells *in vitro* (Baratelli *et al.* 2005). PG synthesis is also induced by seminal plasma in the porcine uterus, with concurrent angiogenesis extending throughout the preimplantation period (Kaczmarek *et al.* 2010, 2013).

Although individual immune mediators of the endometrial response to seminal plasma have been identified, few studies have focussed on trying to paint a comprehensive picture of the large number of mediators involved, particularly in terms of characterising the global immunomodulatory responses involved at the level of the transcriptome. Various studies have focussed on profiling gene expression over the course of the implantation window (particularly in women following ovarian stimulation) in order to identify those genes most likely to be involved in the establishment of endometrial receptivity prior to embryo transfer and in relation to the implantation problems allied to endometriosis (Kao *et al.* 2002, 2003, Riewijk *et al.* 2003, Giudice 2004, Burney *et al.* 2007). To date, however, only one has explored global gene expression in the cervix following stimulation with seminal plasma: Sharkey *et al.* (2012) performed expression microarray analysis on biopsies of *pre-* and *post-coital* human cervix and reported that seminal fluid activated pathways, including the inflammatory response, immune response, immune cell

trafficking, cell movement and antigen presentation. Within these various pathways, CSF2, IL6 and IL8 mRNA were prominently upregulated *post-coitum*, as were COX2 and various MMPs, suggesting active leukocyte recruitment and ECM remodelling (Sharkey *et al.* 2012). Unfortunately, these observations were based only on a single time point in this putatively dynamic process. The aim of the present study was therefore to characterise the endometrial inflammatory response to seminal plasma *in vivo* in detail throughout the pre-implantation period by using a genome-wide screening approach using a murine model. Furthermore, the individual response of the epithelial and stromal cellular compartments to seminal plasma was examined to identify differences in gene expression.

Materials and methods

Mouse husbandry and mating protocol

Ethical approval was not required for this study as all work was conducted in compliance with the ethical and legislative framework set out in the Animals (Scientific Procedures) Act, 1986 (UK). Individually housed male (aged 10–12 weeks), and group-housed (10/cage) nulliparous female (aged 6 weeks) CD1 mice were used for these experiments. Animals had *ad libitum* access to water and the Standard Expanded Beekay diet (B&K, Grimston, Aldborough, UK) and were kept with a ratio of 14 h light:12 h darkness, (05:30 on; 19:30 off). Humidity and temperature were maintained at 55–65% and $21.5 \pm 1^\circ\text{C}$ respectively. Whitten effect-synchronised females had their oestrus confirmed by vaginal cytology prior to being naturally mated to CD1 stud males (one female:one male). Five successful copulations were recorded prior to leaving pairs together overnight in order to confirm successful intercourse. Females were then group-housed according to the time of mating, before being sacrificed under Schedule 1 of the Animals (Scientific Procedures) Act, 1986 at 24, 48, 72 and 96 h after first coitus ± 1 h ($n=3/\text{group}$). Controls were provided by naturally cycling oestrus and dioestrus females (again assessed on the basis of vaginal cytology, $n=3/\text{group}$).

Uterine tissue processing and staining

Within 5 min of culling, the ovaries, uterine horns and vagina were mounted in optimal cutting compound (OCT), frozen in isopentane slush in a liquid nitrogen bath before storage at -80°C . Specimens were later sectioned ($12\ \mu\text{m}$) at -16°C on a Leica CM3050S Cryostat (Leica Biosystems, UK). Sections were mounted onto Arcturus polyethylene naphthalate (PEN) membrane glass slides (Applied Biosystems) and stained with buffered ethanolic cresyl violet staining solution, as previously described, with minor modifications (Cummings *et al.* 2011). Sections were briefly rehydrated in a series of ethanol washes (95, 75 and 50%) prior to staining with cresyl violet in 75% ethanol buffered with 20 mM Tris-HCl pH 8.0 for 40 s. Sections were then dehydrated in a series of ethanol washes (50, 75, 95 and 100%). All solutions were prepared using RNase-free water and molecular-grade absolute ethanol. Slides were

stored at -80°C under dessicant prior to laser capture microdissection (LCM).

