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Regulatory T Helper Cells in Pregnancy and their Roles in Systemic *versus* Local Immune Tolerance

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Abstract

Problem: During pregnancy, the maternal immune system needs to adapt in order not to reject the semi-allogenic fetus.

Method: In this review, we describe and discuss the role of regulatory T (Treg) cells in fetal tolerance.

Results: Treg cells constitute a T helper lineage that is derived from thymus (natural Treg cells) or is induced in the periphery (induced Treg cells). Treg cells are enriched at the fetal-maternal interface, showing a suppressive phenotype. In contrast, Treg cells are not increased in the circulation of pregnant women, and the suppressive capacity is similar to that in non-pregnant women. However, aberrations in Treg frequencies and functions, both systemically and in the uterus, may be involved in complications of pregnancy.

Conclusions: Treg cells seem to have distinguished roles locally versus systemically, based on their distribution and phenotype.

Key words - Cytokines, Reproductive immunology, Tolerance, T helper cell

Introduction

Regulatory T (Treg) cells have important roles in directing immune responses; they prevent autoimmune disease, maintain immune homeostasis and modulate immune responses during infection. Given the importance of immune regulation in pregnancy, it is not surprising that Treg cells have been the focus of many investigations in healthy pregnancy and in complications of pregnancy. While there is a steady increase in the number of reviews on Treg cells, including recent ones highlighting subsets¹ and an increasingly recognized complexity of this population², there is a limited number of reviews in the field of reproductive immunology. While recent reviews in particular emphasized the “immunological attack on the conceptus, spermatozoa and oocytes”³, mouse and human reproduction⁴ and Th1/Th2/Th17/Treg cells⁵, our aim was to give a thorough and general background to T cell and Treg cell biology and to highlight what is known about Treg cells in human pregnancy including the detailed but important issue of how to determine their presence and frequency. We also consider the important question related to their distinguished and possible different roles in local *versus* systemic immunity.

T helper cells are specialized cells with the main purpose to regulate and modulate immune responses

The term “helper” stems from the ability of T cells to help B cells in producing antibodies. Subsequently T helper cells were found to “help” a large number of immune cells by augmenting their effector functions, for example macrophages in performing phagocytosis of bacteria and CD8 T cells and natural killer (NK) cells in cytotoxic killing of infected cells. Importantly, T helper cells do not simply augment effector functions, they also drive the immune response in certain directions, and they also turn off immune responses. The turning-off is of equal importance as the turning-on ability since over-activity of immune responses leads to several states of immune pathology such as autoimmunity and allergy. In the case of pregnancy, an overactive immune response is involved in several complications of pregnancy. Indeed, the immune system has a very difficult task in pregnancy; it should both be modulated in order not to reject the semi-allogenic fetus, but at the same time it must strongly protect the mother and her fetus from infections. While the defense against infections is in general well preserved, there is an increased susceptibility for some infections during pregnancy, like toxoplasmosis and listeriosis and risk of increased severity of others, like influenza and varicella⁶. These infections are controlled by T cells, and it seems that T cell immunity at a general level is down-regulated, while the innate immunity is up-regulated, probably as a compensatory mechanism^{7,8}.

T helper cell subsets and cytokine profiles

T helper cells exert their effects by secretion of signaling molecules, cytokines. Based on which cytokines they secrete, T helper cells are divided into subsets with different cytokine profiles and corresponding effector mechanisms (**Figure 1**). The Th1/Th2 paradigm has been used in an over-simplified manner. Often cytokines have been classified according to the pathology studied. For example in pregnancy, Th1 mediated responses were shown to induce abortions^{9,10} and therefore Th1 was regarded as “the bad” immune reaction. Accordingly, all cytokines counteracting Th1 responses were regarded as Th2, like IL-4 and IL-10. It is here important to emphasize that although originally launched as a cytokine produced by Th2 cells, IL-10 is not strictly a Th2 cytokine, but rather a more general immunomodulating cytokine. Indeed IL-10 inhibits Th1, but in fact it also inhibits Th2 immunity, as it does inhibit several other inflammatory mediators (for review see¹¹). Moreover, although T cells do secrete IL-10, this cytokine is mainly derived from antigen presenting cells; dendritic cells, macrophages and B cells. IL-10 is therefore better classified as an anti-inflammatory cytokine¹¹. In the simplified Th1/Th2 model, pro-inflammatory cytokines are often regarded as belonging to the Th1 subset in fields where Th1 is the disease-promoting response, for example in organ-specific autoimmunity and in pregnancy. Of note, inflammation is a hallmark of other T helper subsets like Th2 and the most recently established T helper subset, Th17¹¹. Consequently, pro-inflammatory cytokines like tumor necrosis factor (TNF) are present also in Th2-mediated pathology such as allergic inflammation, and the pro-inflammatory cytokine IL-6 is for example involved in development of Th17. Therefore it is important to view cytokines from different perspectives and distinguish the T helper (Th1/Th2/Th17) perspective from the pro- versus anti-inflammatory perspective. However, the most relevant approach is to denote the referred cytokine by name rather than to its belonging to a certain group. Here, IL-2 deserves some extra attention since it is often referred to as a Th1 cytokine, although it is a growth factor necessary for activation of all T helper subsets¹¹.

