[Frontiers in Bioscience 13, 2266-2274, January 1, 2008]

The characterization and role of regulatory T cells in immune reactions

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1. ABSTRACT

Regulatory T cells (Tregs) having CD4+CD25+Foxp3+ or CD4+IL-10+ (Tr1) phenotype and capable of inducing anergy towards self- and alloantigens play an important role in autoimmunity, as well as in tolerance of allografts, pregnancy and cancer. Both thymus-derived T CD4+CD25+Foxp3+ natural cells and peripherally-induced T CD4+CD25+Foxp3+ cells prevent migration of effector immunocytes to target organs and inhibit their cooperation with antigen-presenting cells. The suppressive function of CD4+CD25+Foxp3+ Tregs depends on interactions between stimulatory (IL-2, CTLA-4) and inhibitory (GITR, CD28) signals, on stimulation of indoleamine 2,3-dioxygenase (IDO) activity in dendritic cells via CD80/CD86 molecules, and finally on cell-cell inhibition of effector cells by membrane-bound TGF-beta. Anergy of effector cells caused by Tregs could provoke them to secretion of IL-10/TGF-beta in mechanism of "bystander suppression". Tr1 cells constitute the distinctive Tregs population which originates from IL-10-primed naïve T cells or from T cells induced by tolerogenic IL-10/TGF-beta-expressing dendritic cells. The suppressive activity of Tr1 cells is based on local IL-10/TGF-beta secretion in the peripheral tissues. Tregs have a privileged place in the net of immunological interactions which makes them a possible common target for therapeutic interventions in different diseases.

2. INTRODUCTION

Integrity of immune system depends on recognition and defense against foreign antigens with simultaneous assurance of un-responsiveness to self-antigens. The concept of specialized T cells indicating exclusively suppressor activity, although had been present in immunological discussions for a long time, disappeared from literature in 1980s due to conflicting results. Although some previous reports suggested the existence of T cell populations suppressing cytotoxic responses, there were Sakaguchi *et al.* (1) who for the first time reported on naturally occurring T CD25+ cells capable of regulating tolerance towards self-antigens. From that time growing evidence has been shown that regulatory T cells of different phenotype and function could play an important role in self-tolerance and autoimmunity, as well as in tolerance of allografts, pregnancy and cancer tissues.

3. NATURAL T CD4+CD25+FOXP3+ REGULATORY CELLS

3.1. Phenotype and function of natural Tregs

Regulatory natural T CD4+CD25+ ^{high} cells are defined as a subpopulation of thymus-derived T lymphocytes representing about 1-3% of T CD4+ cells, capable of mediating immune tolerance (2, 3). Regulatory T cells express surface markers of activated, antigen-

primed, memory T cells: CD25, CD45RB^{low}, CD62L, CD103, cytotoxic T lymphocyte antigen-4 (CTLA-4), and glucocorticoid-induced tumor necrosis factor receptor (GITR). Disruption of thymic maturation or peripheral maintenance of natural Treg cells predisposes to strong autoimmune reactions (4). Regulatory T cells might act multi-directionally by preventing migration of effector T CD4+CD25- , T CD8+ , natural killer (NK) cells , natural killer T cells (NKT) or B cells to target organs, inhibiting their cooperation with antigen-presenting cells (APC) and thus inducing anergy (3, 4, 5, 6). They are also capable of killing effector cells or APCs via direct cell-cell contact. It was suggested that perforine-dependent mechanism could account for this, however, recent data indicate that it is rather a granzyme-B-dependent pathway (7, 8). Lipopolysaccharide (LPS) activated T regulatory cells can also inhibit reactive oxygen intermediates and cytokine secretion by neutrophils and promote their apoptosis. This kind of suppressive Treg cell capacity is modulated via toll-like receptor-4 (TLR-4) present on their surface (9) together with other members of TLR family (TLR-5, TLR-7, TLR-8) (10).

