Pharmacological Research 129 (2018) 237–250

Contents lists available at ScienceDirect

Pharmacological Research

journal homepage: www.elsevier.com/locate/yphrs

Review

# Molecular adjuvants that modulate regulatory T cell function in vaccination: A critical appraisal

Alexander Batista-Duharte*, Damiana Téllez-Martínez, Deivys Leandro Portuondo Fuentes, Iracilda Zeppone Carlos

*São Paulo State University (UNESP), School of Pharmaceutical Sciences, Department of Clinical Analysis, Araraquara, SP, Brazil*

ARTICLE INFO

*Article history:*
Received 1 September 2017
Received in revised form
20 November 2017
Accepted 21 November 2017
Available online 22 November 2017

*Keywords:*
Regulatory T cells
Adjuvants
Vaccine
$CD25^+FoxP3^+$
Immune regulation
Autoimmunity

ABSTRACT

Adjuvants are substances used to enhance the efficacy of vaccines. They influence the magnitude and alter the quality of the adaptive immune response to vaccine antigens by amplifying or modulating different signals involved in the innate immune response. The majority of known adjuvants have been empirically identified. The limited immunogenicity of new vaccine antigens and the need for safer vaccines have increased the importance of identifying single, well-defined adjuvants with known cellular and molecular mechanisms for rational vaccine design. Depletion or functional inhibition of $CD4^+CD25^+FoxP3^+$ regulatory T cells (Tregs) by molecular adjuvants has become an emergent approach in this field. Different successful results have been obtained for specific vaccines, but there are still unresolved issues such as the risk of autoimmune disease induction, the involvement of cells other than Tregs and optimization for different conditions. This work provides a comprehensive analysis of current approaches to inhibit Tregs with molecular adjuvants for vaccine improvement, highlights the progress being made, and describes ongoing challenges.



**Contents**



*Abbreviations:* ADCC, antibody dependent cell cytotoxicity; CD, cluster of differentiation; mAb, monoclonal antibody; CsA, cyclosporine; CTLA-4, T-lymphocyte antigen 4; GITR, glucocorticoid-induced tumor necrosis factor receptor (TNFR) family related gene; CCR4, C-C chemokine receptor type 4; CY, cyclophosphamide; CTL, cytotoxic T cells; DC, dendritic cells; DEREG, depletion of regulatory T cells; Fc(IgG), constant region of immunoglobulin G; FOXP3, forkhead box P3, (Tregs transcription factor); FR-4, folate receptor 4; GM-CSF, granulocyte-macrophage colony-stimulating factor; GVHD, graft-versus-host disease; IFNγ, interferon gamma; IL-, interleukin; IPEX, immune dysregulation, polyendocrinopathy, enteropathy X-linked syndrome; IDO, indoleamine 2,3-dioxygenase; LAG-3, lymphocyte activation gene-3; LLO, listeriolysin O; MDSCs, myeloid-derived suppressor cells Gr1 + CD11b+; Mab, monoclonal antibody; NAD+, nicotinamide adenine dinucleotide; NICD, NAD-induced cell death; NK, natural killer cell; NKT, natural killer T cell; NO, nitric oxide; Nrp-1, neuropilin-1; OVA, ovalbumin; PD-1, programmed cell death protein 1; RORγT, the Th17 transcription factor; RENCA, renal cancer cells; Tbet, the Th1 transcription factor; Teffs, effector T cells; TGFβ, transforming growth factor beta; Th, T helper cells; TLR, toll-like receptor; Tregs, $CD4^+CD25^+FoxP3^+$ regulatory T cells; nTregs, natural Tregs cells; iTregs, induced Tregs; Ts1, Suppressor T cells 1; TSDR, T regulatory cell–specific demethylation region.

