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Revival of the regulatory T cell: new targets for drug development

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Compelling new evidence supports the idea that regulatory T cells play a major role in our immune system. Several subsets of these regulators have been identified recently. Differences in the phenotypical and functional characteristics of these subsets have immunological implications. From our growing knowledge of the field of immunology, we could potentially generate a new class of therapeutic agents that target immune-related diseases.

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▼ The challenging task for the immune system is to mount an optimal defense against foreign agents as well as host cells, while at the same time avoiding damage to somatic tissues. To achieve this feat, the immune system has developed several checkpoints and regulatory systems. First, repertoire selection in the thymus prevents strongly self-reactive T cells from entering the periphery [1]. Thymic antigen-presenting cells (APC) mediate the deletion of T lymphocytes that have T cell receptors (TCR) with high avidity for self-antigen–MHC (major histocompatibility complex) complexes. However, repertoire selection does not preclude all T cells recognizing self-antigen from passing into the periphery [2], thus the entry of a limited number of these cells results in the formation of a highly diverse TCR repertoire in the periphery. Second, T cells must encounter their antigen under the correct stimulatory conditions to become activated [3,4]. If there are insufficient co-stimulatory signals, for example, CD28–B7 interactions, between T cell and APC, the T cell will either ignore the antigen or be eliminated through activation-induced cell death (AICD) [5]. AICD involves apoptosis and can be triggered by death receptors [e.g. Fas (CD95)], tumor necrosis factor (TNF) or the absence of survival cytokines [6–9]. In addition, T cell elimination can be effected through induction of T cell anergy, the molecular mechanism of which is poorly understood but is thought to involve the negative regulator of T cell activation, cytotoxic T lymphocyte-associated antigen 4 [CTLA-4 (CD152)] [10–12]. *In vitro*, T cell anergy can occasionally be overcome by high concentrations of interleukin (IL)-2 [13] and, in these cases, is a reversible process. However, the extent to which T cell anergy is reversible *in vivo* is unknown. Third, in the 1970s it was proposed that T cells could act as regulatory cells and suppress immune effector cells [14]. However, when none of the proposed suppressor genes was located, suppressor immunology was considered an over-interpretation of data of dubious quality [15]. As a result, suppressor T cells were not thought to be involved in the immunological process. However, this changed in 1995 when Sakaguchi and colleagues [16] reported a subset of $CD4^+$ T cells that constitutively express the IL-2 receptor α-chain (CD25) to exert immunosuppressor activity. Subsequently, $CD4^+CD25^+$ regulatory T (Treg) cells have received exponentially increasing attention from immunologists [17].

Subsets of regulatory T cells

Several subsets of Treg cells can be distinguished but their overlapping features can cause confusion. One possible explanation for the several discrepancies observed in the literature is that researchers investigating different T cell subsets often use similar classifications for these subsets (e.g. $CD25^+$ Treg cell and suppressor T cell refer to the same subset). To prevent further confusion, clear descriptions with consistent nomenclature must now be put into place. Several Treg cell subsets are listed in Table 1.

Naturally occurring regulatory $CD4^+CD25^+$ T cells

The naturally occurring $CD4^+CD25^+$ Treg cell subset is produced in the thymus as a functionally distinct subpopulation of T cells [18].

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Table 1. Subsets of regulatory T cells

Origin	Nomenclature	Surface molecules	Target	Specificity	Mode of suppression
Naturally occurring Treg cells	Intrinsic Treg cells	$CD4^+$, $CD25^+$, $GITR^+$, $CTLA\text{-}4^+$	Effector lymphocytes	Probably self-antigens	Via direct cell–cell contact
Induced Treg cells	Th3, Tr1, induced Treg cells	$CD4^+$, $CD25^+$ $GITR^+$, $CTLA\text{-}4^+$	Effector lymphocytes	Foreign or self-antigens	IL-10, TGF-β, via direct cell–cell contact
	Anergic T cells	$CD4^+$	APC	Any	Downmodulation of DC maturation
	Ts cells	$CD8^+$, $CD28^-$	APC	MHC class I restricted	Induction of tolerogenic DC via inhibitory receptors ILT3 and ILT4
	DN Treg cells	$CD3^+$, $CD4^-$, $CD8^-$	Syngeneic $CD8^+$ T cells	Involvement of Ly-6A	Fas-mediated killing of target CTL

Abbreviations: APC, antigen-presenting cell; CTLA, cytotoxic T lymphocyte-associated antigen; DC, dendritic cell; DN, double negative; GITR, glucocorticoid-induced tumor necrosis factor receptor; IL, interleukin; ILT, immunoglobulin-like transcript; MHC, major histocompatibility complex; TGF, transforming growth factor; Treg, regulatory T; Ts, suppressor T.