Treg cells require T-cell receptor (TCR) stimulation to exert suppression, however, once activated they mediate inhibitory effects without antigen specificity (11). Interleukin-2 (IL-2) is also specifically required for the generation and maintenance of Treg cells, as was shown by using of anti-IL-2 antibody (12, 13) or disrupting IL-2 signalling pathway (14). Deficiency of IL-2-receptors (CD25 and/or CD122) caused "IL-2 deficiency syndrome" characterized by proliferative inflammatory disorders with autoimmune component (15). High doses of IL-2 upregulate CD25 expression on CD4+CD25+ cells and in the presence of TCR stimulation trigger *in vitro* proliferation of natural Tregs (16). Interleukin-2 is also belived to support survival of Tregs inside secondary lymphoid tissues (17). Activated T CD4+CD25^{low} cells are probably the source of IL-2 in peripheral tissues *in vivo* (18). Antagonizing of Treg function could be obtained by signalling through GITR on the surface of effector T cells which is mediated by GITR ligand (GITR-L) on DCs' surface, or alternatively by secretion of IL-6 produced by activated DCs (19, 20). Treg cells produce soluble GITR (sGITR) molecules which could interfere with GITR-L on DCs and block GITR/GITR-L interactions thus providing enhancement of suppression by Tregs (21). Another marker of mouse and human Treg cells is a transcription factor forkhead box protein 3 (Foxp3), which regulates both their development and function. Endogenous expression of Foxp3 was limited to CD4+CD25+ Treg cells, and little or no expression was seen in other T cell subsets, B lymphocytes, NK and NKT cells (22, 23). Therefore Foxp3 is considered as specific CD4+CD25+ Treg marker. It was shown that Foxp3-knockout mice displayed severe Treg deficiency and predisposition to autoimmune disorders and that due to mutations of human Foxp3 gene lethal autoimmune IPEX syndrome (X-linked immunodysregulation, polyendocrinopathy, enteropathy) developed (23).

3.2. Natural Tregs suppressive activity and its regulators

The function of natural thymically derived T CD4+CD25+ regulatory cells depends on modyfing properties of CTLA-4 and CD28 molecules, which act either as enhancers (CTLA-4) or inhibitors (CD28) of Treg suppression (24). Whereas CD28 function seems to be well undesrstood and refers to generation and homeostasis of Tregs, the CTLA-4 role does not appear to be essential, as CTLA-4-knockout mice still possess T cells of Foxp3 phenotype which are capable of suppression (25). However, another experiments indicated that blocking antibodies against CTLA-4 inhibited Treg suppressive properties (26). Recent studies suggest, that the potential function of CTLA-4 may be stimulation of IDO activity in dendritic cells (DCs) via CD80 and CD86 costimulatory molecules (27). The next possible function of CTLA-4 may be the alteration of T cell adherence to APCs through modulation of leukocyte function-associated antigen-1 (LFA-1) - intercellular adhesion molecule-1 (ICAM-1) interactions (28). The mechanisms of suppression exerted by Tregs include also membrane-bound TGF-beta which can influence the effector cells via direct cell-cell contact (29). According to some observations (30) this mechanism of suppression is produced by subpopulation of T CD4+CD25+^{high} cells with increased HLA-DR expression (CD4+CD25+^{high} DR+). Another kind of Tregs activity is called "bystander suppression". In this mechanism initial direct cell-cell contact with Tregs causes anergy of T CD4+CD25- cells and promotes them to secretion of TGF-beta and IL-10 which both are capable of inhibiting further effector reactions (31, 32, 33). This type of suppression is attributed to the second Treg subpopulation without HLA-DR expression (CD4+CD25+^{high} DR-) (30). It was also suggested, that Treg-induced indoleamine 2,3-dioxygenase (IDO) activity in DCs could in turn influence Treg cell function by up-regulating heme oxygenase-1 (HO-1) through 3-hydroxyanthranilic acid (HA), a byproduct of tryptophan metabolism (34). Heme oxygenase-1 activity seems to be important for Treg activity, as carbon oxide (CO) being a product of HO-1 is able to exert antiproliferative effects on T cells independently of their antigen specificity, as well as to downregulate pro-inflammatory cytokines secretion (35, 36). Blocking HO-1 abrogates both the regulatory activity and suppressive properties of Foxp3+ Treg cells (37). The conclusion is, that Treg cells exert their immunoregulatory properties by influence on interactions between T effectors and DCs, and that CTLA-4, IDO and HO-1 are with a high probability engaged in this process (24)(Figure 1).

