Antigen-specific oral tolerance for the treatment of inflammatory and allergic diseases

Huibregtse, I.L.

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Recent advances in clinical practice Immunopathogenesis of IBD: insufficient suppressor function in the gut?

Huibregtse I.L. 1, van Lent A.U.G. 2, van Deventer S.J.H. 1

1Center for Experimental and Molecular Medicine, Academic Medical Center, University of Amsterdam, Amsterdam, the Netherlands
2Department of Immunology, Academic Medical Center, University of Amsterdam, Amsterdam, the Netherlands
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**Summary**

Activation of mucosal T cells occurs in inflammatory bowel disease and is inhibited by various subsets of "regulatory" T cells, which can be functionally and phenotypically characterized. Regulatory T cells can be generated within the thymus or in peripheral tissues.

In mice, a loss of regulatory T cell activity results in inflammatory bowel disease. Regulatory T cell activity has not been thoroughly studied in inflammatory bowel disease, but currently available data do not provide evidence for a loss of regulatory T cell activity in human inflammatory bowel disease.

Regulatory T cells can be generated or activated *in vivo* and *in vitro* using a variety of approaches, including specific small molecules, cytokine-mediated activation, and gene therapy. These cells have therapeutic activity in the preclinical setting.

**Immune activation and suppression in inflammatory bowel diseases**

The intestinal immune system is in a constant state of controlled inflammation, and there is substantial evidence that a loss of control is an important pathogenic mechanism in inflammatory bowel diseases (IBD). A major current working hypothesis defines Crohn’s disease as a dysregulated immune response towards components of the intestinal flora, leading to chronic intestinal inflammation (1). The causes for this inappropriate response can be attributed to (a combination of) defects in the epithelial barrier, the innate immune response or the adaptive immune response.

Animal experiments as well as clinical data indicate that the immunopathogenesis of Crohn’s disease (CD) and ulcerative colitis (UC) differ at the level of T cell differentiation and activation, although the governing mechanisms responsible for these differences have been incompletely defined. In both diseases, activation of T cells is evident, but pathogenic T cells in Crohn’s disease predominantly produce IFNγ, (membrane bound) TNFα and IL-23, whereas ulcerative colitis is characterized by production of IL-5 and IL-13 (2;3).

The increased production of “Th1” type cytokines in Crohn’s disease is likely related to increased activation of mucosal dendritic cells (DC) and macrophages, and the pivotal function of membrane-associated (Toll like receptor -TLR-) and intracellular (NOD family) receptors in the activation of
these antigen-presenting cells (APC) has now been well established. Both receptors are key mediators of innate host defence, crucially involved in maintaining intestinal homeostasis (4). In healthy subjects, the colonic mucosa harbors ‘non-inflammatory’ dendritic cells, expressing low levels of TLR2 and 4 and producing cytokines such as IL-10, contributing to a non-inflammatory environment(5;6), but in the mucosa of CD patients the production of IL-12 is greatly increased (7-9). DC in both UC and CD have an activated phenotype with higher levels of the activation markers CD40, CD86 and produce more IL-12 and IL-6 compared to controls (5;10). The causes for this excessive activation are presumably diverse and have been incompletely defined. A minority of CD patients has inactivating mutations within the susceptibility gene NOD2. The NOD2 protein is normally stimulated by its natural ligand MDP, a degradation product of bacterial peptidoglycan (PGN) (11-13). Some studies have shown increased activation of NFκB, which in antigen presenting cells causes increased transcription of IL-12. Indeed, dendritic cells from CD patients with NOD2 mutations produce increased amounts of IL-12 after stimulation with PGN, most likely via loss of inhibition of the simultaneously activated TLR2 pathway (Zelinkova et al. submitted). It should be noted that other studies have shown impaired activation of NFκB in CD patients with NOD2 mutations, suggesting decreased activation (14;15). Hence, it remains unclear what mechanism is responsible for the excessive Th1 profile in Crohn’s disease and whether the underlying genetic defects lead to initial decreased immune activation with failure to clear pathogens, or whether these mutations directly increase activation of immune cells such as DC. Abnormal activation and expression of TLR receptors may also be linked to inflammatory bowel disease: Associations with TLR4 and TLR5 signalling with their bacterial ligands LPS and flagellin, respectively have been reported (16;17), enhanced expression of TLR2 and TLR4 on DCs was observed (5) and recent studies suggest that CD is also associated with TLR9 promoter polymorphisms (18).