Laser capture microdissection

LCM of selected groups of cells was performed on a Zeiss Palm Microbeam UV laser capture microscope, equipped with PALM@Robo Software version 3.2 (Carl Zeiss, Herts, UK). Samples were captured with cut energy set to 51–60% and laser speed of $20\ \mu\text{m/s}$. Excised areas were captured into adhesive cap tubes (Carl Zeiss), with the average area excised per sample being $3.23 \times 10^6\ \mu\text{m}^2$. Both luminal epithelial and stromal compartments were independently microdissected from the same tissue specimens in order to avoid the risk of RNA cross-contamination (Supplementary Figure 1, see section on supplementary data given at the end of this article).

RNA preparation and microarray analysis

RNA extraction was performed using Qiagen RNeasy plus micro extraction kits (Qiagen). The extraction protocol was based on the manufacturer's instructions with minor modifications; one extra RPE and 80% ethanol wash step was incorporated in order to reduce impurity carryover and improve RNA quality. Samples were eluted from the spin column by addition of $14\ \mu\text{l}$ RNase-free water, with $0.5\ \mu\text{l}$ RNeasySecure (Ambion) immediately added to the eluate, which was then heated to 60°C for 10 min to inactivate RNase. Samples were concentrated using a Savant SpeedVac concentrator (Eppendorf, Hamburg, Germany) for 30 min or until dry, then resuspended in $4\ \mu\text{l}$ RNase-free water and stored at -80°C until use. RNA quality and quantity was assessed using Agilent RNA 6000 Nano kit (Agilent, UK) on a 2100 Bioanalyser (Agilent) equipped with 2100 Expert Software using the RNA Eukaryote Total RNA Nano function, and only samples with a RIN value of seven and above were considered suitable for fluorescent cRNA generation.

Fluorescent cRNA was prepared using Agilent One Colour Low Input Quick AMP labelling kit with 10 ng input RNA. Purification of RNA was performed using the RNeasy Mini kit (Qiagen) following the manufacturer's instructions. Replicates were hybridised in different batches to avoid experimental bias. Fluorescent cRNA quality was assessed using a NanoDrop 1000 (Thermo-Scientific UK). Cyanine (Cy3) concentration (pmol/ μl), RNA 260/280 ratio and cRNA concentration (ng/ μl) were also recorded, and yield and specific activity were calculated for each sample. Samples were hybridised to Agilent SurePrint G3 Mouse GE $8 \times 60\text{ k}$ microarrays as per the manufacturer's instructions. Hybridisation was performed in a hybridisation oven (Agilent) for 17 h at 65°C and a rotation speed of 20 r.p.m. Subsequent washes were completed within 1 h. Slides were scanned immediately to reduce potential variations in signal intensities due to environmental contaminants.

Scanning and feature extraction

Microarrays were scanned using an Agilent 'C' scanner (Agilent), with a scan resolution of $3\ \mu\text{m}$. Feature extraction

was performed using Feature Extraction version 11.0.1.1 (Agilent Technologies). Sample quality was assessed utilising inbuilt quality-control metrics. Eleven of these were applied to the microarray, covering aspects such as alignment, signal intensity and reproducibility, providing a range within which a microarray is considered 'good'. Microarrays falling outside of the manufacturer's 'good' criteria were evaluated for exclusion from the final analysis.

Data analysis

Data were visualised and analysed using GeneSpring version 12.5 (Agilent Technologies). Data were normalised to the 75% signal intensity of all probes on the array as recommended by the manufacturer. Samples were examined by the inbuilt principal components analysis (PCA) to identify major outliers. Sample size and desired fold difference were calculated using the Microarray Sample Size Computation tool available at <http://bioinformatics.mdanderson.org/microarraysamplesize/>. The calculation was based on 5 5821 entities, with an acceptable false positive rate of 5%, power (percentage of differentially expressed entities likely to be detected by the experiment) of 0.8, and a s.d. of 0.7. Due to the sample size, a fivefold change cut-off was applied to the data. Pathway analysis was performed using the condition averages in order to identify immune pathways active in the murine endometrium. *P* values were corrected for multiple comparisons using the Benjamini and Hochberg false discovery rate (FDR). The results from the pathway analysis were used in subsequent cluster analyses in IBM SPSS (Version 19). The analysis was performed for epithelium and stroma individually, based on a two-step cluster analysis using Ward linkage. At each iteration, the Δ coefficient was calculated, with the largest Δ coefficient defining the number of clusters. Identified mRNA species in each cluster were visualised in GraphPad Prism (Version 6).