4. PERIPHERALLY INDUCED T CD4+ CD25+ FOXP3+ REGULATORY CELLS

4.1. Phenotype and induction of peripheral Tregs

Apart from natural Treg cells originating in thymus, population of Tregs can be induced from mature peripheral T CD4+CD25- cells. Peripherally-induced Tregs are phenotypically indistinguishable from thymus-derived cells, including the expression of Foxp3 protein (24). The presence of Foxp3 on natural T CD4+CD25+ cells is dependent on thymic maturation, however, experimental *in*

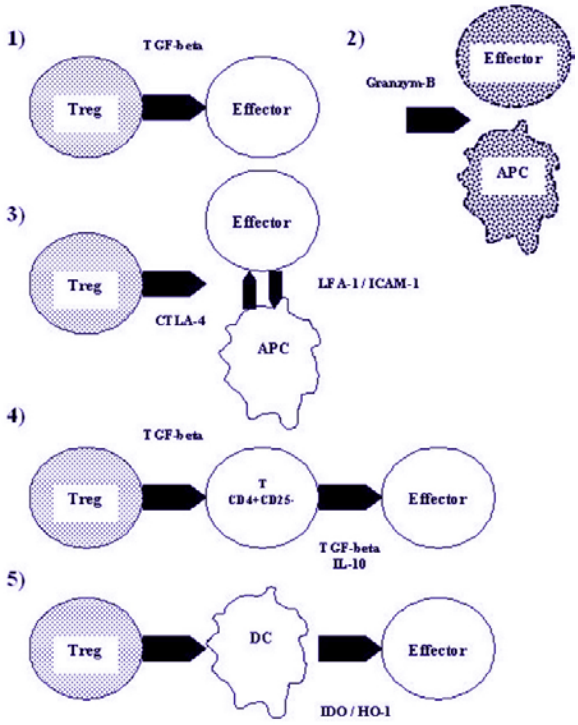

Figure 1. Possible mechanisms of suppressor activity exerted by natural CD4+CD25+Foxp3+ T regulatory cells (Tregs). 1) Natural CD4+CD25+Foxp3+ Tregs can inhibite effector cells via direct cell-cell contact mediated by membrane-bound TGF-beta (29); 2) Tregs are capable of killing effector and antigen-presenting (APC) cells via direct cell-cell contact using granzyme-B pathway (8); 3) Tregs can inhibite adherence of effector cells to APCs via modulation of LFA-1 / ICAM-1 interactions by CTLA-4 molecule (28); 4) Tregs by cell-cell contact mediated by membrane-bound TGF-beta cause anergy of T CD4+CD25- cells and force them to IL-10 / TGF-beta secretion in mechanism of so called "bystander suppression" (31, 32, 33); 5) interaction between Tregs and dendritic cells (DC) induce indoleamine 2,3-dioxygenase (IDO) and heme oxygenase-1 (HO-1) secretion which both have anti-proliferative effects on T cells (34, 35, 36).

vitro stimulation of peripheral T CD4+CD25- lymphocytes in the presence of TGF-beta can also up-regulate Foxp3 and results in acquisition of suppressive function (38, 39, 40). Moreover, retroviral transduction of Foxp3 to T CD4+CD25- cells converts them into functional T CD4+CD25+ Tregs (41). However, TGF-beta alone is not fully effective in mediating Treg induction and IL-2 is important co-stimulator which directs the allo-stimulated CD4+CD25- T cells into Tregs (42). Other cytokines may also participate in regulation of Treg function. In *in vitro* studies IL-4 and IL-13 have been shown to act as converters of naïve T CD4+CD25- cells into CD4+CD25+ Treg cells (43). The Foxp3 expression seems also to be dependent on suboptimal peripheral T cells stimulation with low doses of antigen as well as on the type of APCs employed to stimulate T cells (44).