Additionally, defective apoptosis of T cells has been suggested to play a role in the pathogenesis and chronic state of inflammation in CD. Lamina Propria T cells from CD patients were shown to be resistant to activation induced cell death (AICD), whereas LP T cells from healthy controls readily underwent apoptosis (19;20). The latter would clarify the effective therapeutic action of anti-TNFα agents such as Infliximab in CD, as this reagent was shown to induce apoptosis through binding to membrane-bound TNF-α (21).

Although the precise cellular and molecular pathways involved remain to be elucidated, these findings give solid and abundant evidence for increased stimulation or dysregulation of the innate immune system
in inflammatory bowel disease that -in the case of CD- results in the induction of hyperactive T cells, which is probably necessary for the initiation of chronic mucosal inflammation. Increased activation of the innate and adaptive mucosal immune systems is tightly controlled by various regulatory circuits, and it is possible that defects in such mechanisms that normally downregulate intestinal inflammation are insufficient in inflammatory bowel diseases. This review discusses evidence for abnormal regulation of T cell activation in Crohn’s disease, as well as data pertaining to the existence and functional activity of regulatory T cells in the intestinal mucosa. We also consider the potential therapeutic application of regulatory T cells in IBD.

**Regulatory mechanisms in the gut**

The immune system controls activation of the innate as well as the adaptive arms, through various means primarily including induction of anergy, apoptosis of activated immune cell, and activities of regulatory CD4⁺ T cells. In addition, several other regulatory mechanisms are operational in the gut mucosa, which are CD8 T cells, γδ T cells and NKT cells, that are highly correlated with their surrounding epithelial cells, and IL-10 secreting B cells, immature DC and plasmacytoid DC. Intra-epithelial CD8 T lymphocytes, γδ T cells and NKT cells are mucosal T cell subsets with a restricted T cell receptor repertoire that are in close contact with mucosal epithelial cells (EC). There is evidence that these interactions can lead to the induction of T cells with regulatory capacities: For example, interactions between human intestinal EC and peripheral blood T-cells cause expansion of CD8⁺CD28⁻ T cells with regulatory activity (22). These cells are present in the LP of healthy individuals, but not in the LP of patients with IBD (23), suggesting that these cells prevent the pathogenesis of IBD. Intra-epithelial lymphocytes have been reported to down-regulate excessive inflammation caused by infection and autoimmunity in epithelial tissues and their protective ability has been shown in several murine models of colitis (24-28). Accumulating evidence implicates additional cell types in immunoregulation. Recent studies have revealed a protective role of IL-10 producing B cells in murine CD4⁺ T cell colitis (29) and an inhibition of Ag-specific T-cell proliferation by plasmacytoid DC. This DC population has significant presence in mucosal tissue and is, like steady state LP immature DCs, able to induce a non-anergic state of T-cell unresponsiveness that involves the differentiation of regulatory T-cells (30;31)
These data indicate that mucosal immune activation is regulated at various levels by different cells that down-regulate immune responses. A rapidly expanding body of evidence indicates that the most important among these regulatory cells reside within the CD4+ T-cell population, and these will be the further focus of this review.

Regulatory CD4+ T cells: Phenotype, function and regulation

Once T cells are activated through engagement of the T cell receptor, they do not distinguish between “self” and “non-self”. It is now clear that the human immune system regards antigens expressed by the normal gut flora, as “self”. Because activated T cells that recognize self-antigens induce significant tissue damage, it is important to either prevent their activation, or control proliferation. It is long known that most high-
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affinity self-reactive T cells are clonally deleted within the thymus, but this system is leaky and by itself insufficient to prevent autoreactivity. Hence, prevention of autoreactivity is also continuously controlled outside the thymus, and this “peripheral tolerance” is critically dependent on the presence of regulatory T cells.

Although, in general, microbes mount strong immune responses, the resident gut flora is unable to activate T cells in healthy individuals. Thus there should be a mechanism by which potentially detrimental immune responses in the gut are prevented.

