Immunological role of vitamin D at the maternal–fetal interface

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Abstract

During pregnancy, immune activity is tightly regulated so that antimicrobial protection of the mother and fetus is balanced with the need for immune tolerance to prevent fetal rejection. In this setting, the maternal–fetal interface, in the form of the uterine decidua, provides a heterogeneous immune cell population with the potential to mediate diverse activities throughout pregnancy. Recent studies have suggested that vitamin D may be a key regulator of immune function during pregnancy, with the fetal–maternal interface representing a prominent target. Among its non-classical actions are potent immunomodulatory effects, including induction of antibacterial responses and modulation of T-lymphocytes to suppress inflammation and promote tolerogenesis. Thus, vitamin D may play a pivotal role in normal decidual immune function by promoting innate responses to infection, while simultaneously preventing an over-elaboration of inflammatory adaptive immunity. Research to date has focused upon the potential role of vitamin D in preventing infectious diseases such as tuberculosis, as well as possibly suppressing of autoimmune disease. Nevertheless, vitamin D may also influence facets of immune function not immediately associated with primary innate responses. This review summarises our current understanding of decidual immune function with respect to the vitamin D metabolism and signalling, and as to how this may be affected by variations in maternal vitamin D status. There has recently been much interest in vitamin D supplementation of pregnant women, but our knowledge of how this may influence the function of decidua remains limited. Further insight into the immunomodulatory actions of vitamin D during pregnancy will help shed light upon this.

Key Words

- ▶ vitamin D
- ▶ decidua
- ▶ fetal–maternal interface
- ▶ immunity
- ▶ monocyte
- ▶ T cell
- ▶ uterine natural killer cell

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Introduction

Immune function in pregnancy

From initial implantation of the conceptus, the maternal uterine endometrium undergoes 'decidualisation' to support placental development and function. The resulting

decidua is a tissue formed from the maternal endometrium, originating from epithelial and stromal cells, and is characterised by invasion from the extra-embryonic fetal-derived trophoblasts and close 'cell–cell

juxtaposition' of these different tissues (Fig. 1). The principal function of the decidua is to facilitate early fetal–maternal exchange of nutrients, gases and waste, while also acting as a secretory source of an array of steroid hormones, cytokines and growth factors (Gellersen *et al.* 2007, Salamonsen *et al.* 2007, Ramathal *et al.* 2010). However, the decidua also plays a key role in protecting pregnancy against maternal immune surveillance (Warning *et al.* 2011), and this feature will be the focus of the current review.

Pregnancy presents a unique immune challenge for the maternal host. Systemically, significant gestation-dependent immune adaptations arise throughout pregnancy and characterisation of these is a major focus of materno-fetal immunology research (Moffett & Loke 2006). Specifically, in the mother, there is a shift towards immune tolerance to accommodate the conceptus (Alijotas-Reig *et al.* 2014). As a result, the maternal–fetal interface is a prime area of immune regulation, and cellular immunity within the decidua is importantly

Figure 1

Proposed immunomodulatory effects of vitamin D at the maternal–fetal interface. Illustration of both the innate and adaptive leukocyte decidual subsets with the potential capacity for intrinsic 1,25-dihydroxyvitamin D ($1,25(\text{OH})_2\text{D}$) synthesis from 25-hydroxyvitamin D (25OHD), including uterine NK cells (uNK), macrophages, dendritic cells (DCs), T cells and B cells. Their postulated pro-tolerogenic effects specifically at the fetal–maternal interface, in the presence of $1,25(\text{OH})_2\text{D}$, are illustrated. Notably, uNK cells predominate and demonstrate reduced cytotoxic potential. 'M1' macrophage subtypes exist, demonstrating enhanced antibacterial, pro-inflammatory actions in the presence of $1,25(\text{OH})_2\text{D}$. However, the pro-tolerogenic 'M2' subsets predominate, displaying both enhanced proliferative and tissue remodelling capacities. Immature DCs are sparse; $1,25(\text{OH})_2\text{D}$ may augment their reduced capacity for antigen presentation

and enhanced expression of anti-inflammatory cytokines, such as IL10. Immunosuppressive CD8^+ and CD4^+ T cells coexist and they are suspected key producers of $1,25(\text{OH})_2\text{D}$. Postulated pro-tolerogenic effects include a shift from a Th1 to a Th2 phenotype, increased T regulatory cell (Treg) production and suppression of T helper 17 cell (Th17) activity. Finally, albeit infrequent in number, B cell proliferation and differentiation may be inhibited in the presence of $1,25(\text{OH})_2\text{D}$. 1,25D, $1,25(\text{OH})_2\text{D}$; 25D, 25OHD; M, macrophage; DC, dendritic cell; HLA, human leukocyte antigen; Ig, immunoglobulin; IL, interleukin; 25OHD, 25-hydroxyvitamin D; $1,25(\text{OH})_2\text{D}$, 1,25-dihydroxyvitamin D; VDR, vitamin D receptor; uNK, uterine natural killer cell; Th, T helper cell; Treg, T Regulatory cell; CD, cluster of differentiation; Ag, antigen; $\text{IFN}\gamma$, interferon gamma; $\text{TNF}\alpha$, tumour necrosis factor alpha.

different from that within the maternal circulation. Cellular infiltration is a key feature of immune function within decidua, and leukocytes comprise at least 40% of the total decidual stromal cell population (Bulmer *et al.* 1988, 1991). The leukocyte subtypes present include decidual (uterine) natural killer (uNK) cells, macrophage subtypes, CD4⁺ and CD8⁺ T-lymphocytes (including T-regulatory cells (Tregs)) and antigen-presenting cells (APCs) such as dendritic cells (DCs) (Arck & Hecher 2013). Although well classified anatomically, these immune cells and, in particular, their function are the focus of much contemporary research. There has been renewed interest in the role of hormones as key regulators of decidual immune cell function (Oreshkova *et al.* 2012). The current review will focus on one of these hormones, vitamin D, and its possible roles in fetal–maternal immune tolerance, and the prevention of adverse events in pregnancy.