4.2. Regulatory molecules for peripheral Tregs function

Many studies indicate the presence of functional interplay between TGF-beta, CTLA-4 and Foxp3 on Treg cells. Molecule CTLA-4 appears to modify TGF-beta expression, and in opposite, TGF-beta affects CTLA-4 expression (45, 46). Although TGF-beta together with T-cell activation signal are effective stimulators of Foxp3 induction, this process additionally involves CD28-dependent signalling, as has been presented by experiments on *in vitro* Treg generation using agonistic CD28 antibodies (47). Mature DCs (mDCs) expressing higher levels of CD86 were found to be less effective in pro-suppressory activity compared with immature DCs (iDCs) which have a predominant CD80 expression (24). Results of the studies performed on human Tregs indicate that proliferation of peripheral Treg cells depends on interactions with mDCs expressing high levels of CD86 and CD28 molecule. Costimulatory CD80 molecule is less important in this regard (24), however, CD80-CTLA-4 interactions between T cells and iDCs stimulate suppression (26). Therefore, it seems reasonable to conclude, that CD86 and CD80 costimulatory signals have different ability to interact via CD28 or CTLA-4 molecules, and that both balance in CD80 and CD86 expression on DCs and their type (mature vs. immature) can regulate suppressive capacity (56)(Figure 2). The conclusion is, that DCs are able to expand functional antigen-specific Tregs from the polyclonal repertoire, and that these peripheral Treg cells are much more effective than natural polyclonal CD4+CD25+ Tregs in regulating immunity (47). Dendritic cells mediate their action in peripheral tissues or secondary lymphoid organs invaded by pathogens or tumor cells. In these localisations DCs can interact with other immunocyte populations which are able to modulate the number and function of DCs. It was found that in autologous setting NK cells following activation with IL-2 were able to kill myeloid iDCs (48), and that during NK - DC cell-to-cell interactions NK cells secreted TNF-alpha capable of inducing DC maturation (49). Mature DCs secrete IL-12 which directly stimulates NK cell activity and induce activation of T lymphocytes which in turn produce IL-2 thus indirectly stimulating cytolytic activity of NK cells (50). The NK-cell-mediated killing of iDCs was inhibited by TGF-beta (51) as well as L-kynurenine, a tryptophan catabolite produced by IDO expressed on tumor cells or activated DCs (52, 53). These data suggest that NK cells homing into peripheral tissues and secondary lymphoid organs play a possible role in preventing tolerance during T-cell priming by elimination of iDCs which could induce immune anergy through Treg cells (50, 54). Another mechanism of preventing Treg-dependent tolerance is potentially based on regulatory properties of IFN-gamma. Proliferation of Th lymphocytes as well as IFN-gamma expression by T cells inside lymphatic nodes correlated inversely with the number of resident Tregs (55). There are antigen-specific IFN-gamma-producing T CD8+ cells which could control the generation or activation of T CD4+CD25+ cells either by modulation of DCs function (up-regulation of TLR or alternatively downregulation of TGF-beta expression), or direct inhibition of Tregs via IFN-receptor (56). The potent source of IFN-gamma antagonizing Treg function could also be gamma/delta T cells (57).

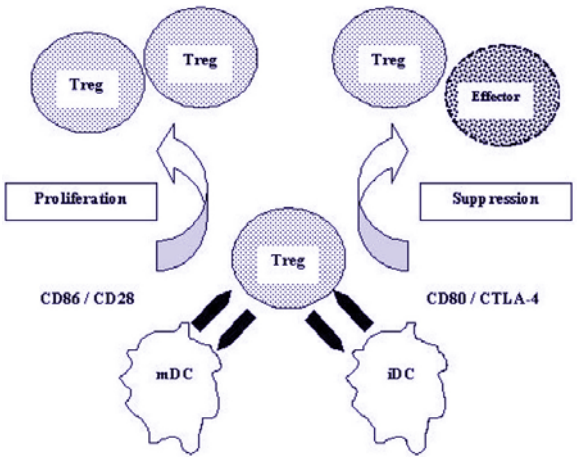

Figure 2. Mechanisms of dendritic cell (DC)-dependent expanding of antigen-specific peripheral CD4+CD25+Foxp3+ T regulatory cells (Tregs). Peripheral antigen-specific CD4+CD25+Foxp3+ Tregs are induced from T CD4+CD25- cells in TGF-beta- and IL-2-dependent manner (38, 39, 40, 42). These Treg cells by interaction with mature dendritic cells (mDC) through CD86 / CD28 molecule pathway are "pushed" to proliferation activity, but interaction between immature dendtitic cells (iDC) via CD80 / CTLA-4 molecule pathway enhances their suppressor potential (24, 26). Therefore balance of CD80 / CD86 expression on DCs and their type (mature / immature) are both regulators of peripheral Tregs behavior (56).