The Th1/Th2 paradigm in pregnancy

The Th1/Th2 hypothesis was the dominating explanation model for immune regulation during pregnancy for at least a decade, and this model still provides, along with more recently added mechanisms, a foundation for explaining fetal tolerance.

The T cells found at the fetal-maternal interface show an activated/memory phenotype^{12,13}, indicating that they are in fact primed. The decidual T cells from healthy pregnancy, as compared with spontaneous abortion cases, secrete more IL-4 and IL-10¹⁴ as well as the pregnancy facilitating leukemia inhibitory factor (LIF) and

colony stimulating factor 1 (most commonly known as macrophage (M)-CSF¹⁵. Further, as compared with blood, more T cells in decidua produce IL-4 whereas fewer produce IFN- γ ¹⁶. These findings of dominating Th2 and anti-inflammatory cytokines in human decidua are in line with Wegman's observations in the murine system^{9,10}, which initiated the Th1/Th2 paradigm in reproductive immunology. The most recently established T helper lineage, Th17, when estimated by their chemokine receptor expression¹⁷, was found to be present in the decidua at low levels as compared with blood¹⁸. Conversely, Nakashima *et al.* found an increase in decidua of Th17 cells as measured by induction of IL-17 producing cells after polyclonal stimulation¹⁹. It is possible that the difference is accounted for by differences in methodology; as resting cells, representing the homeostatic tolerogenic environment, Th17-producing cells are scarce and inactive, whereas under challenging conditions, such as after stimulation, T cells become activated and produce IL-17.

Systemic immune changes have been even more extensively studied, although not resulting in a consistent pattern. One reason for the inconsistency may be differences in methodological approaches; cytokine expression or secretion induced by various stimuli *versus* spontaneous cytokine secretion *versus in vivo* produced cytokines detected as circulating levels in serum or plasma. Polyclonally stimulated peripheral blood mononuclear cells from early normal pregnant women produced higher levels of IL-4 and IL-10, and lower levels of IL-2, IFN- γ and TNF than women with recurrent miscarriage^{20,21}. However, no differences in IL-4 and IFN- γ producing cells were found as compared with non-pregnant women, an observation also done by others²² but disputed by even others showing higher IL-4 activity in first or third trimester pregnancy as compared with non-pregnant women²³. Further, IL-10 was shown to be higher in pregnant as compared with non-pregnant women and women with recurrent spontaneous abortion²³, which is in accordance with Hanna *et al.* implying IL-10 as the main pregnancy facilitator²⁴.

Spontaneous *ex vivo* secretion of cytokines is perhaps more representative of *in vivo* conditions. Using the sensitive ELISPOT technique, or in situ hybridization on resting circulating cells from pregnant women, pregnancy was shown to be associated with more IL-4 and IFN- γ production in all trimesters of pregnancy as compared to post-partum or non-pregnant controls^{25,26} indicating that healthy pregnancy involves priming of both IL-4 and IFN- γ secretion at the systemic level.

To conclude, at the local level, normal pregnancy is characterized by a skewing towards tolerogenic and less aggressive mechanisms in contrast to complicated pregnancy. However, at the systemic level, normal pregnancy seems to prime several arms of the immune system. As seen at the local level, several studies imply skewing of the systemic immunity towards proinflammatory/Th1 immunity in complications of pregnancy.

Regulatory T cells

Given the regulatory roles of all T helper cells, there are many cell types that could potentially be defined as regulatory T cells. After the era of Th1 and Th2, came the suggestion of Th3 cells, identified as TGF- β secreting cells being induced in the mucosal environment that is intrinsically rich for TGF- β , IL-10 and IL-4²⁷. TGF- β is a pluripotent cytokine with pre-dominating anti-inflammatory and tissue remodeling properties¹¹.

Type 1 regulatory (Tr1) cells constitute another regulatory population, being more tightly connected to IL-10 in terms of their induction and to IL-10, but also TGF- β , in terms of their output²⁸. Although IL-10 secreting cells are of major importance, the Tr1 cells have been hard to study due to the lack of a common transcription factor or other specific marker²⁹. Yet, they show promising results in cell-based treatment of transplantation reactions³⁰.