Results

Pathway analysis

Pathway analysis revealed 217 curated pathways active within the murine uterine tract. Of these pathways, 46 were significant at $P < 0.05$, with significance defined by the inbuilt GeneSpring algorithm, which created a composite score of the number of mRNA species matched and the extent of the response of those species (Table 1). Those relating to immune networks were selected in both endometrial epithelial and stromal cell compartments active within the murine endometrium: PG synthesis and regulation ($P < 0.001$), cytokines and inflammatory response ($P < 0.001$), MMPs ($P = 0.01$), inflammatory response pathway ($P < 0.05$) and the TGFB signalling pathway ($P < 0.05$).

Cluster analysis: epithelial compartment

Genes encoding cytokine proteins and MMPs highlighted in the pathway analysis were selected for

subsequent cluster analysis. These elements fell into eight clusters (Fig. 2). Cluster 1 was defined by peaks in expression on days 1 and 3 post-mating with reduced expression on day 2 (*Cxcl3*, *Il10*, *Il12a*, *Mmp9*, *Mmp20*, *Mmp25*, *Tnf*). Cluster 2 exhibited a similar pattern of peaks on days 1 and 3, with a less dramatic reduction in expression on day 2 (*Bmp4*, *Egf*, *Il7*, *Il13*, *Mmp1a*, *Mmp3*, *Mmp16*, *Mmp28*). Cluster 3 generally showed a peak in expression at oestrus and day 2 post-mating with a reduction in expression on day 1 (*Ccl5*, *Csf3*, *Il1b*, *Il15*, *Inhb1*, *Mmp11*, *Mmp14*, *Mmp23*, *Thbs*). Cluster 4 demonstrated a slight peak at dioestrus and day 1 post-mating (*Ifng*, *Mcp1*, *Mmp2*, *Mmp19*, *Tgfb1*, *Tgfb2*, *Tgfb3*). Cluster 5 showed a dip in expression at oestrus, while remaining relatively constant at other time points (*Csf2*, *Cxcl2*, *Il2*, *Il4*, *Il5*, *Il12b*, *Itbp1*, *Mmp10*, *Mmp12*, *Mmp13*, *Mmp21*, *Mmp24*). Cluster 6 exhibited minor peaks on days 2 and 4 post-mating, although many elements showed large variation (*Cxcl1*, *Il1a*, *Lif*, *Mmp8*, *Mmp27*). Cluster 7 was characterised by peaks at dioestrus, days 1 and 4 post-mating, with a dramatic reduction in expression at days 2 and 3 (*Csf1t1*, *Csf1t3*, *Mmp7*). Finally, cluster 8 elements exhibited huge variations in expression at each time point, and therefore could not be ascribed to any particular pattern.

Cluster analysis – stromal compartment

mRNA entities fell into seven clusters (Fig. 3). Cluster 1 comprised three mRNA species (*Csf1t1*, *Csf3*, *Mmp23*) that exhibited a peak at oestrus. Cluster 2 was characterised by a slight increase in expression on day 1 post-mating, with a subsequent decrease on days 2–4 (*Bmp4*, *Csf1t3*, *Csf2*, *Cxcl2*, *Cxcl3*, *Egf*, *Ifnb1*, *Il1a*, *Il2*, *Il4*, *Il5*, *Il6*, *Il7*, *Il10*, *Il11*, *Il12a*, *Il12b*, *Il13*, *Mmp1a*, *Mmp3*, *Mmp8*, *Mmp10*, *Mmp13*, *Mmp12*, *Mmp16*, *Mmp20*, *Mmp21*, *Mmp24*, *Mmp25*, *Mmp28*, *Tnf*). Cluster 3 comprised only *Mmp7*, which showed the highest expression at oestrus with the lowest expression on day 3 post-mating. Cluster 4 increased in expression post-mating and remained high on days 2–4 (*Ifng*, *Il15*, *Mmp2*, *Mmp19*, *Tgfb1*, *Tgfb2*, *Tgfb3*). Cluster 5 peaked on days 1 and 2 post-mating with a subsequent decrease on days 3 and 4 (*Ccl5*, *Inhb1*, *Il1b*, *Mcp1*, *Mmp9*, *Thbs1*). Cluster 6 peaked on day 1 with a subsequent fall to baseline levels of expression (*Mmp11*, *Mmp14*). Cluster 7 was characterised by peaks in expression at dioestrus and day 3 post-mating (*Cxcl1*, *Itbp1*, *Lif*, *Mmp27*).