Vitamin D and human pregnancy

Vitamin D is a secosteroid classically recognised for its key role in bone metabolism and calcium homeostasis. Since the 1980s, many important ‘non-classical’ extra-skeletal functions of vitamin D have been described, including anti-proliferative, pro-differentiative and potent immunomodulatory actions (Haussler *et al.* 2008). A central feature of these extra-skeletal actions is the recognition that they probably involve autocrine/intracrine metabolic mechanisms that are distinct from the ‘classical’ endocrine renal generation of active 1,25-dihydroxyvitamin D (1,25(OH)₂D) characteristic of skeletal actions of vitamin D. Specifically, many non-classical actions of vitamin D are associated with localised metabolism of precursor 25-hydroxyvitamin D (25OHD), with the resulting active 1,25(OH)₂D signalling via endogenous vitamin D receptors (VDRs) (Hewison *et al.* 2007, Hewison 2010). While intracrine synthesis of 1,25(OH)₂D provides many advantages for tissue-specific regulation of vitamin D function, it is also likely to be more sensitive to variations in concentrations of the substrate 25OHD. As 25OHD is the principal marker of vitamin D ‘status’ in any given individual, this has raised the question as to how vitamin D sufficiency or deficiency may affect tissues in which there is significant metabolism of 25OHD. Prominent among these tissues is the placenta, where both fetal and maternal tissues have been shown to synthesise ‘active’ 1,25(OH)₂D (Gray *et al.* 1979, Weisman *et al.* 1979).

The implications of vitamin D deficiency are far reaching. Classically, severe vitamin D deficiency is

recognised for its negative effect on bone mineralisation, as manifested by rickets in children and osteomalacia in adults (Holick 2007, Holick & Chen 2008). In more recent studies, low serum levels of 25OHD have been associated with common cancers, allergic disorders, infections, autoimmune disorders and cardiovascular disease (Hyp-ponen *et al.* 2001, Cannell *et al.* 2006, Munger *et al.* 2006, Grant & Mohr 2009, Brehm *et al.* 2010, Zittermann *et al.* 2011). However, the questions as to what constitutes optimal or adequate vitamin D status and what is the magnitude of vitamin D-deficiency in populations across the globe are a subject of debate (Holick 2007). The 14th Workshop Consensus on Vitamin D concluded that a global vitamin D deficiency epidemic exists (defined by a serum 25OHD below 12 ng/ml or 30 nM) and represents a major health problem (Henry *et al.* 2010). By contrast, the current Institute of Medicine (IOM) classification of vitamin status is as follows: deficiency as 25OHD <20 ng/ml (50 nM), and vitamin D insufficiency as 25OHD <30 ng/ml (75 nM) (Holick *et al.* 2011). In 2010, the IOM recommended an increase in the minimal daily intake of vitamin D too by at least 600 IU (600 IU = 15 µg)/day, and they also recognised that at least 1500–2000 IU/day of vitamin D may be needed to maintain a blood level of 25OHD >30 ng/ml, with a maximum upper level of 4000 IU/day for adults, including pregnant women (Holick *et al.* 2011).

One group who appear to be at particular risk of vitamin D deficiency are pregnant women and women breastfeeding for long periods (Holick 2007, Mithal *et al.* 2009); with those living at high latitude with reduced sunlight exposure, dark skin pigmentation, or with a high BMI, and poor dietary intake at the greatest risk of low vitamin D status (Mithal *et al.* 2009, Holick *et al.* 2011). At present, no separate consensus regarding optimum vitamin D levels in pregnancy has been reached and general IOM standard cutoff values are currently in use. In 2006, 7% of pregnant or lactating women in a large national database in the USA were reported as being vitamin D deficient (<30 nM (<12 ng/ml) serum 25OHD), and 28% as vitamin D ‘inadequate’ (<50 nM (<20 ng/ml) serum 25OHD) (Looker *et al.* 2011). In other studies, Bodnar *et al.* (2007a) showed that 83% of pregnant US black women and 47% of pregnant white women were vitamin D insufficient (<32 ng/ml serum 25OHD) at delivery, with similar values for neonates. Prevalence rates for vitamin D deficiency (<20 ng/ml serum 25OHD) are highly variable elsewhere: 100% of Somali immigrants in Sweden and 98% of Omani women, compared with 24% of Western Canadian women and 7% of women from North Carolina

(reviewed in Urrutia & Thorp (2012)). Concerning the UK, The National Diet and Nutrition Survey reported that 28% of the female population aged 19–24 years had low serum vitamin D levels (<25 nM (<10 ng/ml)) (Public Health England and Food Standards Agency 2014). Furthermore, serum 25OHD concentrations <50 nM (<20 ng/ml) were reported in 49.5% of pregnant women in a recent UK-based prospective cohort study (Gale *et al.* 2008).