After the discovery of a distinct regulatory CD4⁺CD25⁺ T cell population in mice, much focus turned to this population, also in the field of reproductive immunology.

Origin and migration of Treg cells

It was the work of Sakaguchi and colleagues that led to the discovery of natural CD4⁺CD25⁺ regulatory T cells (nTreg cells), being present in naïve mice³¹. They showed that transfer of spleen and lymph node cell suspensions, depleted of CD25⁺ expressing CD4⁺ cells, to athymic recipient mice caused CD4⁺ cell dependent development of organ-specific autoimmune diseases such as gastritis, oophoritis and thyroiditis. Notably, inflammatory disease was prevented by co-transfer of CD4⁺CD25⁺ cells. Subsequently it was shown that these cells migrate from thymus as a distinct cell subset³².

In humans, the cell type corresponding to natural Treg cells was identified a few years later³³⁻³⁸. Since humans are constantly exposed to antigens, and CD25, which is the α -chain of the IL-2 receptor, is also an activation marker³⁹, Treg cells have not been as easily identified in humans as in mice. However, Treg cells showing a naïve phenotype can be found among the naïve cells in neonate cord blood and also in thymus⁴⁰, indicating that similar to the situation in mice, nTreg cells in humans are generated in the thymus and are present before the encounter of antigen.

Suppressive cells with a phenotype identical to natural Treg cells can be induced from non-regulatory CD4⁺CD25⁻ cells, and are named induced (i)Treg cells. In mice,

TGF- β induces FOXP3 expression, which is tightly linked to suppressive function⁴¹ (see below). Although not as clear-cut in the human situation, also here iTreg cells are present and show a suppressive phenotype^{42,43}.

Natural Treg cells originate in the thymus but soon leave this site to patrol the body as a part of the peripheral tolerance to self as well as to non-self antigens. Generally, as naïve cells (CD45RA⁺), Treg cells express receptors associated with homing in lymphoid tissues such as CCR7 and CD62L⁴⁴ allowing them to participate in suppression at an early stage of immune responses. Upon activation and expression of CD45RO, Treg cells reduce the levels of lymphoid homing receptors and begin to express receptors coupled with non-lymphoid homing⁴⁴. Treg cells can express a large number of chemokine receptors (CCR2, CCR4, CCR5, CCR6, CCR8, CCR9, CCR10, CXCR3, CXCR5 and CXCR6, for review see²), thereby allowing them to migrate to a large set of inflamed tissues and suppress different types of effector cells at that site.

Phenotype of human Treg cells and the role for FOXP3

In humans, Treg cells have been characterized to express high levels of CD25³³, hence called CD25^{high} or CD25^{bright}. However, since the CD25 molecule is a marker of activation, the CD25^{high} population is at risk of being contaminated by activated cells lacking suppressive function, underscoring the need for a better marker of true suppressive Treg cells.

FOXP3 is a transcription factor involved in immune regulation. Its importance was evident in Scurfy mice that show massive autoimmune activation due to a mutation in the gene coding for the scurf protein, FOXP3⁴⁵. Humans show a homologous defect; Immune dysregulation Polyendocrinopathy, Enteropathy, X-linked syndrome (IPEX), leading to severe multi-organ autoimmune diseases⁴⁶.

In both mice and humans, it was soon found that CD4⁺CD25⁺ regulatory T cells expressed FOXP3 and that this protein was expressed already in thymus. Furthermore, FOXP3 could confer suppressive function to non-Treg cells upon retroviral transfer and, consequently, FOXP3 was important for lineage commitment to the Treg subset^{42,47,48}.

In mice, FOXP3 seems to be an absolute marker of Treg cells whereas in humans, FOXP3 is not completely specific for cells with suppressive function. Rather, FOXP3 expression seems to be a self-limiting, natural consequence of activation. Thus, activated CD4⁺ cells start transient FOXP3 expression, which is not linked to suppressive function^{49,50}. Importantly, stable expression of FOXP3, as under non-

inflammatory conditions, is still coupled to suppressive function⁴⁹. Further, stable FOXP3 expression and suppressive function is associated with epigenetic DNA modifications, demethylations that make the FOXP3 locus accessible for transcription. In contrast, non-Tregs, induced to express FOXP3 via T cell receptor (TCR) or TGF- β stimulation, did not display FOXP3 demethylations^{51, 52}.