This subset is present in the periphery of a naïve host and therefore can be referred to as intrinsic Treg cells. When stimulated via their TCR, $CD4^+CD25^+$ Treg cells are non-proliferative [19]. On induction by their TCR, these Treg cells strongly inhibit the activation of effector T cells in a cell-contact dependent manner [20–22]. The transfer of T cell populations, from which the $CD25^+$ subset has been depleted, into T cell-deficient mice caused severe autoimmune disorders, for example, thyroiditis, gastritis, insulin-dependent diabetes mellitus and colitis [16,23,24]. After addition of the Treg cells to the recipient mice, the autoimmune effects were strongly suppressed. The depletion of the $CD25^+$ T cell subset for extended periods in mice that are prone to autoimmune diseases induces or boosts autoimmune disease [25]. In addition, $CD4^+CD25^+$ Treg cells have been reported to suppress natural killer T (NKT) cells [26]. Notably, although CD25 appears to be an important marker for Treg cells, it is not exclusively expressed by Treg cells. Resting, mature, naturally occurring Treg cells display a cell surface phenotype typical for activated helper T (Th) cells; they express CD25, CTLA-4, glucocorticoid-induced tumor necrosis factor receptor (GITR) and CD45RO at the cell surface. Recently, it was shown that the constitutive expression of activation markers by Treg cells is a consequence of the continuous exposure to self-antigen [26,27]. Other molecules associated with Treg cells are αEβ7-integrin [28] and membrane-bound transforming growth factor-β (TGF-β) [29].

Induced regulatory T cells

It has become clear that the subclass of induced Treg cells encompasses more than one type of T cell. Treg cells that originate from naïve $CD4^+$ T cells and are induced in the periphery are termed Th3 or Tr1. The proliferation of Th3 or Tr1 cells is the result of exposure to their antigen in the presence of IL-10 and/or TGF-β. T cell clones generated in the presence of IL-10, such as those derived from non-activated (immature) antigen-presenting dendritic cells (DC) or tumor cells, produce a cytokine profile different from that of Th1 or Th2 cells in that IL-5, IL-10, TGF-β, and interferon (IFN)-γ are produced but not IL-2 or IL-4 [30–33]. After repetitive stimulation with immature DCs, alloreactive T cells display an irreversibly reduced proliferation, lose their ability to produce IFN-γ, IL-2 and IL-4 and differentiate into nonproliferating, IL-10-producing T cells [34]. Furthermore, in co-culture experiments, alloreactive Treg cells inhibited the antigen-driven proliferation of Th1 cells in a contact- and dose-dependent, but antigen-nonspecific manner [34]. Others have reported that culturing naïve $CD4^+$ T cells in the presence of TGF-β resulted in the generation of contact-dependent $CD4^+CD25^+$ suppressor T cells that did not require cytokines for suppression [35].

T cells stimulated under particular anergy-inducing conditions can suppress T cell responses. 'Anergic' T cells mediate this suppressive effect via modulation of the T cell-activating capacity of the APC [36,37]. These anergic T cells inhibited the maturation of DCs, but the stimulatory ability of mature DCs was not affected. In contrast with intrinsic or induced Treg cells, which target effector T cells directly, anergic T cells mediate their effect via the APC. Furthermore, it was demonstrated that T suppressor (Ts) cell lines can be generated by *in vitro* immunization of human peripheral blood mononuclear cells (PBMCs), with