4.3. Peripheral Tregs recruitment into target organs

The proper Treg function depends on adequate recruitment and movement towards target peripheral organs. The study performed on cardiac transplant model showed, that generation of peripheral CD4+CD25+Foxp3+ allospecific Tregs occured inside the draining lymph nodes and was dependent on lymph node homing selectin (CD62L) expression on these cells. Blocking of CD62L with monoclonal antibodies resulted in Treg displacement and subsequent graft rejection (58). Among various chemokines produced by B cells and activated DCs, CCL4 is the chemokine which preferentially mediates recruitment of Treg cells. Its role was confirmed in the study where depletion of CCL4 resulted in augmented autoimmunity comparable to that obtained after Treg depletion (59).

5. TYPE 1 REGULATORY T CELLS

5.1. Characterization of type 1 regulatory T cells

One of the subpopulations of Treg cells which phenotype and mechanisms of action have been recently revealed are type 1 regulatory T cells (Tr1). These cells were first described as IL-10-producing Tregs (60). Knowledge about their antigen and functional characteristics has been obtained from studies concerning *in vitro* expansion on both murine and human pathogen-specific Tr1 cells (61, 62, 63). Tr1 cells proliferate poorly after polyclonal TCR-mediated or antigen-specific activation (64). On the other hand it has been revealed that markers such as: CD40L, CD69, CD28, CTLA-4, HLA-DR can be expressed on Tr1 after TCR-mediated activation (65). In the anergic phase of Tr1 cells both Th1 and Th2 cytokine receptors are expressed (66, 67). The absence of the CD25 or Foxp3 expression upon antigen or polyclonal stimulation of Tr1 cells may be the most important difference between Tr1 cells and CD4+CD25+ Treg cells (65, 68, 69). It was found, that no Tr1 cells within the $CD25+^{high}$ population were present during analysis of CD4+CD25+ Tregs (70). Tr1 cells proliferate from naïve cells during the peripheral adoptive phase of immune reaction while natural CD4+CD25+ Tregs have well defined antigen specificity obtained during intra-thymic maturation, or similarly to Tr1 cells, can be induced from naïve cells as peripheral CD4+CD25+ Tregs (71). Actually, no specific marker for Tr1 cells has been discovered. Because many types of human T helper cell subsets can secrete IL-10 it is important to measure the amounts of Th1-associated (IFN-gamma) and Th2-associated (IL-4) cytokines as well as the IL-10 and TGF-beta production in order to properly define Tr1 cells (72). Another type of Tr1 cells (called CD8+ Tr1-like cells) has been also described. These cells also display regulatory properties exerted by IL-10 and can be generated by stimulation of naïve T CD8+ lymphocytes with IL-10-modulated myeloid DCs or activated plasmacytoid DCs (73, 74, 75). However, their precise role needs to be established.