Defining vitamin D deficiency during pregnancy remains a major challenge. Maternal 25OHD levels do not appear to change significantly across pregnancy (Ginde *et al.* 2010). By contrast, serum levels of $1,25(\text{OH})_2\text{D}$ increase by approximately twofold during the first trimester, and peak during the third trimester (Brannon & Picciano 2011). Renal synthesis of $1,25(\text{OH})_2\text{D}$ by the mother plays a key role in this elevation of maternal serum $1,25(\text{OH})_2\text{D}$ levels, but as outlined above, there is also evidence of local $1,25(\text{OH})_2\text{D}$ generation in both the placenta (fetal) and decidua (maternal) (Gray *et al.* 1979, Weisman *et al.* 1979). Early in pregnancy, the maternal decidua and fetal trophoblast cells show induced expression of both VDR and 1α -hydroxylase (CYP27B1), the enzyme that catalyses conversion of 25OHD to $1,25(\text{OH})_2\text{D}$. By contrast, the vitamin D catabolic enzyme, 24 -hydroxylase (CYP24A1), shows decreased expression in placental/decidual tissues across gestation (Zehnder *et al.* 2002, Evans *et al.* 2004). In this way, the placental/decidual tissues have the potential to generate significant amounts of $1,25(\text{OH})_2\text{D}$ without appreciable catabolic inactivation. Studies using non-pregnant rats demonstrated that conversion of 25OHD to $1,25(\text{OH})_2\text{D}$ was absent following nephrectomy, whereas synthesis of $1,25(\text{OH})_2\text{D}$ was still observed in pregnant rats (Weisman *et al.* 1978). This suggests that some $1,25(\text{OH})_2\text{D}$ produced by the placenta can spill over into the maternal circulation. Despite this, it is not clear that placental/decidual CYP27B1 plays a significant role in elevating maternal circulating levels of $1,25(\text{OH})_2\text{D}$. A single case report of a pregnant woman on chronic haemodialysis reported only mildly elevated circulating concentrations of $1,25(\text{OH})_2\text{D}$ relative to pre-pregnancy, with these levels being substantially lower than normal in pregnant women (Turner *et al.* 1988). Observations such as this suggest that the maternal kidneys are likely to be the most important site for maternal $1,25(\text{OH})_2\text{D}$ production during pregnancy. If this is the case, then the question arises as to whether there are alternative physiological functions of locally generated $1,25(\text{OH})_2\text{D}$ within the maternal and fetal utero-placental tissues.

One possibility is that $1,25(\text{OH})_2\text{D}$ generated by either the placenta or decidua acts to facilitate the transport of calcium and/or phosphate across the placenta to support fetal skeletal development (reviewed in Kovacs & Kronenberg (1997)). It has previously been postulated that the observed increase in $1,25(\text{OH})_2\text{D}$ levels in pregnancy represents a normal physiological response to increased calcium requirements (Lund & Selnes 1979, Kooh & Vieth 1980), and that either a pregnancy-specific, or calcium homeostatic stimulus, such as oestrogen (Murphy *et al.* 1980), human placental lactogen (Josinovich 1969), or parathyroid hormone (Garabedian *et al.* 1974), which concomitantly increase throughout pregnancy, is implicated in this process. However, other groups have presented evidence suggesting that, in pregnancy, both calcium mobilisation from bone and intestinal transport occur by a vitamin D-independent mechanism (Van Cromphaut *et al.* 2003). Furthermore, calcium supply to the fetus is not negatively influenced by vitamin D deficiency (Halloran & DeLuca 1980). Other reports have indicated that expression of the VDR is not required for fetal-placental calcium homeostasis (Kovacs *et al.* 2005). Similarly, mineralisation of fetal bones occurs relatively late in pregnancy, while the induction of VDR and CYP27B1 in both maternal and fetal placental tissues occurs early in gestation, and thus alternative actions for intracrine $1,25(\text{OH})_2\text{D}$ have to be taken into consideration now (Evans *et al.* 2004). These include possible effects on fetal development through *homeobox* gene expression (Du *et al.* 2005) and placental vascularisation (Woodham *et al.* 2011). Studies by ourselves and others have supported an immunomodulatory function for vitamin D within the placenta and the specific role of maternal decidua in this process becomes the major focus of the current review.

Vitamin D and placental immunology

A role for vitamin D as a potential modulator of immune responses during pregnancy was first postulated over 50 years ago, with specific reference to implantation (Chambon 1951), but the specific mechanisms by which this may be achieved have only recently gained further attention (Reichrath & Querings 2005). In a similar fashion, a potential role for the placental vitamin D system in promoting anti-inflammatory responses against bacteria and, more recently, viral pathogens have been proposed (Mehta *et al.* 2009, Baeke *et al.* 2010). This has occurred in large because of recent advances in our understanding of how vitamin D interacts with the immune system: it is now clear that vitamin D can

influence both the innate and adaptive (acquired) arms of the immune system (Adams & Hewison 2008, Hewison 2011). The remainder of this review will explore these interactions in more detail with specific reference to the individual groups of immune cells present in the decidua.

Decidual macrophages

Macrophages are the second most abundant immune cell type in the decidua, representing ~20% of decidual immune cells (Salamonsen *et al.* 2007, Bulmer *et al.* 2010). Derived from blood monocytes they execute a wide array of critical immunological functions including phagocytosis and the subsequent intracellular killing of internalised pathogens, antigen presentation and cytokine secretion (Fujiwara & Kobayashi 2005). As such, they play a pivotal role in orchestrating the innate and adaptive arms of the immune system.

