Immunological properties of human decidual macrophages – a possible role in intrauterine immunity

Uma Singh¹, Grant Nicholson¹, Britta C Urban², Ian L Sargent¹, Uday Kishore²,³ and Andrés López Bernal⁴

¹Nuffield Department of Obstetrics and Gynaecology, University of Oxford, John Radcliffe Hospital, Headington, Oxford OX3 9DU, UK, ²Weatherall Institute of Molecular Medicine, University of Oxford, John Radcliffe Hospital, Oxford OX3 9DS, UK, ³Institute for Medical Microbiology, Faculty of Medicine, Justus-Liebig-University, Frankfurter Strasse 107, 35392 Giessen, Germany, ⁴Clinical Science at South Bristol (Obstetrics & Gynaecology), Level D, St Michael’s Hospital, Southwell Street, Bristol BS2 8EG, UK

Correspondence should be addressed to A López Bernal; Email: a.lopezbernal@bristol.ac.uk

Abstract
Our aim was to investigate the contribution of decidual macrophages, which constitute an important immune component of the decidua in late gestation, to intrauterine defence mechanisms. Using flow cytometry we examined the ability of decidual macrophages, isolated from term decidua, to bind and phagocytose fluorescence-labelled bacterial and yeast bioparticles. We also assessed their ability to generate superoxide radicals and tumour necrosis factor-α following lipopolysaccharide challenge. Decidual macrophages bound bacterial and yeast particles in a dose-dependent manner, which subsequently led to phagocytosis. These macrophages also produced superoxide radicals and the pro-inflammatory cytokine TNF-α when challenged with bacterial lipopolysaccharides. These results suggest a role for decidual macrophages in pathogen recognition and clearance during pregnancy, and, therefore, they are likely to protect the fetus against intrauterine infections which might otherwise lead to preterm labour.


Introduction
Decidua is the functional layer of the endometrium of pregnancy and is largely shed during parturition. The decidua contains a variety of immune cells, including T and B lymphocytes, granulocytes, natural killer cells and macrophages (Bulmer et al. 1988, Vince et al. 1990). However, the immunoregulatory contributions of each of these cells during pregnancy are not yet fully defined.

Decidual macrophages (DMs) are considered to be involved in protecting the fetus against intrauterine infections, which probably contributes to more than a third of total preterm deliveries occurring between 23 and 36 weeks gestation (Lettieri et al. 1993). Pathogens such as Group B Streptococcus, Escherichia coli (E. coli), Neisseria gonorrhoeae and Chlamydia trachomatis are known to cause chorioamnionitis during pregnancy. Bacterial lipopolysaccharide (LPS) introduced into amniotic fluid can stimulate DMs to generate phospholipase A2 and, hence, increased production of prostaglandins E2 and F2α (Casey et al. 1989) which may lead to preterm labour. DMs may also produce cytokines such as IL-1, TNF-α and IL-6 in response to an infection and therefore cause an intrauterine inflammatory reaction that could provoke preterm parturition (McGregor et al. 1988). Moreover, during implantation DMs have an important role in the regulation of apoptosis which is critical for the invasion of the developing embryo (Abrahams et al. 2004) and have several biochemical functions that favour local immunological tolerance against fetal tissues (Heikkinen et al. 2003).

In order to further understand the role of DMs in intrauterine immunity, we have examined their ability to interact with pathogenic bacteria and yeast zymosan. Using fluorescein-isothiocyanate (FITC)-labelled bacterial particles and flow cytometry, we have observed that DMs bind bacteria in a dose-dependent manner, which subsequently leads to phagocytosis. These DMs also produce superoxide radicals and the pro-inflammatory cytokine TNF-α when
challenged with bacterial LPS, suggesting an important role for decidual macrophages in bacterial recognition and clearance during pregnancy.

**Materials and Methods**

**Tissue**

Term placenta was collected after spontaneous or caesarean delivery with informed consent and processed within 45 min of delivery. All women delivered healthy singleton infants.