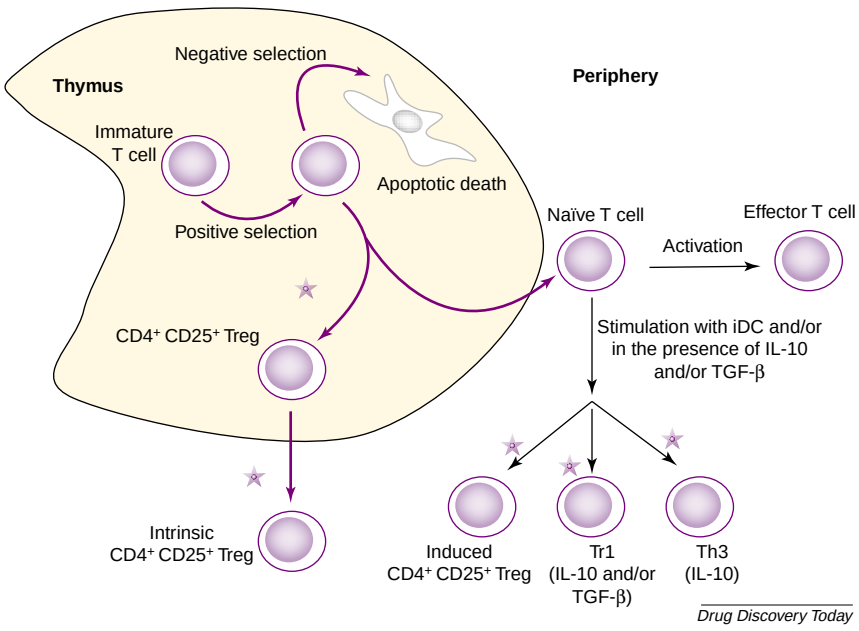

Figure 1. Development of regulatory T (Treg) cells in thymus and periphery. Positive selection ensures that only T cells bearing a relevant TCR (capable of interacting with MHC molecules) develop. Negative selection subsequently eliminates T cells with high-affinity TCR for peptide-MHC complexes, thereby preventing these T cells from entering the periphery and causing autoimmune diseases. In addition, the thymus generates Treg cells. Treg cells generated in the thymus recognize self-peptide–MHC complexes with high-affinity TCR and escape from apoptotic death during negative selection. These T cells differentiate into the naturally occurring (intrinsic) $CD4^+CD25^+$ Treg cells. Naïve Th0 cells can also differentiate into Treg cells by a process that requires immature and tolerizing APC during priming of the response. Possible targets for drug development are indicated by a star. Abbreviations: APC, antigen-presenting cell; iDC, immature dendritic cells; IL, interleukin; MHC, major histocompatibility complex; TCR, T cell receptors.

synthetic peptides or soluble proteins coupled to beads [38]. Ts cells have also been found in allografts [39]. These Ts cells express the $CD8^+CD28^-$ phenotype, require APC to effect suppression and display antigen specificity that is restricted by self-MHC Class I molecules. Recently, Chang *et al.* [40] reported that $CD8^+CD28^-$ T cells downregulate immune responses through induction of tolerizing DCs. The inhibitory receptors ILT3 (immunoglobulin-like transcript 3) and ILT4, which are expressed by DCs, interacted with an unidentified ligand that was expressed by Ts, which rendered the DCs tolerogenic. Ts cells do not act through the direct inhibition of effector T cells.

Zhang and colleagues [41,42] demonstrated that $CD3^+$, $CD4^-CD8^-$ double negative (DN) T cells can suppress allogeneic immune responses *in vitro* and *in vivo* by killing activated syngeneic $CD8^+$ T cells. Furthermore, this research identified that the inhibition of Ly-6A (Sca-1), which has high levels of expression on DN T cells, led to a significant reduction in DN T cell-mediated killing. Although DN T cells were found to inhibit the graft-specific T cell response following transplantation, the function of these cells in maintaining peripheral tolerance in a normal environment remains to be elucidated.

Thymic education of regulatory T cells

Positive and negative selection were thought to be the main events taking place in the thymus. However, several recent reports have indicated a third process in the thymus – Treg cell generation [43–46]. In a transgenic mouse model, self-specific $CD4^+CD25^+$ Treg cells already carrying a suppressor phenotype were shown to emerge from the thymus [47]. Mice that are transgenic for the influenza antigen hemaglutinin (HA) and for a specific T cell receptor directed against HA have a population of HA-specific Th cells, of which ~50% display a suppressor phenotype. In a follow-up study, Jordan and colleagues [45] showed that T cells expressing a high-affinity TCR for a self-peptide had matured in the thymus to become $CD4^+CD25^+$ Treg cells. Interestingly, T cells expressing a low affinity TCR for the same peptide did not undergo selection into the suppressor pathway, which indicated that the specificity of TCR for self-peptides is involved in the selection of $CD4^+CD25^+$ regulatory thymocytes by a process that is different from positive selection and deletion (Figure 1). This finding, which has been confirmed by other reports [43,44], strongly suggests that Treg cells can recognize self-antigen and emphasizes their importance in the prevention of autoimmune damage inflicted by auto-reactive T cells.