Regulatory CD4⁺ T-cells represent a population of lymphocytes with the ability to suppress both adaptive and innate immune responses (fig 1) (32-34), and these characteristic make them important for both maintenance of immunological tolerance and control of anti-microbial responses. Various types of regulatory T cells have been identified (table 1), but because specific phenotypic markers have long lacked, it is uncertain to what extent these Treg constitute separate lineages. Nonetheless, regulatory T-cells can be divided into two major groups, the so-called ‘naturally occurring’ regulatory T-cells and ‘adaptive’ regulatory T-cells, containing the so-called Tr1 and Th3 cells (35).

**'Naturally occurring’ regulatory T-cells**

Most CD4⁺ T cells that recognise auto-antigens in the thymus with high affinity are either clonally deleted, or differentiate into a ‘naturally occurring’ regulatory T cell (Treg). This cell is characterised by a unique phenotype and potent suppressive function towards auto-reactive peripheral T cells. Thymus-derived Treg constitute about 5-10% of mouse and 1-2 % of human peripheral CD4⁺ T cells. Initially, these cells were identified by their CD4⁺CD25⁺ phenotype, but an increasing number

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| Table 1: Characteristics of the different regulatory T-cells subsets. |
|-----------------------------|-----------------------------|
| **Feature**                 | Naturally Treg | Adaptive Treg |
| **Subpopulations**          | CD4⁺CD25⁺      | Tr1           | Th3           |
| **Site of induction**       | Thymus         | Periphery     |
| **Mechanism of action**     | Cell-cell contact, cytokine independent | Cytokine dependent |
| **Characterization**        | CD25⁺ and Foxp3⁺ | IL-10         | TGF-β         |
| **Specificity**             | Self-antigens in the thymus | Tissue specific antigens and foreign antigens |
| **Protection demonstrated** | Transfer colitis SCID | Transfer colitis SCID | Neutralizing TGF-β antibodies |
A recent review of Treg markers has been reported (Table 2). Several membrane-expressed molecular markers such as CD25 (IL-2 receptor α chain), glucocorticoid-induced TNFR family-related protein (GITR) and cytotoxic T-lymphocyte associated antigen 4 (CTLA-4) are constitutively expressed on Treg, but can also be observed on activated non-regulatory T cells, and it was not until the discovery of the *foxp3* gene (*FOXP3* in humans) that a unique marker for murine Treg was identified. Mutations in *foxp3* result in severe autoimmune reactivity in both mice and humans, leading to respectively the scurfy or IPEX (Immune dysregulation, Polyendocrinopathy, Enteropathy, X-linked) syndrome. The *foxp3* gene was identified as a master regulatory gene; it is constitutively and specifically expressed in natural Treg and plays an indispensable role in their development and function. Furthermore, forced expression of *foxp3* can convert naïve peripheral blood T cells to Treg cells (36). The specificity of *foxp3* in mice is clear; it is solely expressed in regulatory T cells and the scurfy mutation is always related to defective suppressive function. Conversely, the expression of *FOXP3* in humans is not restricted to Treg and can be induced upon activation of conventional T cells, albeit at much lower levels than in natural Treg. To add to the confusion, it has been reported that IPEX patients have varying degrees of disease severity and not all patients have dysfunctional Treg (37). Even with these restrictions, there is general consensus that *FOXP3* expression is highly correlated to the suppressive function of CD4+ CD25high T cells.

It has recently become apparent that expression of the α-chain of the IL-7 receptor, CD127, allows an unambiguous flow cytometry-based distinction of regulatory T cells (CD127low) Treg cells and non-regulatory T cells (CD127high) within the CD4+CD25+ populations. CD127low cells were strongly suppressive in functional suppressor assays and expression of *FOXP3* protein was highly correlated to a CD127low phenotype(38;39). These findings are important, because human regulatory T cells can now be accurately identified and isolated.