While FOXP3 is located in the nucleus, it is not a feasible marker for sorting of viable cells. It was therefore an interesting observation that FOXP3 by binding to the promoter of the IL-7 receptor α -chain (CD127), lead to the down-regulation of its expression, thereby giving the FOXP3-expressing cells a phenotype with low expression of CD127 (CD127^{low})⁵³. It was shown that while FOXP3 expression was positively correlated with suppression, CD127 was negatively correlated with suppressive function. Thus, viable and suppressive Treg cells could be sorted by combining the CD25^{high} and CD127^{low} phenotypes^{53, 54}. However, there are also other proper ways of gating viable Treg cells; a combination of CD25^{high} with a lower expression of CD4 (CD4^{dim})⁵⁵ results in a highly suppressive population, as does the combination of CD25^{high} with CD45RA⁵⁶, see below.

Other Treg markers and heterogeneity of Treg cells

A number of other markers for Treg cells have been suggested, but never became fully established; membrane-bound TGF- β (mTGF- β)⁵⁷ and its latency associated peptide (LAP); CD27⁵⁸; Neuropilin-1 (Nrp1)⁵⁹ and; glucocorticoid induced tumor necrosis factor receptor (GITR), although the latter was linked to suppressive function⁶⁰.

Markers that are still in use include CTLA-4, which is also associated with the suppressive function of Treg cells⁶¹. In resting Treg cells, CTLA-4 is found intracellularly, but it is rapidly upregulated following activation³⁵.

There are also a number of markers that seem to divide Treg cells into different subpopulations. About one third of the Treg cells express human leukocyte antigen (HLA)-DR⁶², which has been suggested as a marker of a terminally differentiated subset in the effector Treg cell pool (reviewed in¹). CD39 was shown to be expressed by a subpopulation of FOXP3⁺ Treg cells⁶³. CD39 is an ectonucleotidase that, alongside other enzymes, convert adenosine tri-phosphate (ATP), released during for example tissue damage, to adenosine⁶³, thereby leading to suppressive activity. Another marker associated with suppressive function is inducible T cell co-stimulator (ICOS), which divides Treg cells into subsets with different modes of actions⁶⁴. Helios is a recently discovered marker that seems to distinguish thymus-derived Helios expressing natural Treg cells from peripherally induced Treg cells,

which do not express the Helios marker⁶⁵. Interestingly, by using the Helios marker it was estimated that 70% of Treg cells in lymph nodes were of thymic origin, in both mice and humans.

A very relevant division of Treg cells is based on the expression of CD45RA and CD45RO. Being reciprocally expressed on all T cells, including Treg cells, they denote naïve versus memory phenotype of cells. Although applied to the Treg populations previously, it was recently convincingly shown that CD45RA/RO were useful Treg markers when combined with FOXP3 or CD25⁵⁶. In this way, naïve Treg cells are defined as CD45RA⁺ (they are also CD45RO⁻), showing a medium level of Foxp3 expression whereas memory Treg cells lack CD45RA (but express CD45RO) and show a high expression of Foxp3. Both populations show suppressive activities, although most pronounced in the CD45RO⁺ memory phenotype population. Interestingly, the naïve CD45RA⁺ Treg population expresses the recent thymic emigrant marker CD31 to a high extent, indicating a truly naïve phenotype. In contrast, the CD45RO⁺ Treg population does not express CD31 and seems to emanate from the naïve Treg population. The designation “memory” may be misleading, rather the CD45RO⁺ population seems to constitute an “effector” population derived from the naïve population.

Functions of Treg cells, how do they suppress?

Much information on Treg suppressive mechanisms in humans comes from *in vitro* assays³³⁻³⁸. Typically, CD4⁺ cells lacking the CD25 molecule, CD4⁺CD25⁻ cells, are used as responder cells. These responder cells are cultured alone or in combination with different amounts of Treg cells. As expected, the responder cells will proliferate and secrete cytokines in response to a polyclonal stimulus such as anti-CD3/CD28 antibodies. However, in the presence of Treg cells, both proliferation and secretion of cytokines such as IFN- γ and IL-2 is inhibited. Interestingly, Treg cells themselves are fairly unresponsive to stimulation. Treg cells have been shown to produce cytokines such as IL-10, but also IL-4 and TGF- β , at least under stimulation^{34, 36}.

Cytokines have been the prime candidates when unraveling suppressive mechanisms of Treg cells. However, several *in vitro* assays, for example by separating Treg cells and responder cells, indicate that Treg cells act, at least in part, in a contact-dependent way^{34-36, 66}. Conversely, in mice cytokines seem to play a more pronounced role in Treg mediated suppression⁶⁷. In infections, IL-10 blockade, TGF- β blockade or adoptive transfer of Treg cells from IL-10 deficient mice disables suppressive immune responses⁶⁸. Further, Treg mediated suppression of graft rejection largely depends on IL-10, in addition to CTLA-4⁶⁹. Thus, it is possible that cytokines are important mediators of Treg suppression *in vivo* but not *in vitro*⁶⁷.