Function of $CD4^+CD25^+$ regulatory T cells

The mechanism(s) of action of intrinsic $CD4^+CD25^+$ Treg cells is still unknown. *In vitro*, activated Treg cells strongly inhibit proliferative responses of $CD4^+$ or $CD8^+$ T cells via a mechanism that is independent of TGF-β or IL-10 production [21,22]. Furthermore, blocking expression of CTLA-4 or CD25 does not inhibit the suppressive activity of these Treg cells [48,49]. Several studies have suggested that suppression occurs via a cell–cell contact-dependent mechanism that is independent of the production of a soluble factor (e.g. cytokines) [19,20,22,23]. In addition, Treg cells

downregulate co-stimulatory molecules (e.g. CD80 and CD86) on DCs [50], the mechanism of which has yet to be elucidated. *In vivo*, intrinsic Treg cells might interact with several steps of the T cell activation pathway. DCs presenting antigens to Treg cells, as well as effector T cells, probably create the platform for this regulation. On initiation by their TCR, Treg cells can prevent maturation of the DCs, as well as activation of Th cells. Suppression of Th activation results in inadequate DC activation and cytotoxic T lymphocyte (CTL) priming (Figure 2). Therefore, the induction of effective immune responses in the presence of Treg cells requires a balance between the inhibitory and stimulatory signals relayed by Treg and Th cells. In addition, the activation state of the APC determines the subsequent tolerization or activation of the effector T cell responses. Treg cells were recently found to display surface expression of the GITR family-related gene (also known as TNFRSF18) [51,52]. Triggering of GITR prevented Treg cell activation and subsequent expression of the suppressive phenotype. GITR–ligand expression has been described for DC, macrophages and B cells [53]; GITR–ligand expression could be induced on DCs that have experienced a danger signal in their original micro-environment. DCs that express GITR–ligand might inactivate Treg cells and, as a result, induce effective immune responses. In the absence of sufficient inflammation or danger signals, DCs might not express GITR–ligand, which would enable Treg cells to be actively involved in maintaining peripheral tolerance.

CTLA-4 and regulatory T cells

It is widely accepted that CTLA-4 signaling results in inhibition of effector T cell responses [3,54,55]. Recent research has shown that CTLA-4 signaling and intrinsic Treg cells represent two alternative pathways for suppressing autoreactive T cell immunity [56]. The blocking of the two regulatory pathways had a synergistic effect on the enhancement of auto-reactive CTL activation and tumor rejection. One T cell surface marker that appears to have an altered function in Treg cells, compared with effector T cells, is CTLA-4. Human and murine $CD25^+$ Treg cells intracellularly express CTLA-4 and expression is elevated after activation and culture [48,57,58]. The role of CTLA-4 in Treg cells remains an enigma. *In vivo* studies [57,59,60] have suggested an essential role for CTLA-4 on Treg cells at some stage of the Treg-mediated suppression process. However, these conclusions were based on models in which Treg and effector T cells were present simultaneously during CTLA-4 inhibition. Therefore, these experiments cannot distinguish between suppressor T cell and effector T cell. Indeed, using a mouse model to study the role of Treg cells in allograft acceptance, Sanchez-Fueyo and colleagues [61] showed that CTLA-4 blocking antibodies acted on the naïve rather than the Treg cell population.