### Table 2: Cell surface and intracellular markers constitutively expressed by thymus derived natural Treg.

<table>
<thead>
<tr>
<th>Species</th>
<th>Cell surface</th>
<th>Intracellular</th>
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<tbody>
<tr>
<td><strong>Murine</strong></td>
<td>CD25+, CD122+, CD69+, CD44+, CD45RBlow, GITR+, CD103+(αE-integrin), CD134+(OX-40), CD54+(ICAM)</td>
<td>CTLA-4+, Foxp3+</td>
</tr>
<tr>
<td><strong>Human</strong></td>
<td>CD25high, CD122+, HLA-DR+(50%), CD45RO+(80%), CD95high, CD45RBlow, CD38low, partly CD62low, GITR+, CD127low</td>
<td>CTLA-4+, FOXP3+</td>
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The use of markers has been recently reported (Table 2). Several membrane-expressed molecular markers such as CD25 (IL-2 receptor α chain), glucocorticoid-induced TNFR family-related protein (GITR) and cytotoxic T-lymphocyte associated antigen 4 (CTLA-4) are constitutively expressed on Treg, but can also be observed on activated non-regulatory T cells, and it was not until the discovery of the *foxp3* gene (*FOXP3* in humans) that a unique marker for murine Treg was identified. Mutations in *foxp3* result in severe autoimmune reactivity in both mice and humans, leading to respectively the scurfy or IPEX (Immune dysregulation, Polyendocrinopathy, Enteropathy, X-linked) syndrome. The *foxp3* gene was identified as a master regulatory gene; it is constitutively and specifically expressed in natural Treg and plays an indispensable role in their development and function. Furthermore, forced expression of *foxp3* can convert naïve peripheral blood T cells to Treg cells (36). The specificity of *foxp3* in mice is clear; it is solely expressed in regulatory T cells and the scurfy mutation is always related to defective suppressive function. Conversely, the expression of *FOXP3* in humans is not restricted to Treg and can be induced upon activation of conventional T cells, albeit at much lower levels than in natural Treg. To add to the confusion, it has been reported that IPEX patients have varying degrees of disease severity and not all patients have dysfunctional Treg (37). Even with these restrictions, there is general consensus that *FOXP3* expression is highly correlated to the suppressive function of CD4+ CD25high T cells.

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Treg were originally thought to be anergic when stimulated \textit{ex vivo}, yet adoptive transfer studies using (DO11.10) TCR transgenic CD4$^+$ CD25$^+$ cells have clearly demonstrated the ability of these cells to expand \textit{in vivo} upon T cell receptor stimulation (40). Recently, it has been shown that human Treg can be greatly expanded \textit{ex vivo} by TCR stimulation in the presence of high concentrations of IL-2, since CD25 is functionally essential as a key component of the high affinity IL-2 receptor (41,42), largely increasing their potential for therapeutic manipulation. The exact mechanism of suppression by regulatory T cells remains uncertain. \textit{In vitro}, the suppressive function can be assessed by co-culturing Treg with conventional CD4 (or CD8) T cells (the ‘responder’ T cells) in a Mixed Leucocyte Reaction (MLR). The proliferation of conventional T cells in such assays is induced via TCR-stimulation by allogeneic PBMCs or agonistic anti-CD3 antibodies. In the presence of regulatory T cells, in ratios below to 1/10, the proliferation of the responder T cells and their cytokine production is strongly suppressed. In these \textit{in vitro} studies the suppressive function is cell-contact dependent and independent of cytokines. However, mouse studies have proven the suppression to be dependent on cytokines like TGFβ and IL-10. The mechanisms responsible for these differences between \textit{in vivo} and \textit{in vitro} results remain to be fully explained.

The regulatory T cell executes suppressive function as soon as it is activated via its T cell receptor, either aspecifically (CD28, CD3), by its natural (HLA class II presented) ligand, or by foreign antigens that are cross-reactive to self-antigen receptors (43) in the periphery. Treg not only suppress proliferation, but also downregulate activation, differentiation and even effector function of multiple immune cells including CD4$^+$ and CD8$^+$ T cells, natural killer cells, and dendritic cells(44-46).

APC are able to regulate Treg activation by differential expressed costimulatory molecules and MHCII molecules. Although the precise mechanism of CTLA-4 expression and its involvement on Treg is not known (47), it is thought that engagement of CTLA-4 by CD80/CD86 on DC activates Tregs, whereas interaction with CD28, in the context of TCR activation, downregulates suppression. In addition, activation of GITR by GITR-ligand (which is expressed on DC) downregulates Treg function (48). An integrin that is expressed by DC, CD103, is also involved in T-cell polarization, promoting a positive balance of regulatory T cell over effector T cell activity in the intestine (49). Interestingly, the very same stimuli (i.e. LPS) that cause APC such as DC to become activated and present antigen, have a direct effect on Treg. Murine CD4$^+$CD25$^+$ Treg express Toll-like receptors 1, 2 and 4-8, and activation of TLR-4 and TLR-5 by LPS and flagellin, respectively activates Treg and increases suppressor
function in vitro (50-52). Therefore, TLR activation of Treg seems to counteract uncontrolled activation of T cell proliferation. Conversely, natural ligands for TLR8 and TLR2 can reverse Treg function (53;54). The functional importance of Tregs is underscored by many observations in mice, where depletion of the CD4+CD25+ population precipitates diseases characterized by autoreactive T cells (55-59).