Differences in endometrial epithelial and stromal expression

Many mRNA species displayed differences in expression between endometrial epithelial and stromal cells despite exhibiting similar pathway involvement. The majority

Table 1 Pathways active in murine endometrial epithelial and stromal cells.

Pathway	WikiPathways ID	P value	Number of matched entities	Number of pathway entities
GPCRs, Class A rhodopsin-like	WP189 62834	0.00	208	231
GPCRs, other	WP41 54691	0.00	150	210
GPCRs, other	WP41 62667	0.00	150	210
Cytoplasmic ribosomal proteins	WP163 62833	0.00	77	80
Peptide GPCRs	WP234 41308	0.00	63	70
Metapathway biotransformation	WP1251 41349	0.00	117	143
Monoamine GPCRs	WP570 48232	0.00	32	33
Monoamine GPCRs	WP570 60231	0.00	32	33
Calcium regulation in the cardiac cell	WP553 47774	0.00	120	150
Striated muscle contraction	WP216 41273	0.00	40	45
Complement and coagulation cascades	WP449 41301	0.00	53	62
Prostaglandin synthesis and regulation	WP374 41394	0.00	29	31
Small Ligand GPCRs	WP353 41279	0.00	18	18
Selenium	WP1272 58514	0.00	22	31
Cytokines and inflammatory response (BioCarta)	WP222 53571	0.00	26	30
Selenium	WP1272 59028	0.00	21	31
Complement activation, classical pathway	WP200 47967	0.00	16	17
Cytochrome P450	WP1274 48227	0.00	35	40
Myometrial relaxation and contraction pathways	WP385 47969	0.00	120	157
Blood clotting cascade	WP460 62696	0.00	19	20
Glucocorticoid and mineralcorticoid metabolism	WP495 62838	0.00	13	13
SIDS susceptibility pathways	WP1266 58254	0.00	49	61
SIDS susceptibility pathways	WP1266_58281	0.00	49	61
GPCRs, Class B secretin-like	WP456 41317	0.01	20	22
Nuclear receptors in lipid metabolism and toxicity	WP431 47744	0.01	26	30
Glucuronidation	WP1241 59029	0.01	11	18
Irinotecan pathway	WP475 48258	0.01	11	11
Matrix metalloproteinases	WP441 41300	0.01	25	29
Macrophage markers	WP2271 53132	0.01	10	10
XPodNet – protein–protein interactions in the podocyte expanded by STRING	WP2309 58142	0.01	573	836
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Oxidative stress	WP412 41381	0.02	24	29
GPCRs, Class C metabotropic glutamate, pheromone	WP327 41361	0.02	14	15
Folic acid network	WP1273 48256	0.02	20	27
Inflammatory response athway	WP458 57463	0.03	25	30
TGFβ signalling pathway	WP113 41270	0.03	41	52
Metapathway biotransformation	WP1251 59060	0.03	22	143
Hedgehog signalling pathway	WP116 41332	0.03	19	22
Folic acid network	WP1273 60224	0.03	19	27
Glycolysis and gluconeogenesis	WP157 43573	0.03	38	48
Endochondral ossification	WP1270 41292	0.03	48	62
Serotonin and anxiety-related events	WP2140 58159	0.03	12	13
Glycolysis and gluconeogenesis	WP157 51735	0.04	39	50
ESC pluripotency pathways	WP339 42897	0.04	81	111
Retinol metabolism	WP1259 51364	0.04	31	39
E ₂ metabolism	WP1264 48250	0.04	11	13

showed greater relative expression profiles in stromal cells as compared to epithelial cells, with the exception of *Mmp7* and elements of the PG pathway, which were consistently more highly expressed in epithelial cells.

CSFs varied in expression in each cell type, with notable differences in both transcript variants of *Csf1* (Fig. 4). This mRNA peaked on days 1 and 4 post-mating in the epithelium, while stromal expression remained relatively constant. *Csf2* showed a minor peak in expression on day 1 post-mating in both epithelium and stroma, with epithelial expression peaking on day 4 while stromal expression slowly declined. Of the CSFs, *Csf3* showed the greatest difference in expression

between epithelium and stroma, with a dramatic drop in epithelial expression on day 1 followed by a peak on day 2 post-mating.