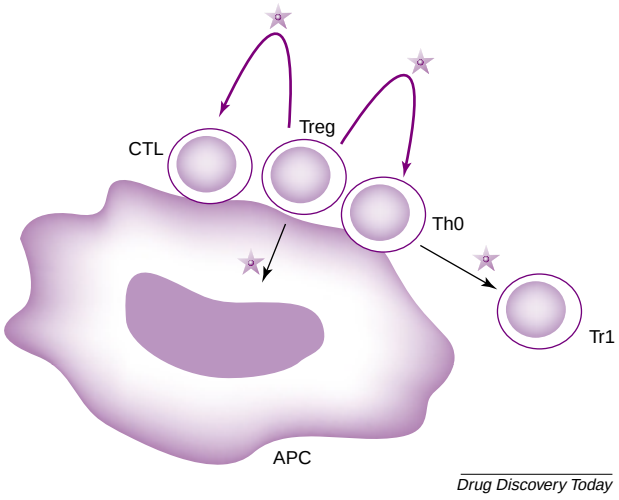

Figure 2. Interaction of regulatory T (Treg) cells with the immune system. Treg cells that recognize antigen presented by APC suppress other lymphocytes, for example, CTL and Th cells, through a mechanism that requires direct cell–cell contact. Several subtypes of Treg cells have been shown to inhibit the expression of co-stimulatory molecules presented by APC. Naïve Th0 cells, activated in the presence of intrinsic Treg cells, can differentiate into anergic Treg cells that suppress via secretion of IL-10 or TGF-β (infectious tolerance). Possible targets for drug development are indicated by a star. Abbreviations: APC, antigen-presenting cell; CTL, cytotoxic T lymphocyte; IL, interleukin; TGF, transforming growth factor; Th, helper T.

In an *in vitro* study, Nakamura *et al.* [29] showed that co-stimulation with CTLA-4 enhanced Treg cell activation. These results suggest that the effect of CTLA-4-signaling on Treg cells is the opposite of that on effector T cells. Other reports show that blocking CTLA-4 does not result in removal of suppression as mediated by Treg cells [48,49,62], which indicates that CTLA-4 does not mediate suppression, but is involved in the control of the activation process of Treg cells.

Infectious tolerance

Suppression mediated by previously activated human $CD25^+$ Treg cells is fixation resistant and independent of membrane-bound TGF-β [63]. However, suppression does require $CD25^+$ Treg cell activation and subsequent protein synthesis before fixation. Importantly, the incubation of intrinsic Treg cells with Treg cell-depleted $CD4^+$ T cells resulted in anergized $CD4^+$ T cells that in turn inhibited the activation of conventional, freshly isolated $CD4^+$ Th cells (Figure 2). The infectious suppressive activity expressed by the anergized $CD4^+$ T cells was cell–cell contact-independent and partially mediated by soluble TGF-β. The induction

of suppressive properties in conventional $CD4^+$ Th cells represents a mechanism that underlies the phenomenon of infectious tolerance, which might explain the conflicting data on the role of TGF-β in $CD25^+$ Treg cell-mediated immunosuppression [64].

Clinical applicability

The absence of intrinsic Treg cells results in autoimmune disorders such as thyroiditis, gastritis, insulin-dependent diabetes mellitus and colitis [16,17,23,30]. Furthermore, several studies have indicated an active role for Treg cells during allograft acceptance and/or tolerance [61,65,66]. In addition, the prevention of co-stimulatory interactions (e.g. CD28–B7 and CD40–CD154) can induce tolerance in which the tolerized allo-specific $CD4^+$ T cells acquire regulatory activities [67]. Therefore, boosting intrinsic Treg cell activity or stimulating Treg cell induction offers a promising option for improving the treatments available for autoimmune-related illnesses and allograft acceptance in transplantation.

This approach could be highly applicable to the field of immunotherapy of cancer. Limited Treg cell depletion for shorter time periods induced the rejection of immunogenic tumors without giving rise to life-threatening autoimmune disease [68,69]. It is possible that the lack of suppressive regulation by the Treg cells was sufficient for the immune system to mount an effective anti-tumor response. In this case, new $CD25^+$ regulatory thymic emigrants that prevented the initiation of autoimmune disease were developed. Recent research has shown that CD25 depletion and blocking of CTLA-4 act synergistically to promote immunity to a melanoma vaccine [56]. This result illustrates that the temporal elimination of a Treg cell subset strongly enhances the vaccination efficiency (with regard to anti-tumor immune responses) and encourages the further investigation of Treg cell depletion combined with vaccination strategies in humans. In addition, targeting (induced) Treg cell effector mechanisms, for example, IL-10 and/or TGF-β production, affords further opportunities for blocking suppressive T cells, thus favoring effector T cell responses.