‘adaptive’ regulatory T-cells

Apart from the CD4+CD25+ thymus-derived Treg, there is evidence for the existence of Treg that are induced in the periphery, the so-called ‘adaptive’ regulatory T cells. In mice and humans, peripheral conventional CD4+ T cells were shown to differentiate into CD4+CD25+ Treg under the influence of TGF-β in addition to TCR mediated signals (60-62). Alternatively, adaptive Treg which are phenotypically distinct from Treg from intrathymic origin have been identified, and known as the Th3 and Tr1 T cell subsets. These generally do not express CD25 or foxp3 and are characterised by the secretion of the immunosuppressive cytokines TGF-β and IL-10, respectively. Although their functions are complex and incompletely understood, it seems that their suppressive activity is critically dependent on the production of regulatory cytokines.

A classical example of a peripheral regulatory cells is the Th3 Treg that secretes predominantly TGF-β together with varying amounts of IL-4 and IL-10, and mediates oral tolerance(63;64). The main immuno-suppressive effect of TGF-β is the inhibition of Th1 responses via down-regulation of IL-12β2 chain expression, and TGF-β itself is required for differentiation of TGF-β producing cells. Th3-like cells have been shown to be important in some cases of allergy and in autoimmune diseases (65;66).

Tr1 cells were initially isolated from human SCID chimera and subsequently derived by culturing naïve T cells in the presence of high concentrations of IL-10. They secrete high levels of IL-10, a cytokine that inhibits Th1 induction by down-regulation of IL-12 and suppresses the production of pro-inflammatory effector cytokines. Tr-1 cells are anergic, functionally suppressive in vitro, generally produce low levels of TGF-β and IL-5 but no IL-4, and are critically dependent on IL-10 for their function and development (67). In SCID patients transplanted with HLA mismatched haematopoietic stem cells the number of Tr1 cells correlated with tolerance of the host to the graft (37).

The proliferation of murine Tr1 cells in vivo is induced by plasmacytoid DC that express low numbers of CD11c and costimulatory molecules, and secrete large amounts of IL-10 (68). Human Tr1 cells can be induced ex vivo with the pharmacological immunosupressant vitamin D3 and
dexamethasone (69) and by immature (CD83-) DC (70). In contrast to the latter observations, we have demonstrated that induction of Treg that result from activation of monocyte-derived CD11c+ DC by probiotic bacteria requires full maturation of the DC (Braat H et al. submitted). Induction of these Treg is dependent on production of IL-10 by the mature DC, and although these Treg also secrete IL-10, this is not required for their regulatory function.

In summary, peripheral regulatory T cells comprise a heterogeneous group of T cells that secrete immunomodulatory cytokines that have been implemented in various inflammatory conditions.

**Regulatory T-cells in experimental colitis**

It is well known that adoptive transfer of T cells depleted of CD4+CD25+ cells in immunodeficient mice causes multiorgan autoimmunity in the recipient animals (71) and many studies have demonstrated that depletion of CD4+CD25+ T cells in mice aggravates T cell-mediated models of inflammation (72), including colitis (73). Conversely, Treg clearly have anti-inflammatory effects in various murine models of IBD (fig 2). For example, the induction of colitis that results from transfer of CD4+CD45RB^high T-cells into immunodeficient mice can be prevented by co-transfer of the antigen-experienced CD4+CD45RB^low T-cells. Thymus-derived Treg are CD45RB^low, and it is now thought that the CD4+CD25+ Treg present in the CD4+CD45RB^low subset are responsible for this regulatory activity (74;75). Co-transfer with isolated CD4+CD25+ T-cells prevents the induction of colitis, which is reverted by the addition of monoclonal anti-CTLA-4, -IL-10R or -TGF-β antibodies. Not only do CD4+CD25+ T-cells prevent the induction of colitis, they also can reverse established colitis and wasting disease, indicating their importance in controlling ongoing immune-mediated inflammation (76).