As well as being able to phagocytose pathogens such as bacteria and viruses, macrophages and their precursor monocytes can also sense pathogen-associated molecular patterns (PAMPs) by utilising pattern-recognition receptors (PRRs), such as toll-like receptors (TLRs) (Takeda & Akira 2005). In 2006, studies to identify monocyte genes regulated in response to the tuberculosis pathogen *Mycobacterium tuberculosis* showed induction of CYP27B1 and VDR expression following *M. tuberculosis* PAMP-sensing by TLR2/1 (Liu *et al.* 2006). This observation suggested that these cells promote cell-specific activation of vitamin D in response to *M. tuberculosis*, with the resulting 1,25(OH)₂D binding to endogenous VDR. This 'intracrine' system allows vitamin D to modulate gene expression in response to an immune challenge against *M. tuberculosis*. Potential targets for intracrine activation of vitamin D include the antibiotic protein cathelicidin, which is a direct transcriptional target for the 1,25(OH)₂D-VDR complex (Wang *et al.* 2004, Gombart *et al.* 2005). Functional analyses showed that 25D-mediated induction of cathelicidin in monocytes promotes intracellular killing of *M. tuberculosis* (Liu *et al.* 2006). Thus, although TLR2/1 responses to *M. tuberculosis* initially involve activation of monocyte CYP27B1 and VDR gene expression, the efficacy of subsequent antibacterial activity ultimately depends on the availability of 25OHD for intracrine conversion to 1,25(OH)₂D. In this way, vitamin D status may be a key determinant of monocyte antibacterial responses, and variations in human serum 25OHD have been shown to correlate with induction of monocyte cathelicidin (Liu *et al.* 2006). The conclusion from these studies was that low serum 25OHD is less able to support monocyte antibacterial activity, with an increased

risk of infection. Conversely, supplementation of vitamin D-insufficient individuals may improve monocyte cathelicidin production (Adams *et al.* 2009) and help to protect against infection.

Demonstration of a vitamin D-dependent, pathogen-mediated antibacterial response in monocytes from peripheral blood has raised the possibility that similar innate immune responses will be present in monocytes and macrophages from other tissues (Bacchetta *et al.* 2013). Purification of decidual cells into non-adherent stromal cells and adherent cells, which include decidual macrophages and uNK cells, showed that adherent cells demonstrate a greater capacity for 1,25(OH)₂D production (Kachkache *et al.* 1993). In view of the lack of evidence for functional expression of CYP27B1 in NK cells, including uNK cells, it seems likely that decidual macrophages will play a significant role in the localised generation of 1,25(OH)₂D within the decidua. What is less clear is the immune impact of 1,25(OH)₂D in this setting. One possibility is that decidual monocytes and macrophages will promote the same antibacterial responses observed for equivalent cells from the peripheral blood. Although, to date, this possibility has not been addressed in purified decidual monocytes/macrophages, studies using unpurified first-trimester decidual cells have shown 25OHD- and 1,25(OH)₂D-mediated induction of cathelicidin (Evans *et al.* 2006). It is also important to recognise that intracrine activation of vitamin D promotes antibacterial responses beyond simple induction of cathelicidin and other antibacterial proteins such as β -defensins (Liu *et al.* 2009). These include the induction of autophagy (Shin *et al.* 2011) and possible effects on intracellular iron concentrations via the iron export modulator hepcidin (Bacchetta *et al.* 2014). Crucially, these mechanisms are focused on an intracellular function for vitamin D, and enhanced antibacterial responses to vitamin D are optimal for intracellular pathogens such as *M. tuberculosis*. As yet, it is not clear whether similar pathogens and the associated antimicrobial responses will be evident in the decidua. Indeed, it is possible that decidual vitamin D will support alternative antimicrobial responses including potential antiviral activity (Equils & Hewison 2010).

Beyond their established innate antimicrobial activity, macrophages also play a pivotal role in tissue inflammation and antigen presentation, and both of these facets of immunity are crucial to normal decidual function. In the context of inflammation, macrophages can be categorised according to their functional role, comprising pro-inflammatory and anti-inflammatory subtypes. Analogous to the concept of different groups of

T helper (Th) cells, macrophages activated under the influence of pro-inflammatory cytokines (such as interferon γ (IFN γ) and tumour necrosis factor α (TNF α)) have been termed 'M1', while those induced via exposure to Th2 cytokines (interleukin 4 (IL4), IL10 and IL13) are termed 'M2'. M1 subtypes, which have potent antimicrobial and cytotoxic properties, are subsequently implicated in microbial killing and autoimmune disease, whereas M2 subtypes convey a distinct immunosuppressive phenotype consistent with a role in humoral immunity and repair (Mosser 2003, Gordon & Martinez 2010). This plasticity is demonstrated by acquisition of distinct morphological and functional properties and appears to be tissue specific (Houser *et al.* 2011).

In the decidua, M1 macrophages are postulated to participate in the progression of inflammation, whereas M2 decidual macrophages serve to promote fetal-maternal immune tolerance, tissue remodelling and scavenging of apoptotic cells (Erlebacher 2013a,b). Comparison between first- and second-trimester leukocyte populations has recently found that, although there was a significant decline in total macrophage numbers, a CD163⁺CD206⁺ subset designating alternatively activated pro-tolerogenic M2-like macrophages concomitantly increased (Kwan *et al.* 2014). Microarray gene expression analysis of human 1st-trimester decidua-derived macrophages has also revealed gene expression patterns associated with M1 and M2 macrophages (Gustafsson *et al.* 2008). In addition to their enhanced immune-modulatory potential, decidual macrophages also demonstrated a higher proliferative and tissue remodelling capacity, when compared with their peripheral blood counterparts (Gustafsson *et al.* 2008).