However, there are issues that remain to be resolved before we can fully understand the activity of intrinsic Treg cells. Antigens that are specifically recognized by Treg cells, the mechanism of suppression and the *in vivo* target cells of Treg cells have yet to be identified. The molecular identification of the maturation and homeostasis pathways and function of intrinsic and induced Treg cells should provide pharmacists with targets for the development of chemical compounds that inhibit (for cancer treatment) or activate (for auto-immunity or transplantation) these functions. For example, recently, a new interesting target for immunotherapy was identified. *Foxp3* encodes a transcription factor that is genetically defective in an autoimmune syndrome in humans and mice. *Foxp3* is specifically expressed in intrinsic $CD4^+$ Treg cells [70] and retroviral gene transfer of *Foxp3* converts naïve T cells to a Treg cell phenotype that is similar to that of intrinsic $CD4^+$ Treg cells. Compounds that can activate or inhibit the function of Foxp3 will have enormous therapeutic potential. The development of antibody-based tools (e.g. targeting CD25, CTLA-4 or GITR) that are capable of modulating intrinsic or induced Treg cell activity *in vivo* promises to provide additional therapeutic opportunities.

Immunomodulating drugs

Drugs that act as immunosuppressors are used in the treatment of a wide spectrum of diseases, including allergic disorders, auto-immune disease and immune responses against allo-antigen (graft rejection and graft versus host disease). Immunosuppressive agents, for example, rapamycin, tacrolimus, cyclosporin A and glucocorticoids (dexamethasone) are targeted at suppression of immune responses to decrease disease manifestation. Because these drugs have different modes of action they exhibit distinct suppressive profiles [71]. Interestingly, dexamethasone treatment of DC redirects the DC maturation process from a stimulating phenotype towards the production of the anti-inflammatory IL-10 by APC [72]. The efficacy of Dexamethasone-treated DCs for suppression of unwanted T cell responses is currently being investigated *in vivo*.

By contrast, the use of immunostimulatory agents in the treatment of infectious diseases and cancer is still under intensive investigation. A major problem with the use of many immunostimulatory agents (e.g. IL-2) is that they are poorly tolerated and their use can lead to severe side effects including fever, autoimmune disease fluid retention and even shock. Another example of a class of immunostimulatory agent is compounds that are ligands for the Toll-like receptor (TLR) family, a class of receptors that recognizes pathogen-associated molecular patterns or endogenous inflammatory-associated molecules [73]. To date, ten receptors that recognize different or overlapping ligands have been identified. For example, TLR4 recognizes lipopolysaccharide (LPS) from Gram-negative bacteria and TLR9 recognizes bacterial DNA (unmethylated CpG motifs). TLRs are expressed on macrophages and DCs. Recent research indicates that TLRs are expressed by Treg cells [74]. Furthermore, this research showed that selective expression of TLR4 signals the activation of Treg cells, thus resulting in a modulation of the immune response. However, this study was performed using non-purified LPS. Furthermore,

to date, there have been no findings reported that support these results and, therefore, speculation on the role of TLRs in Treg cells remains. Taken together, it appears that differences in TLR distribution and TLR–induced signaling pathways mediate the activation of a tailor-made defensive response. The identification of TLR ligands has created many new opportunities for fine-tuning the immune response.

Many drugs act through inhibition of a specific pathway. In the future, we envisage that immunodrugs could encompass the antigen involved in the immune disease process in combination with, or even directly coupled to, an immunomodulating agent. Alternatively, the antigen combined with an immunomodulating cocktail of drugs and/or antibodies could be incorporated into vesicles and injected locally at the site of disease. This would circumvent the need for systemic immunosuppression or immunostimulation, thereby reducing unwanted and poorly tolerated side effects. To direct the immune defenses to cure the disease, the mode of action of future immunodrugs will be based on activation or inhibition of a particular cell type (e.g. intrinsic Treg cells). Furthermore, an immunodrug cocktail that simultaneously addresses several components of our immune defenses (e.g. APC, Treg and conventional T cells) should produce sufficient therapeutic effects. Immunodrugs will be relatively easy to produce (in the case of small peptides used in combination with an immunomodulating drug) and to administer. Considering these proposals, we believe that immunodrugs will become the future of the treatment of immune-related illnesses. However, directing the activity of Treg cells will prove to be a crucial factor in the success of immunodrugs.

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