Transforming growth factors (TGFs) were consistently more highly expressed in stroma than in epithelium (Fig. 5). *Tgfb1* exhibited a divergence between stromal and epithelial expression on day 1 post-mating, with stromal expression increasing while epithelial expression decreased. *Tgfb2* showed a similar divergence on day 2 post-mating. Interferons (*Ifnb* and *Ifng*) were both detected in murine endometrium, with a higher relative expression noted in stroma (Fig. 6). There was one exception to this pattern: *Ifnb* epithelial

Figure 2 Cluster analysis of mRNA encoding cytokines and MMPs in murine endometrial epithelial cells. Clusters were identified by a two-step cluster analysis using Ward linkage. At each iteration, the Δ coefficient was calculated, with the largest Δ coefficient defining the number of clusters. Cluster membership: cluster 1 – *Cxcl3*, *Il10*, *Il12a*, *Mmp9*, *Mmp20*, *Mmp25*, *Tnf*; cluster 2 – *Bmp4*, *Egf*, *Il7*, *Il13*, *Mmp1a*, *Mmp3*, *Mmp16*, *Mmp28*; cluster 3 – *Ccl5*, *Csf3*, *Il1b*, *Il15*, *Inhb1*, *Mmp11*, *Mmp14*, *Mmp23*, *Thbs*; cluster 4 – *Ifng*, *Mcp1*, *Mmp2*, *Mmp19*, *Tgfb1*, *Tgfb2*, *Tgfb3*; cluster 5 – *Csf2*, *Cxcl2*, *Il2*, *Il4*, *Il5*, *Il12b*, *Itbp1*, *Mmp10*, *Mmp12*, *Mmp13*, *Mmp21*, *Mmp24*; cluster 6 – *Cxcl1*, *Il1a*, *Lif*, *Mmp8*, *Mmp27*; cluster 7 – *Csf1t1*, *Csf1t3*, *Mmp7*; cluster 8 – *Ifnb*, *Il6*, *Il11*.

expression peaked above stromal expression on day 2 *post-coitum*. Differences were noted in other inflammatory cytokines too: *Tnf*, *Lif* and *Ccl5* were all expressed in endometrial stroma at relatively higher levels than in epithelium, with the exception of *Tnf* and *Lif* on day 4 (Fig. 7). *Ccl5* expression patterns were similar in both compartments *post-coitum*.

Elements of the PG pathway were also implicated: PG synthases were detected in both endometrial epithelium

and stroma (Fig. 8). *Ptgs1* (Cox1) demonstrated a dramatic increase in epithelial expression, which was relatively higher than stromal expression on day 2 *post-mating*; this was sustained until a day 4 drop. *Ptgs2* (Cox2) peaked in the epithelium on day 2 *post-coitum*. The PG E receptors *Ptger1*, *Ptger2*, *Ptger3* and *Ptger4* exhibited differences in expression between endometrial epithelial and stromal cells. Both *Ptger1* and *Ptger3* were more highly expressed in stroma than in epithelium.

Figure 3 Cluster analysis of mRNA encoding cytokines and MMPs in murine endometrial stromal cells. Clusters were identified by a two-step cluster analysis using Ward linkage. At each iteration, the Δ coefficient was calculated, with the largest Δ coefficient defining the number of clusters. Cluster membership: cluster 1 – *Csf1t1*, *Csf3*, *Mmp23*; cluster 2 – *Bmp4*, *Csf1t3*, *Csf2*, *Cxcl2*, *Cxcl3*, *Egf*, *Inhb1*, *Il1a*, *Il2*, *Il4*, *Il5*, *Il6*, *Il7*, *Il10*, *Il11*, *Il12a*, *Il12b*, *Il13*, *Mmp1a*, *Mmp3*, *Mmp8*, *Mmp10*, *Mmp13*, *Mmp12*, *Mmp16*, *Mmp20*, *Mmp21*, *Mmp24*, *Mmp25*, *Mmp28*, *Tnf*; cluster 3 – *Mmp7*; cluster 4 – *Ifng*, *Il15*, *Mmp2*, *Mmp19*, *Tgfb1*, *Tgfb2*, *Tgfb3*; cluster 5 – *Ccl5*, *Inhb1*, *Il1b*, *Mcp1*, *Mmp9*, *Thbs1*; cluster 6 – *Mmp11*, *Mmp14*; cluster 7 – *Cxcl1*, *Itbp1*, *Lif*, *Mmp27*.