In the context of adverse pregnancy, elevated serum concentrations of IL12 (which augment the production of inflammatory cytokines) have also been measured in the peripheral blood of women with pre-eclampsia (PET) (Saito *et al.* 1999). Lower levels of anti-inflammatory IL10 have also been reported in the decidua basalis in cases of PET (Schonkeren *et al.* 2011, Darby *et al.* 2013). Polarisation towards an M2 macrophage subtype has also been observed in normal-term placentas relative to those from women with pregnancies complicated by both preterm birth and PET. M2 macrophage numbers in relation to total macrophages (CD163/CD14 ratio) were lower in placentas from preterm PET pregnancies compared with those from preterm 'controls'. Interestingly, total macrophage numbers were significantly higher in the PET group and positively correlated with PET severity (Schonkeren *et al.* 2011). However, although this

shift towards a pro-inflammatory cytokine environment is described in the context of malplacentation disorders, this is an over-simplification; contradictory information exists as outlined later in this review. Certainly, a more detailed understanding of whether Th1 cytokine activity drives local dysregulation of immunoregulatory mechanisms in PET is required (Heikkinen *et al.* 2001) and, furthermore, whether vitamin D status is important to this. It is interesting to note that, in mouse models of inflammation and pregnancy, loss of the placental vitamin D intracrine system as a consequence of *Vdr* or *Cyp27b1* gene knockout greatly exaggerates pro-inflammatory activity in the placenta (Liu *et al.* 2011).

Dendritic cells

Monocytes can also differentiate into DCs (CD83⁺), which populate the decidua throughout pregnancy (Laskarin *et al.* 2007). In human decidua, DCs are relatively sparse compared with macrophages (~1% of total decidual cells (Kammerer *et al.* 2000)), questioning the functional importance of these cells in the decidua (Rieger *et al.* 2004). Macrophages and DCs share the ability to act as APCs, and both cells may therefore coordinate innate and adaptive immune responses in human pregnancy by balancing maternal immune responses against foreign antigens with protection of the semi-allogenic conceptus (Kammerer *et al.* 2000). First-trimester human CD83⁺ decidual DCs possess T cell immune-stimulatory capacity in *ex vivo* mixed leukocyte reactions and also cluster with T cells *in situ* (Kammerer *et al.* 2000). Decidual DCs appear to be more tolerogenic than their peripheral blood counterparts, with a lower capacity for antigen presentation, reduced expression of co-stimulatory molecules, reduced expression of inflammatory cytokines such as IL12 and enhanced expression of anti-inflammatory cytokines such as IL10 (Laskarin *et al.* 2007, Arck *et al.* 2013). This is consistent with an immature DC phenotype that is generally associated with a more tolerogenic T-cell response (Kammerer *et al.* 2003). Recent data obtained specifically from 1st- and 2nd-trimester decidua support this, as maturation of DC precursors towards the inflammatory CD83⁺ DC subtype appears to be inhibited, given that no change in either CD209⁺ (immature) or mature CD83⁺ populations was observed, whereas the intermediate CD205⁺ DC subtype significantly declined (Kwan *et al.* 2014).

DC activity in decidua may also be modulated through other mechanisms. In murine models of pregnancy, a phenomenon known as 'DC entrapment' has

been reported, where decidual DCs fail to migrate to uterine lymph nodes following their activation (Collins *et al.* 2009). It has been postulated that this may serve to limit adaptive T cell responsiveness towards fetal/placental antigens and restrict immunosurveillance specifically at the maternal–fetal interface.

As yet, it is unclear whether antigen presentation in the decidua is associated with a specific APC type. However, it would appear that both macrophages and DCs can utilise vitamin D through intracrine pathways (Kreutz *et al.* 1993, Hewison *et al.* 2003). *In vitro*, 1,25(OH)₂D inhibits the differentiation and maturation of DCs through binding to the VDR (Griffin *et al.* 2001). Similar to macrophages (Kreutz *et al.* 1993), DCs derived from peripheral blood show differentiation-dependent changes in expression of VDR and CYP27B1 (Hewison *et al.* 2003). Using this intracrine system, 25OHD acts to inhibit DC maturation, as characterised by reduced cell surface expression of major histocompatibility (MHC) class II, CD40, CD80 and CD86 (Griffin *et al.* 2000). As a result, T cells stimulated by DCs in the presence of 25OHD have decreased proliferative capacity and a more tolerogenic phenotype (Hewison *et al.* 2003, Jeffery *et al.* 2012). Both a relative increase in T cell expression of IL10 when compared with IFN γ and enhanced Treg functionality have been reported in vitamin D-treated DCs (Hewison *et al.* 2003, Jeffery *et al.* 2012).

In contrast to the wealth of recent data for DCs from peripheral blood, the exact role of DCs within the decidua is much less clear, particularly with respect to vitamin D responses. In studies of recurrent PET (Huang *et al.* 2008), and recurrent miscarriage (Askelund *et al.* 2004), an overabundance of decidual DCs has been observed, suggesting that DCs may be involved in the aetiology of some adverse events in pregnancy. The presence of an active intracrine system for vitamin D in DCs strongly suggests that variations in maternal 25OHD may have a significant effect on DC recruitment, maturation and antigen presentation. In future studies, it will be important to assess the impact of vitamin D status on decidual DC function. However, in addressing this, it is unclear how effective animal models will be, given that mice lacking uterine DCs do not show obvious reproductive deficits (Huang *et al.* 2008).