In addition to TGF- β and IL-10, studies in mice indicate that the newly discovered cytokine IL-35 has an important role in Treg mediated suppression. After contact with responder cells, Treg cells produced higher amounts of IL-35, consisting of the Epstein-Barr virus-induced gene 3 (EBI3) and p35 (IL12a) subunits^{70, 71}. The IL-35 subunits were both necessary for Treg mediated suppression in a murine model of inflammatory bowel disease, whereas ectopic expression of IL-35 in non-Tregs induced suppressive properties in these cells⁷¹. However, production of IL-35 from human Treg cells was recently dismissed⁷².

Apart from acting through cytokines, several other mechanisms have been put forward as outlined in **Figure 2**. For more detailed information regarding mechanisms, see other recent reviews^{67, 73}.

Regulatory T cells in murine pregnancy

Already in 1982, Chaouat and co-workers demonstrated that splenic cells from multiparous mice lacked cytotoxic activity against paternal cells. This suppression was explained by the presence of antigen-specific regulatory cells, able of inhibiting cytotoxicity as a third party in a mixed leukocyte culture cytotoxicity assay⁷⁴. More than twenty years later, the interest for the revived Treg population, now defined as CD4⁺CD25⁺, was raised and it was soon found that in murine models Treg cells were crucial in both syngenic and allogeneic pregnancies^{75, 76}, reviewed in⁴.

Treg cells are enriched in human decidua

Treg cells seem to be enriched at the fetal maternal interface throughout healthy pregnancy⁷⁷⁻⁸⁰, as shown also by usage of FOXP3 as a Treg marker. It was also demonstrated that Treg cells were localized primarily to the decidua basalis⁸⁰.

How Treg cells are enriched at the fetal maternal interface in mice seems fairly settled, involving chemokine receptors, hormones, seminal factors and paternal alloantigens⁸¹. However in human pregnancy, the enrichment process of Treg cells in decidua remains unknown. Alike the murine model, human Treg cells could be attracted to the fetal-maternal interface. Human chorionic gonadotropin (hCG) and CCL5, released by trophoblast cells, were suggested to attract Treg cells and to enhance their FOXP3 expression, respectively^{82, 83}. Further, estradiol, present at increased concentrations in pregnancy serum and even more in placenta, enhances the suppressive function of FOXP3 expressing Treg cells⁸⁴. Seminal fluid has convincingly been shown to induce migration of Treg cells to the fetal-maternal interface in mice. However, the role for seminal factors in Treg expansion in humans remains unclear.

Yet another unanswered question is the occurrence of paternal/fetus-specific Treg cells. Interestingly, *in vitro* depletion of Treg cells from decidual leukocytes, but not

from peripheral blood leukocytes, lead to enhanced immune reactivity against fetal antigens during normal pregnancy⁸⁰. This effect of decidual Treg cells, together with the finding of reduced frequency of Treg cells in blood, was interpreted as selective migration of fetus-specific Treg cells from peripheral blood to decidua.

In agreement with these data, we found a clear enrichment of decidual Foxp3⁺ Treg cells in first trimester pregnant women¹⁸. We could show that more decidual than circulating Treg cells expressed the proliferation marker Ki-67, suggesting that local proliferation of decidual Treg cells takes place, possibly induced by fetal alloantigens. Further, we could show that decidual Treg cells express the chemokine receptor CCR4. A ligand for CCR4, CCL17, is produced by trophoblasts and stromal cells in first trimester pregnancy and was suggested to mediate recruitment of Th2 cells to decidua⁸⁵. Hence, we suggest a scenario where Treg cells are recruited from the circulation, proliferate on site, and participate in local tolerance of fetal alloantigens.