**Alkaline phosphatase–anti-alkaline phosphatase (APAAP) staining of decidual sections**

Rolls were made from amnion chorionic decidua, as previously described (Sutton et al. 1986). Frozen sections, cut at 7 µm and fixed in acetone, were blocked with rabbit serum (40 µl, 5 min), washed and then incubated with anti-CD14 monoclonal antibody (50 µl, 30 min; Serotec, Oxford, UK). Following washing (50 mM Tris–HCl and 0.15 M NaCl, (pH 7.6), 2 min), slides were first incubated with rabbit anti-mouse serum (DAKO, Cambridge, UK; 30 min), followed by anti-mouse APAAP complex (30 min; DAKO). A solution containing 160 mM Naphthol (Sigma, Dorset, UK), diluted with 200 µl Dimethylformamide (Sigma), 9.8 ml 0.1 M Tris buffer (pH 8.2), 1 mM Levaminsole (Sigma), 1 mM Phe-Gly-Gly and 10 mg Fast Red (Sigma) was applied for 15 min at room temperature. Slides were then counterstained with haematoxylin for 15 sec.

**Preparation of cell suspension from term decidua**

This was carried as described previously (Vince et al. 1990). Decidua, scraped from fetal membranes, were washed with Dulbecco’s PBS to remove red blood cells. The amount of decidual tissue obtained varied between 2 and 9 gm per placenta. All subsequent steps were carried out under sterile condition in RPMI-1640 medium (10 ml/gm tissue) containing L-Glutamine (Gibco-BRL, Basingstoke, UK) and 9 gm per placenta. All subsequent steps were washed with Dulbecco’s PBS to remove red blood cells. 1990). Decidua, scraped from fetal membranes, were washed with Dulbecco’s PBS to remove red blood cells. All subsequent steps were washed with Dulbecco’s PBS to remove red blood cells.

**Preparation of cytopsins**

Cells were diluted in Tris buffered saline containing 0.1% w/v BSA to adjust 0.025 × 10^4 cells per 100 µl. Slides were cytopsin (150 g, 6 min) and stained with anti-CD14 antibodies, as described above for the tissue sections.

**Interaction of decidual macrophages with labelled bioparticles**

E. coli BODIPY FL-conjugated bioparticles (Molecular Probes, Leiden, Netherlands) were suspended at a concentration of 20 mg/ml in PBS containing 2 mM sodium azide and sonicated briefly prior to use. Bioparticles were incubated with opsonising reagent (1 unit per 10 mg of bioparticles) at 37°C for 1 h prior to binding and phagocytosis assays. Decidual cells (1 × 10^6) were incubated with various concentrations of opsonin-treated E. coli (30 min, 4°C). Anti-CD14-RPE (10 µl/tube; Serotec) was added (30 min, 4°C). Cells were then washed with PGB and resuspended in 300 µl PBS plus FCS. In order to distinguish between surface-bound and phagocytosed bioparticles, trypan blue (0.2% v/v) was used as a quenching agent. (Antal-Szalmas et al. 2000). This is added to the cells after incubation with the bioparticles and quenches the fluorescence of the bioparticles bound to the cell surface but not those which have been internalised by phagocytosis. Thus, by measuring the fluorescence with and without trypan blue, it is possible to determine the levels of binding and phagocytosis. For some experiments, Zymosan and Staphylococcus aureus (Staph. aureus; BODIPY FL-conjugated bioparticles) were also used.

**Generation of reactive oxygen intermediates by decidual macrophages**

Decidual cells were first incubated with anti-CD14-RPE (30 min, 4°C). Following washing with PGB, cells were incubated with 5-(and-6)-chloromethyl-2,
7-dichlorodihydro-fluorescein diacetate (CM-H$_2$DCFDA, 20 µM; Molecular Probes) for 15 min at 37°C. LPS (E. coli serotype 026:B6; Sigma) was used as stimulant at different concentrations (30 min incubation at 37°C). The DMs were analysed by flow cytometry using two-colour labelling. Decidual cells, incubated with CM-H$_2$DCFDA without LPS, were used as control.

**Intracellular cytokine staining**

Decidual cells, in 1 ml RPMI-1640 plus 10% v/v FCS and 100 µM monensin (Sigma), were incubated at 37°C (5% v/v CO$_2$) in a non-adherent 24-well tissue culture plate and stimulated with 0.5 µg/ml LPS (Henter et al. 1988). The cultured cells were harvested at various time points, washed, incubated with anti-CD14-RPE (30 min over ice), and then fixed using PBS plus 0.25% w/v paraformaldehyde and 2% w/v glucose (10 min, 4°C). The cells were permeabilised using PBS plus 0.1% saponin (Sigma) and 0.1% w/v BSA (20 min, 4°C) and then incubated with mouse anti-human TNF-α-FITC (Serotec; 30 min over ice). Cells were washed, as described earlier, and then analysed by two-colour FACS (Jung et al. 1993). Decidual cells, stimulated with 0.5 µg/ml LPS without monensin, were used as control.