Decidual/uterine NK cells

In contrast with peripheral blood, in which only 10% of total lymphocytes are of NK origin (characterised by the cell surface antigen CD56), uNK cells account for a major proportion of the stromal leukocyte population in the decidua. uNK cells differ phenotypically from the majority

of peripheral blood NK cells, being CD56-bright, CD16 negative and CD9 positive and showing distinct expression of both inhibitory and activating NK receptors (Trundley & Moffett 2004, Bulmer & Lash 2005, Bulmer *et al.* 2010). They are present in non-pregnant endometrium throughout the menstrual cycle but increase in number in the mid-secretory phase with a further increase in pregnancy to account for 70% of the total decidual stromal immune cell population (Bulmer *et al.* 2010). The number of uNK cells decreases in the second half of pregnancy, although a substantial number persists in the third trimester (Scaife *et al.* 2003).

The mechanisms that underpin the increased number of uNK cells in secretory-phase endometrium and early pregnancy are not fully understood. Recruitment of CD56⁺ cells to the decidua has been suggested, either through increased expression of the chemokine receptors CXCR4 and CCR5 both by decidual cells and invading extravillous trophoblast cells (Hanna *et al.* 2003). There is other evidence that uNK cells develop *in situ* within endometrium, either from haematopoietic stem cells (Lynch *et al.* 2007, Vacca *et al.* 2011) or from immature NK cell precursors (Male *et al.* 2010). In addition, uNK cells actively proliferate in secretory-phase endometrium, with up to 40% of CD56 cells in mid- and late-secretory-phase endometrium co-expressing the cell proliferation marker Ki67 (Bulmer, unpublished observations (Pace *et al.* 1989)). Transforming growth factor β (TGF β) and IL15, both of which are produced by decidua in early pregnancy, are able to promote maturation of peripheral NK precursors towards uNK cells (Keskin *et al.* 2007). Array studies have indicated that uNK cells from early pregnancy and non-pregnant endometrium are distinct from each other and that in turn both populations are distinct from peripheral blood NK cells (Kopcow *et al.* 2010). In addition, uNK cells also demonstrate decreased cytotoxic capabilities and enhanced expression of a range of cytokines, chemokines and growth factors relative to peripheral blood NK cells (Hanna *et al.* 2003, Lash *et al.* 2010a). Indeed, the phenotype and cytokine/chemokine production of uNK cells have been reported to alter at different gestational ages with increased cytokine production and reduced angiogenic growth factor production at 12–14 weeks compared with 8–10 weeks gestational age.

The prominence of uNK cells around the time of implantation and early placentation has suggested a fundamental role in the establishment of successful pregnancy. Furthermore, numbers of uNK cells have been reported to be altered in pre-eclampsia (Williams *et al.* 2009), although results are inconsistent, and several

groups have reported increased uNK cells in mid-secretory-phase endometrium from women with a history of recurrent miscarriage or recurrent implantation failure (Clifford *et al.* 1999, Laird *et al.* 2003, Quenby & Farquharson 2006). As the uNK cells are the predominant decidual leukocyte population in early pregnancy, many roles have been suggested for them. Identification of high numbers of CD56⁺ cells later in third-trimester samples suggests that the role of uNK cells may even be more far reaching than anticipated (Bulmer *et al.* 2010, Lash *et al.* 2010a). However, despite numerous *in vitro* studies of function, the *in vivo* role of uNK cells remains unclear.

The effects of vitamin D on both peripheral blood and uterine NK cells have been poorly defined. In peripheral blood mononuclear cells, 1,25(OH)₂D inhibits NK cell activation and cytotoxic actions in a time- and dose-dependent manner (Merino *et al.* 1989). The cytokines IFN γ and IL2 have been reported to augment NK cytotoxic activity, and both precursor vitamin D and 1,25(OH)₂D inhibit this function (Leung 1989). However, in this latter case, studies were carried out using a heterogeneous mix of immune cells and so the precise mechanism by which the NK cells respond to vitamin D is unclear. NK cells express mRNA for VDR and CYP27B1 (Moran-Auth *et al.* 2013), although it is still not clear whether this represents a functional intracrine system for synthesis of 1,25(OH)₂D by these cells. Studies of vitamin D and uNK cells have also been very limited, but may help to elucidate the mechanisms underpinning some of the associations between vitamin D status and pregnancy outcomes. In studies using partially purified uNK cells from first-trimester human pregnancies, treatment with either 25OHD or 1,25(OH)₂D *ex vivo* promoted antibacterial and anti-inflammatory responses (Evans *et al.* 2006). In view of the fact that vitamin D deficiency in humans (Bodnar *et al.* 2007b) and mice (Liu *et al.* 2013) has been linked to PET, it is interesting to speculate that uNK cells may play a role in the pathophysiology of this complication of pregnancy. uNK cells appear to play a pivotal role in spiral arteriole remodelling (Robson *et al.* 2012) and the regulation of extravillous trophoblast invasion into decidua basalis and superficial myometrium through the production of local cytokines and chemokines (Lash *et al.* 2010b). In mice lacking uNK cells, there is a failure of normal decidual artery modification, and decidual hypo-cellularity/necrosis is observed (Lash *et al.* 2010a). Incomplete spiral arteriolar transformation and failure of extravillous trophoblast invasion are together considered to be responsible for placental underperfusion and may thus underlie the pathogenesis of both PET and intrauterine growth restriction (IUGR).