Peripheral Treg with a Tr1 phenotype also have the capacity to control colitis. Chronic activation of OVA-specific naïve CD4+ T cells in the presence of IL-10 induced Tr1 cells that produced large amounts of IL-10 after exposure to OVA. These cells were able to control colitis induced by pathogenic CD4+CD45RB^high T cells in immunodeficient mice, and this function was dependent on activation of the T cell receptor by OVA (77;78). Mouse strains deficient in IL-10 spontaneously develop chronic enterocolitis, underlining the importance of IL-10 in controlling responses against the commensal flora (79-81). Treatment with recombinant IL-10 in the T-cell transfer model prevents but does not cure established colitis. In contrast, local mucosal delivery of recombinant IL-10 by the
genetically modified bacteria of the strain *L. lactis* seemed to be effective when disease activity was well established, and ameliorated DSS-induced colitis and colitis in IL-10-deficient mice (82). TGF-β is also recognized for its ability to downregulate immune responses. TGF-β1-deficient mice develop a systemic inflammatory response and blockade of TGF-β signalling in T-cells results in T-cell activation and induction of IBD in mice (83). CD4+ Th3 cells protect against uncontrolled inflammation in the gut, and in models of intestinal inflammation TGF-β producing mucosal T cells were shown to reduce disease activity (84-86).

The role of regulatory T cells in inflammatory bowel diseases

Because it has long been difficult to reliably characterize regulatory T cells, all data about their presence and functional characteristics in humans are of recent date, and some of these data require confirmation. Several clinical observations indicate that the CD4+CD25+ population in patients with “autoimmune” diseases such as multiple sclerosis, uveitis and autoimmune polyglandular syndromes (APS)-II is functionally defective(87). In patients with Crohn´s disease, about 6% of both peripheral blood and lamina propria (LP) T cells were found to be CD4+CD25+, and the fraction with a high expression of CD25 (CD25\text{bright}), expressed CTLA-4 and GITR. In contrast to peripheral blood T cells, some expression of CTLA-4 and GITR was found on lamina propria CD4+CD25- T cells. In concordance with this finding, Foxp3 was predominantly transcribed by CD4+CD25\text{bright} LP T cells, and to a lesser extent by CD4+CD25- T cells. When tested for functional properties, it was found that LP CD4+CD25\text{bright} T cells, but not CD4+CD25- T cells did suppress the proliferation (as well as cytokine production) of peripheral blood CD4+CD25- T cells (88;89) but not of LP CD4+CD25- T cells. The inability of LP regulatory T cells to suppress proliferation of LP T cells may be related to the relative anergic and memory phenotype of the latter (85). Likewise, CD4+CD25+ T cells isolated from human colonic MLN in UC display typical features of Treg cells and possess potent suppressor activity in vitro in spite of persistent mucosal inflammation {unpublished results, Saruta M et al, DDW 2006 abstract 599}. At present, these sparse data on regulatory T cells in IBD suggest that the inflammatory pathology in CD patients does not result from an absence or altered functionality of the regulatory T cell population, although the
increase in regulatory T cell numbers and activity may be insufficient to suppress the inflammatory condition (90).

There are no reliable data on the existence of Tr1 or Th3 cells in the human mucosa, or on their functional properties. Interleukin-10 deficient mice develop inflammatory bowel disease, but patients with IBD do not have deficient IL-10 production (91). Remarkably, isolated T-cells from patients with IBD were found to express high levels of SMAD7, a negative regulator of TGF-β signalling, suggesting that impaired responsiveness to TGF-β may be involved in IBD (92;93). The functional role of LP regulatory T cells may be more complex than that of peripheral regulatory cells, because of the necessity specifically suppress immune responses to endogenous bacteria, but not to bacterial pathogens.

**Therapeutic potential of regulatory T cells**

As described above, Treg can prevent and even cure various experimental colitis models. Although their therapeutic potential is without dispute, translation of these data into therapeutic strategies is not straightforward. Furthermore the ability to apply this therapeutic strategy in a human clinical setting will depend on techniques to isolate and transfer adequate numbers of cells. In most mouse models of autoimmune diseases the antigens that induce T cell activation are known, and antigen-specific regulatory T cells are able to potently suppress activation in an antigen-dependent manner. For example, in the NOD model of autoimmune diabetes, islet antigen-specific BDC2.5 Tregs completely prevent diabetes. However, polyclonal Tregs are at least 50-fold less potent than antigen specific Treg and can only be a viable therapeutic option in this context when sufficient numbers are applied (94).