* Corresponding author at: São Paulo State University – UNESP, School of Pharmaceutical Sciences, Department of Clinical Analysis, Rodovia Araraquara-Jaú km 1, Postal Code:14800-903, Araraquara, SP, Brazil.
*E-mail address:* batistaduhartea@gmail.com (A. Batista-Duharte).

https://doi.org/10.1016/j.phrs.2017.11.026
1043-6618/

## 1. Introduction

A vaccine is a biological preparation that induces acquired immunity to a particular disease, providing protection (prophylactic vaccine) or aiding treatment (therapeutic vaccine). Vaccines typically have a basic composition that includes two principal components: one or more antigens derived from a causal agent, responsible for the specificity, and an adjuvant to enhance the immunogenicity and protective efficacy of the vaccine [1].

Over several decades, studies of vaccines have evolved towards the search for well-characterized and highly-purified antigens, such as recombinant proteins and peptides; over the last several years, this search has included the introduction of defined, potent and safer adjuvants for a new generation of vaccines [2]. Aluminum salts were the first substances used as vaccine adjuvants, and they were the only approved adjuvants in preventive human vaccines for decades. However, owing to their relatively weak capacity to induce cellular immunity and an emerging debate regarding possible links between aluminum and certain severe post-vaccination reactions [3,4], the search for alternative adjuvants has become an area of intense investigation. For many years, the search for new adjuvants has been active but empirical, and as a consequence, a growing number of new substances with documented adjuvant activity have been reported in the literature. However, owing to safety concerns, very few adjuvants, such as MF59 and AS03, have been licensed for use in human vaccines [2,5]. For this reason, there has been an increasing trend towards the rational design of vaccines using defined molecules with well-characterized cellular and molecular mechanisms of action. One of the current directions of this approach is the targeting of immune regulatory networks [6].

Regulatory T cells (Tregs) are a heterogeneous subpopulation of T cells that modulate the quality and magnitude of immune responses. These cells play critical roles in the maintenance of immunological tolerance to self-antigens, avoiding autoimmune diseases, infection-induced tissue damage, allergies, transplant rejection and other dangers associated with inflammation [7]. Although Tregs can control excessive inflammation associated with cancer and infection, excessive suppression can also affect immune responses against certain tumors and microorganisms. Thus, balanced Treg function is essential in order to maintain immune homeostasis [8].

Recent studies have revealed considerable heterogeneity and plasticity within Treg populations in different microenvironments [9,10]. Their central role in immune regulation has stimulated an emerging interest in the design and development of new bioactive molecules targeting Tregs for the pharmacological management of immune-mediated diseases [11,12]. Accordingly, numerous studies have focused on the role of Tregs in post-vaccination immune responses as a way to improve vaccine immunogenicity and effectiveness [13–15].

This review summarizes the main strategies that are currently being evaluated to improve vaccines by Treg targeting with defined molecules (molecular adjuvants). Advances and ongoing challenges that remain to be resolved are critically analyzed.

## 2. Origin, development and function of regulatory T cells

T lymphocytes play a central role in cell-mediated immunity. They are called T cells due to their origin and maturation in the thymus. T helper cells (Th cells) are a type of T lymphocyte that helps the activity of other immune cells by releasing cytokines. These cells help or regulate the function of other immune cells. They aid the antibody production by B lymphocytes, are essential in the activation and growth of cytotoxic T cells (CTLs), and promote the activity of innate immune cells, such as the phagocytosis of neutrophils and macrophages and the cytotoxicity of natural killer cells. Th1 cells develop when a specific cellular-mediated immune response is required for the control of infections and tumors. Their main effector cytokine is IFN-γ. Th2 cells are effectors against extracellular parasites, including helminths. Their effector cytokines are IL-4, IL-5, IL-9, IL-10 and IL-13. Th17 cells are defined by their production of IL-17. They play an important role in pathogen clearance at mucosal surfaces, are effective against pathogenic fungi and are also involved in different inflammatory process [7,8].

The existence of T cells with a suppressive capacity was first proposed in 1970 [16]. CD25 was the first phenotypic marker identified for suppressive T cells, found several years later by Sakaguchi in mice [17]. Shortly thereafter, a $CD4^{+}CD25^{high}$ T cell population with suppressive function was also identified in humans by several groups [18–22]. Simultaneously, mutations in the Foxp3 gene were identified as being responsible for an X-linked recessive inflammatory disease in Scurfy mice, which spontaneously develop severe autoimmune/inflammatory illnesses [23]. Furthermore, mutations in the human gene FOXP3, the ortholog of murine Foxp3, were found to be the cause of IPEX (immune dysregulation, polyendocrinopathy, enteropathy X-linked syndrome), a similar human autoimmune disease [24–26]. In 2003, several groups reported that FOXP3 is a key molecule essential for the development and function of Tregs; immediately thereafter, it became the main marker for Treg populations in mice [27–29] and humans [30,31].