5.2. The generation and regulation of Tr1 cells

The population of functionally active Tr1 cells can be generated after priming of naïve T cells with antigen in the presence of IL-10. Although the presence of IL-10 is necessary for the differentiation of Tr1 cells, still it has been found to be insufficient (76). Co-addition of IFN-alpha on *in vitro* model promoted further autocrine IL-10 production resulting in proper differentiation of CD4+ Tr1 cells (77). Co-signaling via CD2 and CD46 molecules also induced proliferation of Tr1 cells. However, it is not really clear, whether these CD3/CD46-stimulated T cells were typical Tr1 cells. Moreover, it has been revealed that Tr1 cells can be expanded in the presence of IL-15 which is an important growth factor for Tr1 cell *in vitro* and seems to be essential for surviving Tr1 cells *in vivo* (reviewed in 68). Addition of rapamycine to T cell cultures also promotes Tr1 expansion (78). Tr1 cells can also be induced by co-culturing of T cells with TGFbeta/IL-10-expressing "tolerogenic" DCs (73, 76, 79) or immature DCs (63). Dendritic cells can influence Tr1 cell function in the IDO- or HO-1-dependent pathway which seems to be functionally connected with IL-10 and has a role in regulating Tr1 cell activation (52, 80, 81). It is possible that IDO+ DCs can induce subsets of Tr1 Treg cells as well as suppress local T responses to antigens which are presented by neighboring IDO-negative DCs in the mechanism of "bystander" suppression (52). It was also found, that plasmacytoid DCs (pDCs) expressed inducible costimulator (ICOS-L), which promoted generation of IL-10-producing T regulatory cells (82). Because of insufficient knowledge about the stimulation factors for Tr1 cells there is a need for further research on this field.

5.3. Tr1 cells suppressive activity

Once activated via the TCR, Tr1 cell can indicate suppressive activity against other antigens. This regulatory

activity is mediated by local secretion of IL-10 and TGF-beta (64). These cytokines inhibit Th1 type CD4+ T cells, negatively influence the naïve and memory T-cell responses and downregulate expression of costimulatory molecules and pro-inflammatory cytokine production by APCs (60, 83, 84, 85, 86). This mechanism of suppression may be partially or completely reversed by addition of an anti-IL-10 and anti-TGF-beta monoclonal antibodies and is independent from the cell-cell contact (60, 68, 87). In certain experimental conditions Tr1 cells may be also stimulated to produce low levels of IL-5 and IFN-gamma but they do not secrete IL-2 or IL-4 (65). It has been suggested that Tr1 cells display an ability to migrate to inflamed tissues and that this migration of Tr1 cells is not dependent on the type of tissue, on the kind of inflammation, or on the presence of the specific antigen. The suppressive effect of regulatory T cells occurs locally (86, 88). The absence of chemokine receptors on Tr1 cells could be viewed as a specific adaptation to control immune responses by circulating throughout the body without being disturbed by chemo-attraction (64), but this concept needs more investigations.

6. PERSPECTIVES

The history of scientific investigations has revealed many times that excessive significance was attributed to the novel mechanisms or drugs which were for some time viewed as remedy for almost all human diseases. The same can happen in the case of T regulatory cells, however, with appropriate dose of criticism Tregs might become a valuable tool in immunotherapeutic approach towards many diseases. First, the precise understanding of Tregs induction, maintenance and function has to be established. The next step is to define Tregs regulatory mechanisms in order to learn how to modulate the Treg activity in certain environment without compromising their function in another place and how to obtain the appropriate level of suppression. Future knowledge about CD80/CD86 pathway, CTLA-4 molecule, GITR/GITR ligand system and cooperation between Tregs and DCs, mast cells, and T or B lymphocytes will help to influence Tregs behavior in different diseases. The future techniques will probably use selective modulation of Treg receptors, modulation of expression of surface molecules and pattern of cytokine secretion by the means of properly constructed monoclonal antibodies or genomic manipulation. The target organs could also be modified in order to enhance or diminish Tregs suppressor abilities. We should also learn more about the rules of Tregs recruitment into certain tissues and organs, which would let us to remove unnecessary Treg activity away from target organs or to redirect it precisely into demanded tissue. Tregs have a privileged place in the net of immunological interactions which makes them a common target for therapeutic interventions in autoimmunity, allergy, allograft tolerance and other situations. From another point of view, the privileged place of Tregs in the diversity of immunological reactions could be also viewed as disadvantage, which brings the possibility of unwanted side effects during therapeutic Tregs manipulation. Therefore, there is a very interesting issue to study and to compare the mechanisms of Tregs regulation and function in different clinical situations, like pregnancy and cancer.

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Key Words: $CD4^+$, $CD25^+$, $Foxp3^+$, T cells, Type 1, Regulatory T cells, Tregs, Tr1 cells, Review

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