Another subtype of NK cells that accumulate with decidua are the CD1d-reactive natural killer T (NKT) cells, which have an invariant T cell receptor rearrangement (Boyson *et al.* 2008). Murine data suggest that NKT cell activation may induce preterm birth as well as pregnancy loss during the period from early gestation to mid-gestation (Boyson *et al.* 2006). As NKT cells are known to activate a number of other leukocyte subsets, it has been hypothesised that activation of NKT cells is the first step in a cascade of adverse anti-tolerogenic effects that may then lead to preterm birth (Boyson *et al.* 2008). As yet, there have been no studies to assess the impact of vitamin D on decidual NKT function and pregnancy outcome. However, in studies carried out in mice, variations in vitamin D status (maternal serum 25OHD) *in utero* have been shown to result in altered NKT cell function of the resulting offspring (Yu & Cantorna 2011). This clearly has important implications for the potential impact of maternal vitamin D status on child health in humans.

T cells

Maternal tolerance of the fetus is generally not compromised by the production of T cells specific for fetal antigens during pregnancy. The mechanisms by which tolerance is maintained have gained much interest in recent years, and an increasingly complex, multi-faceted system has gradually been elucidated. CD3⁺ T lymphocytes (T cells) comprise ~10% of the endometrial stromal leukocyte population in first trimester decidua and are of both helper T cell (CD4⁺) and cytotoxic T cell (CD8⁺) subtypes. In contrast to the peripheral blood where CD3⁺CD8⁺ cells predominate, decidual CD3⁺CD4⁺ T cells are less abundant (CD8⁺:CD4⁺, ratio 3:1). In first-trimester human decidua, ~30–45% of T cells are CD4⁺, and 45–75% are CD8⁺ T cells (Bulmer *et al.* 2010). Both subtypes have immunosuppressive functions and may be involved in mediating materno-fetal tolerance, particularly as pregnancy evolves (Blois *et al.* 2004).

Activation of naïve Th cells by antigen leads to the generation of CD4⁺ Th subgroups with distinct cytokine profiles: Th1 (IL2, IFN γ and TNF α) and Th2 (IL3, IL4, IL5 and IL10), which respectively support cell-mediated and humoral immunity (Abbas *et al.* 1996, Romagnani 2006). A third group of Th cells known to be influenced by vitamin D are IL17-secreting T-cells (Th17 cells). Initially, it was believed that pregnancy reflects an immune setting dominated by Th2 cytokines (Wegmann *et al.* 1993). However, this is not absolute and a local Th2 dominance

has not been consistently demonstrated in term decidual samples (Athanasakis & Vassiliadis 2002). Elevated levels of decidual Th17 cells have been reported in spontaneous abortion (Saito *et al.* 2010) and increased decidual Th1/Th2 ratios in recurrent spontaneous abortion (Piccinni *et al.* 1998). As conflicting data again exist, there appears a more complex T cell response and cytokine paradigm in which reciprocal Treg and Th17 cell pathways are also implicated (Bates *et al.* 2002, Saito *et al.* 2010).

One of the initial observations linking vitamin D to the adaptive immune system was that T cells express VDR, with these levels increasing as T cells proliferate (Nunn *et al.* 1986). As a consequence, initial studies of vitamin D and T cells focused on anti-proliferative responses (Nunn *et al.* 1986, Provvedini & Manolagas 1989, Karmali *et al.* 1991). However, it is now clear that vitamin D also influences the phenotype of T cells, in part by promoting a shift from Th1 cytokine profile to Th2 (Lemire *et al.* 1995, Overbergh *et al.* 2000, Boonstra *et al.* 2001). In this way, it has been proposed that vitamin D could limit potential tissue damage associated with excessive Th1 cellular immune responses by switching to a more humoral Th2 phenotype. However, the validity of this hypothesis is uncertain as *Vdr* gene knockout mice (which lack 1,25(OH)₂D-mediated signalling) have reduced rather than elevated levels of Th1 cells (O'Kelly *et al.* 2002).

Thus, it seems likely that effects of vitamin D on T cells *in vivo* are more complex than thought originally, with other cells such as inflammatory Th17 cells being the potential targets. Th17 cells are important for promoting immune responses to some pathogens, but they have also been linked to inflammatory tissue damage (Bettelli *et al.* 2007, Korn *et al.* 2007). Exposure to 1,25(OH)₂D *in vitro* suppresses Th17 cell development (Colin *et al.* 2010, Palmer *et al.* 2010) and expression of IL17 (IL17A) (Daniel *et al.* 2008) respectively. Paradoxically, *Cyp27b1* gene knockout leads to elevated levels of IL17 (Liu *et al.* 2008). Recent studies using a rat model of pre-eclampsia demonstrated that vitamin D supplementation significantly decreased circulating CD4⁺ Th cells (Darby *et al.* 2013). However, in the same study, cytokine levels from human whole-placenta explants from both normal pregnant women and those complicated by PET following culture in both hypoxic and normoxic conditions were assessed. Expression of Th2-associated IL10 was significantly decreased, and that of Th1-associated IL6 increased in pre-eclampsia explants compared with controls, but this was unaffected by vitamin D (Darby *et al.* 2013).