The majority of human $CD4^{+}CD25^{high}$ Tregs also express FOXP3, but in contrast to mice, human $CD4^{+}CD25^{-}FOXP3^{-}$ T-cells and $CD8^{+}CD25^{-}FOXP3^{-}$ T-cells also show a transient $FOXP3^{+}CD25^{+}$ state during activation [32]. For this reason, the IL-7 receptor (CD127), which is downregulated during Treg maturation, is often used for their identification, and it is now accepted that

$CD4^+CD25^{high}CD127^{low/-}$ represents a more specific phenotype for human Treg identification [33].

There are two main subsets of Tregs: natural Tregs (nTregs), which are thymus-derived, and induced, adaptive or peripheral Tregs (pTregs), which are derived from naive $CD4^+$T cells under a variety of conditions [34]. Characteristically, nTregs have epigenetic demethylation of the T regulatory cell-specific demethylation region (TSDR), a promoter region of Foxp3. Owing to this selective demethylation of TSDR, Foxp3 cannot be switched off; these cells and their progeny therefore cannot revert to effector T cells (Teffs). Neuropilin-1 (Nrp-1), a protein originally characterized based on its functions in the central nervous system, and Helios, an Ikaros transcription family member, are two important molecules involved in the suppressive activity of Tregs. These molecules were originally reported to be constitutively expressed at high levels on nTregs, and they are frequently used to distinguish between nTregs and pTregs [35]. However, it was recently found that high expression of Nrp-1 is not a specific characteristic of many nTregs, and low expression of Helios and Nrp-1 does not exclusively indicate pTregs cells. Thus, the reliability of Nrp-1 and Helios as markers discriminating between nTregs and pTregs, is now controversial [36].

Following antigen presentation and in the presence of high concentrations of IL-2, nTregs express receptors for IFNγ and IL-12 (Th1 cytokines) and are named first-order T suppressor cells (Ts1). If inflammation persists, Ts1 cells are further expanded to Th1-like Tregs that express both FOXP3 and Tbet (the Th1 transcription factor) and produce IFNγ but not IL2 [37].

pTregs arise from peripheral $CD4^+CD25^-FOXP3^-$ T cells that are exposed to antigen in the presence of transforming growth factor-β (TGFβ) without IL-6 or IL-1β. In this microenvironment, FOXP3 expression is triggered, but its expression is not stable, and the expression of RORγT (the transcription factor of Th17 cells) is inhibited. However, they have a $CD4^+CD25^+$ phenotype and express other markers of nTregs, such as CTLA-4 and GITR [37].

Other T cells acquire regulatory functions following antigenic stimulation in specific cytokine milieus. These cells include IL-10-secreting Tr1 cells, IL-35-secreting Tr35 cells, TGFβ-secreting T helper 3 (Th3) cells, certain $CD4^-CD8^-$ T cells, $CD8^+CD28^-$T cells, NKT regulatory cells and T follicular regulatory cells [38,39]. The more important Teff cells that are suppressed by Tregs are Th1, Th2, Th17 and CTLs.

## 3. Mechanisms of immunosuppression by Tregs

Treg-mediated suppression can be accomplished in two ways: by cell–cell contact or by means of soluble mediators and cytokines (paracrine signaling). Several mechanisms have been identified as involved in the immunosuppressive function of Tregs, including (1) inhibition by immunoregulatory cytokines such as TGFβ, IL-10, and IL-35; (2) cytolysis of effector cells by producing granzyme and perforin; (3) metabolic interruption, including inhibition of proliferative response via the IL-2 receptor, cAMP-mediated metabolic inhibition, and immunomodulation mediated by the A2 adenosine receptor; and (4) interactions with dendritic cells that modulates their function and maturation [40]. A thorough description of these mechanisms is provided in Table 1.