Figure 4 Relative CSF mRNA expression in murine endometrial epithelial and stromal cells *post coitum*. *Csf1* (both transcript variants) varied little in the epithelium, while peaking on days 1 and 4 in the stroma. *Csf2* exhibited higher stromal expression at all time points except day 4. *Csf3* exhibited the most variation across cell type and time point.

Ptger1 showed an increase in stromal expression with a concurrent decrease in epithelial expression on day 2 post-mating. *Ptger2* and *Ptger4* were consistently more highly expressed in epithelium than in stroma. *Ptger2* peaked on days 1 and 4 post-mating, while *Ptger4* peaked on day 3 (Fig. 7).

Discussion

This study characterised the murine endometrial epithelial and stromal responses to seminal plasma in the development of maternal immunotolerance to the foetal allograft. Various mediators of this response have been identified. Chief amongst these, CSF2 is secreted into the uterine lumen within hours of *coitus* (Robertson & Seamark 1990, Robertson *et al.* 1996b). This ties in well with the present findings, where *Csf2* expression was noted to rise markedly between oestrus and day 1 in both epithelial and stromal compartments. Relative stromal

expression exceeded that of the epithelium except on day 4, suggesting that stroma may be the principal source of CSF2. This challenges the conventional epithelium-centric view borne from studies based on trypsinised cell cultures or antibody-based panning techniques that may alter epithelial cell phenotype and response (Robertson & Seamark 1990, Robertson *et al.* 1992, 1996b). Moreover, the stromal role is often overlooked in the design of such investigations. *Csf2* mRNA extracted from entire murine uteri *post coitum* has been shown to be upregulated across days 1 and 2 (Robertson *et al.* 1996a), in contrast to the day 2 decline reported here. This may reflect differences in mouse strain or use of the entire organ mRNA pool rather than the more selective approach used herein. *Csf2* was raised on day 1 post-mating in both epithelium and stroma, pointing to its likely involvement in the endometrial response to seminal plasma. Epithelial *Csf2* expression was highest on day 4, suggesting its involvement in endometrial receptivity to the implanting blastocyst. Indeed, CSF2 recruits leukocytes to the endometrium to facilitate implantation, with dendritic cells, regulatory T cells and natural killer cells coordinating trophoblast invasion (Bulmer *et al.* 1991, Gonzalez *et al.* 2012, Hatta *et al.* 2012, Male *et al.* 2012, Chaouat 2013, Erlebacher 2013, Fu *et al.* 2013, Hemberger 2013). Its importance in placentation is highlighted by a study of null mice that showed that absence of CSF2 was associated with late pregnancy loss and delayed foetal growth (Seymour *et al.* 1997). CSF1 is a weak chemotactic agent, recruiting macrophages to the epithelium (Wood *et al.* 1997), such that its protein levels correlate with macrophage numbers (Pollard *et al.* 1987). In the present study, epithelial *Csf1* expression peaked on days 1 and 4, suggesting two waves of macrophage recruitment. Conceivably, the day 1 wave could be accounted for by the role of macrophages as antigen-presenting cells in the induction of maternal immunotolerance alongside their phagocytic clearance of seminal components within the uterine lumen (Olive *et al.* 1987), while on day 4, they may participate in tissue remodelling and increasing epithelial cell adhesiveness in preparation for implantation (Lee *et al.* 2011).

Epithelial cluster 1 transcripts (*Cxcl3*, *Il10*, *Il12a*, *Mmp9*, *Mmp20*, *Mmp25*, *Tnf*) peaked on days 1 and 4

Figure 5 Murine endometrial cell relative TGFβ mRNA expression in the pre-implantation period. All *Tgfb* isoforms were consistently more highly expressed in the stromal compartment post-mating. With the exception of *Tgfb1*, which demonstrated a reduction in stromal compartment expression, all isoforms exhibited a rise in expression in day 1 post-mating.