T cell immune responses to vitamin D are not restricted to Th cells, but also include actions on suppressor Tregs, a group of CD4⁺ T cells known to inhibit the proliferation of other CD4⁺ T cells. Treatment of naive CD4⁺ T cells with 1,25(OH)₂D potently induces the development of Tregs (Gorman *et al.* 2007), and this has been proposed as a mechanism for potential beneficial effects of vitamin D on autoimmune disease and host-graft rejection (Gregori *et al.* 2002, Mathieu & Badenhop 2005, Spach *et al.* 2006). In stimulating Treg development, 1,25(OH)₂D may act directly on VDR-expressing CD4⁺ T cells (Jeffery *et al.* 2009, Urry *et al.* 2009). However, as outlined earlier in this review, it may also act via effects on APCs such as DCs or macrophages (Gregori *et al.* 2001, Dong *et al.* 2003, Adorini *et al.* 2004). In common with helper and Tregs, CD8⁺ cytotoxic T cells express VDR and are sensitive to cytokine regulation by 1,25(OH)₂D (Willheim *et al.* 1999). Recent studies have demonstrated that *Vdr* gene knockout in mice leads to dysregulation of CD8⁺ T cell proliferation and increased risk of inflammatory disease in these animals (Chen *et al.* 2014). Intriguingly, these studies also suggested that these murine CD8⁺ cells are the major site of immune synthesis of 1,25(OH)₂D (Ooi *et al.* 2014).

Fetal protection from rejection by T cells reactive to fetal antigens has been attributed to clonal deletion or suppressed proliferation of T cells. The inhibitory co-stimulatory molecule programmed death 1 (PD1) receptor, the cell surface death receptor FAS and indoleamine 2,3,4 deoxygenase (IDO)-dependent pathways have all been implicated in this process, as their respective ligands are expressed by decidual and fetal trophoblast cells in human and mouse pregnancies (Arck & Hecher 2013). Studies on mice have indicated that although independently these T cell-constraining pathways are not critical for fetal survival and development, their immunoregulatory functions are still important and may be targeted by vitamin D. For example, 1,25(OH)₂D stimulates CD4⁺CD25⁺ FoxP3⁺ T cell production through an IDO-mediated pathway (Correale *et al.* 2009). Preliminary data from our group using decidual cells found that expression of CYP27B1 correlated with TLR4 and IDO (IDO1) expression (Evans *et al.* 2006). Functional responses to 1,25(OH)₂D were studied in the context of uNK cells as opposed to decidual T cells in this instance, but nevertheless data demonstrated a shift towards a pro-tolerogenic cytokine profile.

B cells

Decidual B cells are present during human pregnancy, but in very low numbers compared with T cells

(Rieger *et al.* 2009). Consequently, they have received less research attention, despite the fact that in pregnant women there appear to be distinct differences in circulating B cell subtypes when compared with non-pregnant women (Fettke *et al.* 2014). In particular, IL10-secreting B cells termed B10 cells, which have been linked to protection against autoimmune disease (Matsushita *et al.* 2008), appear to play a key role in pregnancy by promoting tolerance of the developing fetus in mice (Jensen *et al.* 2013). As yet, the function of these cells specifically within the decidua remains unclear.

As with T cells, 1,25(OH)₂D can also suppress proliferation (Shiozawa *et al.* 1985) and immunoglobulin (Ig) production (Provvedini *et al.* 1986) in VDR-expressing B cells. Initially this was thought to be mediated via an indirect, T cell-dependent, mechanism, but more recent reports have endorsed a direct effect of 1,25(OH)₂D on B cells (Chen *et al.* 2007). Moreover, besides confirming the anti-proliferative actions of 1,25(OH)₂D on B cells, these studies demonstrated that 1,25(OH)₂D can inhibit the differentiation of plasma cells and class-switched memory cells (Chen *et al.* 2007), highlighting a potential role for vitamin D in B cell-related disorders such as systemic lupus erythematosus. The implications of these findings for human pregnancy remain unclear as yet. Nevertheless, it is possible to speculate that maternal vitamin D deficiency may lead to dysregulation of decidual B cell function with potential adverse outcomes.

Conclusions

Pregnancy presents a unique immune challenge for the maternal host, with the maternal–fetal interface being the prime area of immune regulation. In this setting, the immune cells within the heterogeneous population of cells that make up the maternal decidua play a key role in maintaining tolerance of the developing fetus while protecting the conceptus against infection and inflammation. Successful maintenance of this complex decidual immune system requires an equally complex set of regulatory factor and, in this setting, vitamin D may play a highly versatile role by promoting antibacterial innate immune responses to infection while suppressing adverse inflammatory adaptive immunity. Several other features of vitamin D support its role as a regulator of decidual immunity. Crucially, the placenta is one of the principal sites for extra-renal synthesis of 1,25(OH)₂D, with both the maternal and fetal sides of the placenta cooperating to maintain high localised tissue levels of this hormone. This is likely to be a key factor in optimising the immune

function of vitamin D, but may also be important in promoting the crosstalk between the maternal decidua and fetal trophoblast. However, it is important to recognise that the success of these decidual responses to vitamin D will be dependent on the availability of substrate 25OHD, so that tissue-specific levels of 1,25(OH)₂D may be compromised under conditions of vitamin D-insufficiency. It is important that these mechanisms are identified in both human and murine pregnancy. The impact of this on immune function during pregnancy, particularly within the decidua, is likely to be a key component of future studies of vitamin D and pregnancy.

Declaration of interest

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

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