**Data analysis**

Experiments were repeated at least three times with tissue from different donors. Results were analysed with PRISM, version 3.02 (Graphpad Software, San Diego, CA, USA) using $t$-tests and Newman-Keuls multiple comparison test.

**Results**

**Identification of decidual macrophages in decidual tissue sections and cell suspensions**

Direct fixing and staining of membrane roll sections, using the APAAP method, revealed scattered CD14$^+$ macrophages in the decidual layer, in addition to being present in the amniotic membrane (Fig. 1a). The percentage of DMs, as identified in cytospin preparations by their surface staining, varied between 10 and 20% (Fig. 1b).

**Macrophages form a significant proportion of decidual cells**

Using a EPICS ELITE flow cytometer (Beckman Coulter Inc, Fullerton, CA, USA), dead cells were identified by gating on the propidium iodide positive population and defining their forward and side scatter characteristics. The average yield of cells obtained from term decidua was 2.9 ± 6x10$^6$ per gram of tissue (mean± s.E.M., $n = 25$). The viability of cells varied between 60 and 90%. Initial characterization of decidual cells showed that 31.7 ± 6.9% were CD45$^+$, 11.9 ± 3.1% were CD14$^+$, 10.6 ± 2.5% were CD64$^+$ (Fc receptor on macrophages), 15.4 ± 4.0% were HLA-DR$^+$ and 11.2 ± 2.3% were GHI 61$^+$ (CD163 protein in tissue macrophages, mean± s.E.M., $n = 3$; Law et al. 1993). For routine experiments, the percentage of cells that were labelled with anti-CD14 antibody compared with the control was calculated. This showed that the decidual macrophage population averaged 13.5 ± 1.6% (mean± s.E.M., $n = 25$) (Fig. 2). These results agree well with previous detailed analysis of immune cell populations in human decidua (Vince et al. 1990).
Decidual macrophages can bind and phagocytose pathogenic bacteria and zymosan

FACS analysis (Fig. 3) revealed that DMs bound *E. coli* in a dose-dependent manner. Binding of *E. coli* bioparticles increased linearly up to a concentration of $250 \times 10^6$ bioparticles per $10^6$ decidual cells/ml (Fig. 4a). Percentage of phagocytosis approached saturation at $200 \times 10^6$ *E. coli* per ml (Fig. 4b). Similar results were obtained with Zymosan and *Staph. aureus* bioparticles (Fig. 4a and b). With Zymosan, the percentage of phagocytosis increased linearly but with *Staph. aureus* it reached saturation at $200 \times 10^6$ bioparticles per ml.

Decidual macrophages generate superoxide radicals following challenge with *E. coli* LPS

Nonfluorescent CM-H$_2$DCFDA was used which, when trapped intracellularly, gets oxidised to yield a fluorescent derivative, oxidized CM-DCFDA, by the oxidative burst intermediates within the stimulated cells (Sacks et al. 1998). Exposure of DMs to *E. coli* LPS resulted in a dose-dependent increase in the mean channel brightness, reflecting increased superoxide radical production. The effect of LPS was biphasic, having a stimulatory effect up to 500 ng/ml ($P < 0.001$) and an inhibitory effect at higher doses ($P < 0.01$; Fig. 5).

Figure 2 Flow cytometry. Digest of term chorio-decidua was labelled with anti-CD14-RPE and analysed by flow cytometry. Colour back gating shows macrophage population (shown in green) on a size and granularity plot. On average (based on 25 experiments), ~13.5% of the cells were macrophages.

Figure 3 Binding and phagocytosis of *E. coli* bioparticles by decidual macrophages determined by flow cytometry. (a) Control (no bioparticles), (b) *E. coli* bioparticles, (c) *E. coli* bioparticles plus trypan blue.
Decidual macrophages produce TNF-α following LPS stimulation

Decidual cells were stimulated with LPS and cultured in the presence of monensin throughout various time points. Monensin is a lipophilic metabolite, which can disrupt ion gradients in biological membranes (Tartakoff 1983), thereby restricting protein secretion. Secretion-competent molecules, such as cytokines, therefore accumulate within the cells and can be detected using labelled antibodies by FACS (Fig. 6). The percentage of TNF-α-producing DMs increased from 2 ± 1% at 1 h to 55 ± 10% at 4 h (P < 0.001). The LPS concentration of 0.5 µg/ml was found to be optimal for TNF-α production by DMs.