Although there is evidence for a role of peptidoglycan and flagellin at the level of dendritic cell stimulation, it is unknown what antigens are involved in the pathogenesis of IBD, excluding the possibility to employ antigen-specific Treg. It is now clear that regulatory T cells do not need to be antigen specific in order to suppress immune responses as a result of so-called bystander suppression (fig 2). A clear example of bystander suppression was demonstrated in the SCID transfer model, where OVA-specific Tr1 cells did suppress the occurrence of IBD after administration of OVA, although OVA is not involved in the immune mediated inflammation in this model (95). Therefore, the OVA-specific Tr1 cells were able to suppress responses induced by other antigens, very likely derived from intestinal bacteria, and this is known as “bystander” suppression. In various situations CD4+CD25+ regulatory T cells, once activated by
their TCR, have been shown to be capable of such antigen-non-specific bystander suppression (96).

Apart from bystander suppression, Treg inhibit the response of conventional CD4 T cells in a contact dependent manner and can even confer suppressive properties to such T cells. This process is known as “infectious tolerance” and results in the conversion of conventional T cells into IL-10 producing Tr1-like cells and TGF-β producing Th3-like cells (97). These concepts of 'infectious tolerance' and bystander suppression are instrumental in providing a context for using Treg as a potential therapy. It has now become possible to produce and expand sufficient CD4+CD25+ and Tr1-like cells for therapeutic application and clinical studies have been initiated. For example in a currently ongoing clinical trial, Roncarolo et al use ex vivo induced Tr1 cells as post-transplant cellular therapy in haematological cancer patients undergoing HLA-haploidentical HSC transplantation.

**Figure 2: Bystander suppression** Presentation of bacterial antigens to naive T cells by dendritic cells results in the generation of Th1 effector cells that migrate into the intestine and cause an inflammatory response. Because the antigens that are involved in IBD are unknown, therapeutic application of Treg requires antigen-non-specific suppression. Bystander suppression is the capacity of Treg to suppress immune responses that are caused by a different antigen. The mechanisms involved include the production of regulatory cytokines, deactivation of DC that attempt to stimulate effector T-cells, or direct contact with the responding T-cell. The concept of bystander suppression has been shown for Tr1, CD4+CD25+, Th3 and CD8+ T cells.
transplantation. After a 10 days ex vivo culture of donor PBMC in the presence of irradiated host PBMC and IL-10, the IL-10-anergized donor T cells are infused in the host. The ultimate goal is to provide immune-reconstitution with donor T-cells that are anergic towards host antigens and contain pre-cursors of host specific Tr1-cells. Although promising, the clinical usage of Tr1 cells for the cure of T-cell mediated diseases is still in a developmental stage (77).

A second important observation has been that regulatory functions can be imprinted in mouse and human T cells by genetic engineering. Unselected peripheral blood naïve mouse T cells become regulatory following transfection with a retroviral vector encoding IL-10, and these cells are able to suppress inflammation by a bystander mechanism (98). Such cells can also be generated from human peripheral T cells (99).

Using similar techniques, FOXP3 can be overexpressed in human CD4+ T cells but the data on the functional efficacy of these generated suppressor T cells is conflicting(100;101).

Finally, it may be possible to induce regulatory T cells in vivo by directing APC such as DC. It appears that the capacity of DC to induce regulatory T-cells depends on the DC instruction and maturation state (102).

Different approaches to render “regulatory” DC(103;104) include ex vivo genetic manipulation, anti-inflammatory cytokine exposure or by direct instruction with tolerogenic compounds. We have recently demonstrated that injection of the Bordetella pertussis-derived filamentous hemagglutinin A (FHA) reduced inflammation in a mouse model of IBD (105). The experimental data of T-cell as well as DC manipulation, along with future investigations needs to determine the exact value of both approaches, but recent advances are very promising.

When considering ex-vivo manipulation or induction of Treg, two major hurdles need to be overcome.