Treg cells are not increased in the circulation of humans

In line with the results in mouse models and according to expectations, the first studies in humans, using the proper tools available at that time, showed that the number of Treg cells was increased in the circulation of pregnant women⁷⁸, peaking in second trimester and declining towards the end of pregnancy and post-partum^{77, 86}. However, investigating Treg cells in humans, in any condition, is complicated by the presence of activated cells sharing phenotypic properties with the Treg population. In our study of Treg cells in the circulation of 2nd trimester women⁵⁵ we first interpreted data in similarity with previous investigators, suggesting an increase in circulating CD4⁺CD25^{high} Treg cells in pregnancy. However, a detailed analysis revealed that a defined population of suspected non-suppressive CD4⁺CD25^{high} cells was responsible for the increase of CD25^{high} cells in pregnancy. Indeed, by analyzing FOXP3 protein and additional markers, with flow cytometry, we were able to show that the increased CD4⁺CD25^{high} population in pregnancy did not show a true Treg phenotype. Sorting of this population confirmed the suspicion that the expanded CD4⁺CD25^{high} population in pregnancy was an activated, non-suppressive, non-Treg population. When excluding this non-Treg CD25^{high} population from the analysis, the frequency of Treg cells did not differ between pregnant and non-pregnant women and importantly, neither did their suppressive function on a per cell basis⁵⁵. Interestingly, the true suppressive CD25^{high} Treg population showed a slightly lower expression of CD4, hence the designation CD4^{dim}CD25^{high} cells. At the same time, similar protocols started showing up in the literature⁸⁷, abandoning the traditional gating strategy³³. Importantly, there are also other more recent reports showing unaltered^{79, 80} or lowered^{88, 89} circulating CD4⁺CD25^{high} frequencies in second trimester. In addition, it was recently also reported that Treg frequencies, defined by

FOXP3⁺, CD25^{high} and CD127^{low}, were lower during pregnancy than post-partum, although the pregnancy samples were only taken in the last trimester approximately one month prior to labour⁹⁰. In a study on patients with multiple sclerosis, it was shown that the frequency of Treg cells decreased during pregnancy compared with postpartum, and in line with previous observations, this was also the case in pregnant control women⁹¹.

How can the maternal immune system both induce fetal tolerance and at the same time sustain a strong anti-microbial response?

Taken together, there is now ample evidence that the circulating Treg population is not increased in size during pregnancy^{55, 79, 80, 88-91} and circulating Treg cells show normal suppressive function during pregnancy⁵⁵. An increase in circulating Treg numbers or suppressive capacity would indeed be dangerous considering their unspecific suppressive capacity and the importance of protecting the mother and her fetus from infections. On the other hand there is a need for fetal tolerance, which would benefit from an increase in Treg cell mediated suppression. This paradox could, at least in part, be explained by differential Treg cell composition and function in the different compartments. While Treg cells in decidua are frequent⁷⁷⁻⁸⁰ and show a stable suppressive phenotype^{55, 80}, the circulating Treg cell pool, on the other hand, is not increased in size and circulating Treg cells show a “normal” phenotype and suppressive capacity⁵⁵. However, a state of fetal tolerance should also be present systemically, for example when considering the presence of fetal cells in the maternal circulation. One smart solution would be if circulating Treg cells showed a high degree of plasticity and flexibility. Indeed, our own unpublished data point in this direction and recent knowledge in T helper cell plasticity has changed the view on T helper lineage commitment and plasticity.

T helper cells, including Treg cells, show a high degree of plasticity

The T helper subsets Th1 and Th2 have been considered lineages with little room for re-differentiation once committed to their respective fate. The new additions to the T helper subset family, Treg and Th17 cells, and the interplay between these subsets, has challenged the view of fixed lineages, for review see⁹²⁻⁹⁴. Treg cells and Th17 cells have been suggested to be in reciprocal relation to each other⁹⁵. Thus, it was hypothesized that high levels of TGF- β promoted Treg cells, whereas lower levels of TGF- β in combination with IL-6 enhanced Th17 development⁹⁶. In mucosal tolerance in mice, retinoic acid (RA) co-operates with TGF- β to promote FOXP3 expression. Reciprocally, RA inhibits TGF- β /IL-6 induced Th17 development⁹⁷. EBI, a subunit of the Treg cytokine IL-35 discovered in mice, forms IL-27 with p28 and IL-27 has been shown to inhibit RORC expression and IL-17 secretion from naive T cells *in vitro*, hence acting with Treg cells to suppress Th17 development⁹⁸.

Treg cells may also be re-programmed into other T helper phenotypes. Interestingly, in humans, resting double-positive FOXP3⁺RORC⁺ cells have been found in tonsils. Further, purified Treg cells from blood, particularly those lacking HLA-DR⁹⁹, can develop into IL-17 producers upon *in vitro* stimulation¹⁰⁰. They may also express transcription factors associated with Th1 (T-bet) and Th2 (GATA3) like immunity¹⁰¹. However, the reduction in suppressive function accompanying a Treg-Th17 shift was shown to be transient⁹⁹.

Treg cells do not only show flexibility towards Th17 development. Murine Treg cells expressing moderate levels of FOXP3 were shown to develop into Th2 or Tr1 like cells, based on their production of IL-4 or IL-10, respectively^{102,103}.