Discussion

In this study, we have examined some of the functional properties of macrophages isolated from choriodecidual tissue at term. Using anti-CD14-RPE labelling and FACS, we identified DMs in decidual cell suspensions which were characterised without having to separate them from the rest of the decidual cell population. Using FITC-labelled *E. coli* bioparticles coated with opsonising reagent (Oben & Foreman 1988, Ragsdale & Grasso 1989), we were able to observe direct attachment of *E. coli* to DMs which subsequently led to their phagocytosis. The uptake of bacteria by DMs was concentration-dependent. We next examined the killing mechanism which DMs were likely to use against phagocytosed bacteria. In order to measure reactive oxygen intermediate production by DMs, nonfluorescent CM-H2DCFDA was used as a marker, which is oxidized to CM-DCFDA by the oxidative burst intermediates within the stimulated cells. When the DMs were stimulated with LPS, there was a considerable amount of TNF-α production by CD14+ cells which peaked at 4 h, consistent with the results previously obtained using monocytes (Henter et al. 1988). TNF-α has previously been shown to be constitutively produced in the decidua and its secretion by decidual cell suspensions has been shown to be enhanced by LPS (McGregor et al. 1988, Casey et al. 1989, Romero et al. 1989, Vince et al. 1992). The decidua comprises several cell types, but our present study shows that DMs are the major decidual cell type producing TNF-α in response to LPS challenge.

The roles of DMs in the maintenance of early pregnancy have been examined in terms of antigen presentation, immunoregulation, and lymphokine production (Abrahams et al. 2004). The DMs present in human early decidual tissue have a capacity for allo-antigen presentation, a higher suppressive activity, and a lower capacity to produce IL-1 following LPS stimulation than peripheral blood monocytes (Mizuno et al. 1994). DMs may represent an inhibitory type of antigen presenting cells and are thought to have an immunosuppressive role in early pregnancy due to their high levels of interleukin 10 and indoleamine 2,3-dioxygenase (Heikkinen et al. 2003, Kudo et al. 2004).
It is considered that the initiation of human parturition in the presence of infection is controlled by the host (Lettieri et al. 1993). Systemic maternal infections (pyelonephritis) or localised infections (deciduitis) can potentially trigger parturition by the activation of the monocyte/macrophage system in the decidua, where the intrauterine or maternal environment becomes hostile and threatens the survival of the fetal–maternal pair, thereby leading to preterm labour (Gómez et al. 1997). Human decidua in the third trimester of pregnancy contains at least four different cell types: stromal cells, macrophages, T lymphocytes and granulocytes (Vince et al. 1990). The contributions of these cells to the overall function of the tissue are still not fully understood. Chorioamnionitis may cause preterm labour by provoking the release of inflammatory mediators in the decidua/fetal membranes and it is likely that activation of prostaglandin release by DMs is involved in triggering labour (López Bernal et al. 1991, López Bernal et al. 1993). Human TNF-α can selectively stimulate prostaglandin F2α production by human DMs (Norwitz et al. 1992).

Our data demonstrate that DMs are functionally involved in the binding and phagocytosis of bacteria and may provide a local defence mechanism in the decidua/fetal membranes. However, in some cases the defence mechanism may fail, leading to intrauterine infection and chorioamnionitis. The macrophages are known to secrete cytokines, including IL-1, IL-6 and TNF-α. The action of TNF-α on human decidua may be mediated by an autocrine or paracrine mechanism, where it is released by DMs on exposure to LPS and may induce stimulation of PGF2α production by macrophages, thereby precipitating preterm labour (Norwitz et al. 1992). Macrophages isolated from the amniotic fluid of pregnant mice respond to fetal surfactant protein A (SP-A) by increasing IL-1β and NF-κB production, and this may trigger uterine activation and labour (Condon et al. 2004). SP-A levels increase sharply in human amniotic fluid towards term (Miyamura et al. 1994) and it would be of interest to study its effect on human DMs.

In conclusion, our data demonstrate, for the first time, that the DMs in term tissues are functional. However, further work is necessary to investigate DM activity at different gestational stages. The incidence of chorioamnionitis is relatively high between 25 and 30 weeks gestation, and it is important to establish whether there are alterations in macrophage populations or in the release of cytokines that contribute to the triggering of infection-associated preterm labour.

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