Firstly, sufficient numbers of the manipulated T cells need to be directed to the gut mucosa, a process known as “homing”, which is directed by specific integrins and by chemokines. It has been reported that cultured CD4+ gut-derived T cells that express high levels of the pivotal gut-homing receptor α4β7, did not home to the gut following injection in healthy individuals (89). However, we have reported that homing –at least in mice and rats- is greatly increased in inflammatory conditions. Also, it has become apparent that the isolation and expansion of CD45RA+ naïve (instead of CD45RO+) CD4+CD25+ T cells is the best strategy for adoptive Treg cell therapies(106).

Secondly, a problem of adoptively transferred Treg may be that the bystander suppression mechanism is that the targets for downregulation cannot be controlled; this problem needs to be solved. Regulatory T cells
may worsen inflammatory disease, because they may interfere with the immune mechanisms that are necessary for clearance of microbial pathogens (107). The non-specific immunosuppressive effects of Treg are a concern when considering therapeutic application. On the other hand such effects may be limited, because effective pathogen-specific immune responses are shown to be Treg resistant (108). However, from this perspective the use of natural occurring CD4+CD25\textsuperscript{high} Treg may be preferred, as in these cells, at least \textit{in vitro}, TLR2 triggering results in a temporal loss of the suppressive Treg phenotype (54).

**Conclusions**

Regulatory T cells are key players of immune regulation, and they have important functions in suppressing unwanted inflammatory responses towards self-antigens, and the antigens of endogenous intestinal bacteria (fig 5). IBD patients do not seem to have a primary absolute defect in regulatory cells, but apparently, the regulatory capacity of these cells is insufficient to down-regulate inflammation.

None of the current therapies for IBD directly targets Treg function or generation, but drugs that are widely clinically used may influence Treg function. For example, corticosteroids may enhance Treg function in asthma and allergic diseases (109-111) but future research needs to determine the exact role of corticosteroids on regulatory T-cell function. Interestingly it was recently shown that anti-TNF antibodies increased the FOXP3 mRNA and protein levels in the CD4+CD25\textsuperscript{high} compartment and restored their tolerogenic function (112). In mice and humans with diabetes treatment with non-agonistic anti-CD3 antibodies resulted in prevention of progression a loss of islet cell function by immunosuppressive mechanisms that included the induction of Treg (113).

None of the previously discussed possible clinical approaches uses a strategy that allows control over regulatory T cells activity. Are such approaches feasible?

Intravenous administration of relatively large doses of regulatory cytokines is not effective (rhIL-10 administration in IBD patients (114)) or found to be toxic (TGFβ). Only a small fraction of the total administered dose of such cytokines reaches the mucosa and results may be better when such cytokines are locally administered. Mucosal delivery of recombinant IL-10 by genetically modified bacteria such as \textit{L. lactis} addresses this problem and was indeed shown to ameliorate DSS colitis and colitis in IL-10-/- mice. Recently, we have demonstrated in a clinical phase 1 trial that this engineered cytokine-excreting organism can be safely administered to
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Crohn’s disease patients and is biologically contained (115). TGFβ can also be locally expressed, for example by delivery of TGFβ-encoding plasmids to mucosal surfaces and this approach is in the phase of pre-clinical trials (116). Contained immunosuppression can also be accomplished by modifying or expanding T cells with regulatory properties ex vivo. This strategy involves a harvesting step that yields peripheral blood T lymphocytes (for example by apheresis) followed by forced differentiation by exposure to cytokines and tolerogenic compounds, or by genetic engineering with a “regulatory” gene. Upon readministration to the patient, such cells can down-regulate inflammation by a bystander mechanism, following specific integrin-mediated homing. It is technically feasible to specifically expand T cells with a predefined T cell receptor, that can be specifically activated by an orally administered antigen, allowing for control of the immune suppression. These strategies are attractive in view of the long lifespan of T cells, and are expected to have long-term effects. Regulatory T cells are extremely potent and production of low amounts of IL-10 by a very small fraction of mucosal T cells (IL-10-engineered T cells that comprised only ~ 0.001% - of all mucosal T cells were effective) a sufficient therapeutical effect can be achieved (117). With the identification of more genes that determine regulatory T cell development, the ability to identify Treg using cell surface markers and with improving transduction methods over time, the possibilities for such approaches will be significantly expanded.

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References


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