Figure 6 Relative *Ifnb* and *Ifng* expression in murine endometrial cell compartments post-mating. *Ifnb* exhibited a sharp peak in expression on day 2 post-mating in the stromal compartment, while *Ifng* showed a peak at this time point in the epithelium.

post-mating. TNFA is key to the acute inflammatory response (Warren *et al.* 1988), accounting for its early expression. CXCL3 controls monocyte adhesion and migration (Smith *et al.* 2005), thus likely mediating macrophage recruitment. Although CXCL3 is induced by IL13 (Ooi *et al.* 2012), their transcripts did not cluster, potentially due to functional redundancy (see below). MMPs also featured prominently in the initial epithelial response to seminal plasma, suggesting some degree of early remodelling and/or neoangiogenic signalling to stroma in preparation for implantation (Rundhaug 2005).

Expression of epithelial clusters 3 (*Ccl5*, *Csf3*, *Il1b*, *Il15*, *Inhb1*, *Mmp11*, *Mmp14*, *Mmp23*, *Thbs1*) and 6 (*Cxcl1*, *Il1a*, *Lif*, *Mmp8*, *Mmp27*) was downregulated on day 1 post-mating. This is surprising because *Ccl5*, *Csf3*, *Cxcl1* and *Il15* are leukocyte chemotactic/differentiation agents whose levels might be expected to parallel leukocyte activity *post coitum* (Schall *et al.* 1990, Bleul *et al.* 1996, Wood *et al.* 1997, Ashkar *et al.* 2003, Kryczek *et al.* 2005). Instead, *Ccl5* and *Il15* expression rose in stroma, suggesting that this compartment may be their principal source. This may also reflect a degree of functional redundancy, a phenomenon partly due to cytokine receptors sharing a common subunit specific to a family of cytokines. For example, IL2 deficiency can be compensated by other cytokines in its family (IL4, IL7, IL9, IL15, IL21) that signal through its receptor by binding to the common γ -chain (Lin *et al.* 1995, Ozaki & Leonard 2002). This family of ILs falls into clusters 3 and 5, whose expression either fails to increase above baseline or is downregulated post-mating, with the exception of *Il7* (cluster 2), which peaked on days 1–3. It is tempting to speculate that *Il7* performs an overarching function on behalf of its family, rendering the others functionally redundant. Alternatively, other mediators may operate as selective suppressors of *Il4*, *Il9*, *Il15* and *Il21* expression although this cannot be determined from our data. The apparent day 1 downregulation of *Il1a* and *Il1b* may be due to the fact that the inflammatory response may peak and fall prior to day 1 sampling. This is conceivable because cytokine expression increases within hours (e.g. <3 h for *Il1b*

in human endometrial epithelial cells *in vitro*; Gutsche *et al.* (2003)). It is however more probable that the inflammatory response itself is modified to induce maternal tolerance to paternal antigens without necessarily involving the stereotypical acute phase cytokine cascade.

Figure 7 Relative expression (mean normalised intensity) of *Lif*, *Tnf* and *Ccl5* in murine endometrial epithelial and stromal compartments. *Lif* expression remained relatively constant except at day 4, while *Tnf* expression profiles were similar in both compartments although with higher expression in the stromal compartment at all days except day 4. *Ccl5* expression was consistently more highly expressed in the stromal compartment.

Figure 8 Murine endometrial expression of prostaglandin pathway elements during the pre-implantation period. Synthases *Ptgs1* and *Ptgs2* peaked on day 2 in the epithelium with a subsequent decline to day 4. Receptor expression varied, with *Ptger1* and *Ptger3* having relatively higher stromal expression, while *Ptger2* and *Ptger4* followed the opposite pattern.

PG-endoperoxide synthases (PTGSs; cyclooxygenases) shape the implantation-related PG response and influence immune function, angiogenesis and tissue remodelling (Salleh 2014). We found that *post-coitum* *Ptgs1* and *Ptgs2* expression localised to the epithelium, a profile reflected by their protein localisation in both post-mating rats and cycling human uteri (Yang *et al.* 2002). PG receptor *Ptger1* and *Ptger3* expression rose in stroma in parallel to epithelial *Ptgs2*, suggesting paracrine interactions between the two compartments. Cluster 2 (*Bmp4*, *Egf*, *Il7*, *Il13*, *Mmp1a*, *Mmp3*, *Mmp16*, *Mmp28*) epithelial expression rose post-mating and was sustained until day 4. EGF and IL7 are functionally similar in inducing immune effector cell proliferation and differentiation (Niu & Qin 2013) while IL13 modulates human monocyte/B cell function and downregulates/induces MMPs based on the physiological setting (Wadsworth *et al.* 2010, Bailey *et al.* 2012, Firszt *et al.* 2013). Their sustained mRNA expression supports their role in inducing immunotolerance and stromal remodelling.