Taken together, T helper cells show a high degree of plasticity. A hierarchy of plasticity was suggested dependent on the stability versus flexibility of the lineage-specific transcription mechanisms⁹³. Here, induced Treg cells were considered the most flexible population, followed by Th17 cells. Although a number of environmental and intrinsic factors decide the status and flexibility of T helper cells, it is reasonable to consider pregnancy as a challenging change in circumstances that may affect T helper and Treg cell plasticity. We therefore hypothesize that Treg cells may show a higher flexibility in pregnancy. Our data (to be published elsewhere) indicates that after stimulation, Treg cells seem to produce higher amounts of transcription factors for different T helper lineages. A higher plasticity of Treg cells in pregnancy would be suitable considering the demands of being suppressive when needed but also being able to quickly switch to a more aggressive phenotype in case of infections.

Treg cells in complications of pregnancy

Despite ambiguities regarding the role of systemic Treg cells in normal pregnancy, aberrations in Treg cell function or frequency have been reported for several complications of pregnancy. Patients experiencing recurrent miscarriages (RM) showed reduced levels of circulating CD4⁺CD25^{high} cells, both during^{78,104,105} and after pregnancy⁸², the latter finding also confirmed by the more specific marker FOXP3. A reduction of Treg cells, as determined by CD4⁺CD127^{low}, was noted in blood as well as in decidua¹⁰⁶ (Wang JRI 2010). Interestingly this reduction was inversely correlated with an increase in Th17 cells both in blood and decidua. It was also shown that Treg cells from women with RM had a decreased ability to suppress Th17 cells¹⁰⁷. Further, Arruvito *et al.* found diminished expansion and decreased suppressor function of induced FOXP3 expressing Treg cells¹⁰⁸. Recently, it was proposed that low levels of CD25⁺ FOXP3⁺ cells could be used to predict miscarriage in women with a history of previous failures¹⁰⁹. Taken together, data indicates that

defects in numbers or function of Treg cells may be involved in the development of RM.

Also preeclampsia has been associated with diminished Treg numbers and/or function. When defined as CD4⁺CD25^{+/high}, preeclamptic women showed reduced circulating Treg numbers^{110, 111} or levels similar^{112, 113} to those of healthy pregnant women. Studies using FOXP3 protein as Treg marker support the notion of reduced Treg numbers^{88, 114-116} in preeclampsia. In one report, the lower Treg number was accompanied by an increase in Th17 cells¹¹⁶. Further, a lower expression of FOXP3⁺ cells has also been found in the placenta from women with preeclampsia¹¹¹.

Conclusions

During human pregnancy, there is an enrichment of Treg cells in the decidua, presumably by recruitment from blood. Speculatively, these Treg cells are recruited and expanded by their recognition of fetal antigens, although further proof is needed here. Decidual Treg cells show a stable and highly suppressive phenotype and may be important for fetal tolerance. In contrast, the circulating Treg population is not increased in size and show a normal suppressive phenotype in healthy pregnancy. The precise role of the circulating Treg cells is not clear. We propose that they may be flexible cells able to switch between tolerance and anti-microbial activity. The definition of Treg cells is extremely important in order to correctly estimate the size of the Treg population, a notion that may explain inconsistencies in the literature. Despite this obstacle, there is evidence that a defect in Treg number or function may be involved in failures of pregnancy and pregnancy-associated diseases. The continuous increase in knowledge of Treg biology and function will further enlighten their role in pregnancy and pregnancy complications.

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Legend to figures:

Figure 1

A schematic and simplified view of T helper cell development and differentiation to different cell subsets. Naïve CD4⁺ T cells emigrate from thymus. Depending on the environment, in particular the cytokine being present, T helper cells upon activation can turn into different subsets through lineage-specific transcription factors like Tbet (Th1), GATA-3 (Th2) or RORC (Th17). For some not fully established T helper subsets (Th3 and T regulatory subset 1, Tr1) no specific transcription factor has been defined. Regulatory T cells, expressing the transcription factor FOXP3, emigrate from thymus (natural (n) regulatory T cells), but they can also be induced in the periphery (induced (i) regulatory T cells). Depicted are also chemokine receptors representative of each subset.

Figure 2.

Proposed suppressive mechanisms for Treg cells acting on responder cells (such as effector T cells and antigen presenting cells APCs). Expression of FOXP3 poses the key switch to development of a regulatory phenotype. Surface-associated mechanisms include, from left to right; (1) Treg cells use ecto-enzymes **CD39** and **CD73** to cleave ATP to adenosine, which acts on the anti-inflammatory adenosine receptor A2a on effector T cells. About 60% of FOXP3-expressing Treg cells express CD39 and these cells are suppressive whilst those lacking CD39 are not; (2) Treg cells, when activated, express **CTLA-4**, which interacts with CD80/86 thereby affecting T cells directly or indirectly thorough APCs; (3) **Soluble cytokines IL-10, TGF- β** and (in mice) IL-35 exert immunomodulatory and suppressive effects. In addition, membrane-bound (m) TGF- β may have a role in suppression, at least in mice; (4) Treg cells may also suppress via **lysis of the target cells** via a perforin-dependent cytotoxic pathway; (5) Since Treg cells express high levels of the IL-2 receptor α -chain CD25, Treg cells may act as an **IL-2 sink**, consuming available IL-2 molecules at the expense of T effector cells; (6) Treg cells (at least in mice) are able to induce expression of the enzyme **IDO** in dendritic cells via the action of CTLA-4, leading to tryptophan starvation and induction of apoptosis in surrounding effector T cells.