As regards the stromal response, cluster 5 (*Ccl5*, *Il1b*, *Inhb1*, *Mcp1*, *Mmp9*, *Thbs1*) was of most interest; expression peaked on days 1 and 2 post-mating and fell on days 3 and 4. In contrast to the epithelial

response, the increase in *Il1b* expression suggests that its stromal function may relate to its role in governing cell proliferation/differentiation, inducing PTGS2 expression and regulating MMPs (Rossi *et al.* 2005), in agreement with the rise in day 2 stromal *Ptgs2* expression. IL1B also likely influences the steroid hormone microenvironment through its suppression of steroid sulphatase activity (Matsuoka *et al.* 2002), thereby reducing stromal oestrogenic effects so as to create an environment favourable for nidation. The remaining cluster 5 members mediate leukocyte recruitment and extracellular remodelling in preparation for implantation, as discussed earlier.

Cluster 4 (*Ifng*, *Il15*, *Mmp2*, *Mmp19*, *Tgfb1*, *Tgfb2*, *Tgfb3*) stromal gene expression levels rose on day 1 and remained high thereafter, in contrast to the epithelium. While these agents regulate epithelial proliferation/remodelling in the menstrual cycle (Gold *et al.* 1994, Godkin & Dore 1998), this apparent discrepancy may reflect a paracrine stroma:epithelial relationship, such that bidirectional communication is essential to coordinate the preparatory changes for implantation. TGFB1 can inhibit proliferation and induce PTGS2/prolactin expression in human endometrial stromal cells *in vitro* (Chang *et al.* 2008), pointing to a role in tissue remodelling in preparation for decidualisation, as supported by the present data. MMP2 and 9 cleave latent TGFB into its active form (Yu & Stamenkovic 2000), accounting for our observation that *Mmp2* clusters with all TGFB isoforms in both epithelium and stroma. As such, MMP:TGFB signalling pathway interactions may participate in creating the immunopermissive environment of implantation. In contrast with our findings, a murine study showed TGFB2 expression to be confined to the epithelium during the preimplantation period (Das *et al.* 1992), which may reflect post-transcriptional processes. *Ifng* and *Tgfb* isoform transcript clustering in stroma was expected given that these cytokines display antagonistic relationships (Eickelberg *et al.* 2001, Higashi *et al.* 2003, Yu *et al.* 2006). In murine and human endometrial epithelium, IFNG has a strong inhibitory effect on TGFB via TGFB type II receptor (*Tgfb2*) downregulation (Yu *et al.* 2006). *Ifng* is unlikely to perform a similar role in stroma since *Tgfb2* was upregulated on day 1 post-mating (data not shown). Instead, the parallel reduction in *Tgfb2* expression in the epithelium points to paracrine stromal:epithelial interactions. A likely endometrial role for both these agents revolves around regulating T cell function: IFNG induces T cell apoptosis/inhibits their proliferation, while TGFB promotes naïve T cell differentiation into Tregs (Chen 2011). IFNG also has anti-proliferative effects on human endometrial epithelial cells *in vitro* (Tabibzadeh *et al.* 1988) and an inhibitory effect on stromal VEGF production (Kawano *et al.* 2000), which is at odds with *post-coital* endometrial events occurring *in vivo*.

To our knowledge, this is the first study to characterise comprehensively the *post-coitum* physiological changes in endometrial epithelial and stromal gene expression. Immune pathways were most affected and exhibited dynamic, quantitatively and chronologically distinct compartment-specific changes. Since the expression and bioactivity of many of the mediators involved are subject to both post-transcriptional and post-translational regulation, future studies should focus analyses at the protein level to draw more functional interpretations. Moreover, the cluster analysis used herein (and in other studies) is based on pre-defined pathways intrinsic to microarray analytical software, which may be overly prescriptive and prevent the identification of novel, broader network interactions (e.g. cytokine/eicosanoid/hormone interactions in inflammatory networks). This calls for new analytical and modelling strategies to account for these phenomena.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-15-0087>.

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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