FIGURE 1

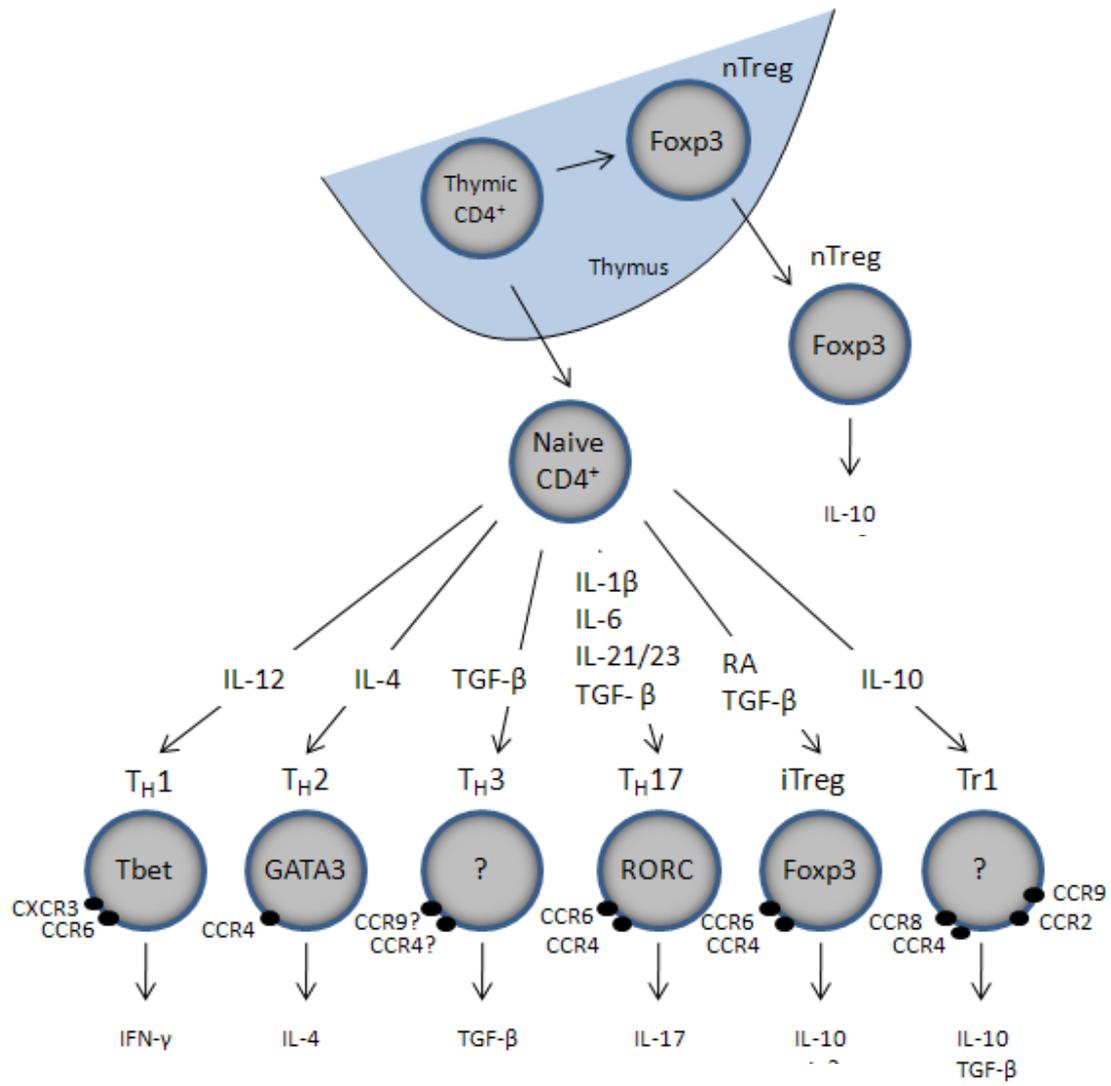


FIGURE 2