## 4. Targeting Tregs in vaccination

Different studies have shown that depletion of Tregs prior to vaccination in murine models enhances the immune responses to certain vaccines, suggesting that Tregs can interfere with the generation of vaccine-induced immunity [41–45]. Over the last several years, two valuable models have been developed for the study of Tregs: transgenic DEREG mice (depletion of regulatory T cells), which express a fusion protein of the receptor for diphtheria toxin (DT) and enhanced green fluorescent protein (GFP) under the control of the Foxp3 locus [46], and B6.Foxp3hCD2 mice, a transgenic mouse model containing a sequence coding for a GPI-linked human CD2-CD52 fusion protein in the 3′UTR of the X-linked Foxp3 gene such that all Foxp3-expressing cells co-express the fusion protein on their cell surface. In B6.Foxp3hCD2 mice, Treg depletion is achieved using ablative anti–human CD2 antibodies [47]. These mice allow selective depletion of total $FOXP3^+$ Tregs without affecting $CD25^+$ Teffs.

Though the role of Tregs in controlling vaccine immunogenicity remains to be fully determined, different approaches are being evaluated for achieving effective modulation of Treg activity post-vaccination. Several immunological characteristics of Tregs, such as their high expression of different markers (e.g., CTLA-4; CD25; CCR4, FOXP3), their active proliferative capacity, and their apoptosis-prone tendency, can be exploited to specifically control their functions [48]. In this way, different molecules are being evaluated to reduce the immunosuppressive effects of Tregs in prophylactic or therapeutic vaccination against different pathological conditions, including cancer and infectious diseases. These molecules, with well-defined molecular targets, can serve as molecular adjuvants enhancing vaccine immunogenicity (Fig. 1). Other molecules, not discussed here, can elicit pharmacological Treg activation and may help control autoimmune diseases and allergies [13,14,49,50].

At least four distinct mechanisms have been described for molecular adjuvants targeting Tregs: 1) Depletion by direct killing of Tregs or reducing de novo generation via differentiation and expansion; 2) Inhibition of Treg function through immune checkpoint blockade; 3) Disrupting homing (migration) of Tregs to inflammatory sites; and 4) Exploiting T-cell plasticity to convert Tregs into IL-17–secreting T cells (Th17) [51] (Fig. 2). The following sections will be devoted to analyzing each of the compounds that have demonstrated significant effects as molecular adjuvants by targeting Tregs.

### 4.1. Metronomic chemotherapy

Chemotherapy, which mainly destroys proliferating cells, may skew Tregs towards becoming Teffs [52]. Low doses (metronomic doses) of cyclophosphamide (CY) and other cytostatic agents have been extensively used to inhibit Tregs for vaccine improvement due to their low cost and wide availability [53,54]. In 1974, Polak and Turk reported that CY reversed immune tolerance induced by two intravenous injections of dinitrobenzene sulfonic acid (DNBSO3) in a guinea pig–sensitization model through inhibition of a putative suppressor T-cell population [55]. Berd and collaborators subsequently demonstrated significant antitumor immune responses with regression of metastases after treatment with a melanoma cell vaccine preceded by CY treatment given in metronomic doses [56,57]. Lutsiak and colleagues evaluated the function of Tregs isolated from untreated and CY-treated mice two and ten days after 2 mg of intraperitoneal CY treatment. CY-treated Tregs decreased the expression of GITR and Foxp3 and exhibited an increased susceptibility to apoptosis with significant impairment in their suppressive capacity, which returned by day 10 after treatment [58]. More recently, Heylmann and collaborators demonstrated that Tregs are more sensitive than CTLs and other T cells to mafosfamide, an active derivative of CY, which does not require metabolic activation. The high sensitivity of Tregs to mafosfamide resulted not only in enhanced induction of apoptosis but also in impaired Treg function, as demonstrated by a decline in the suppressor activity of Tregs in a co-culture model with Teffs and $Helios^+$ Tregs [59]. However, the clinical benefit achieved by CY-mediated depletion of Tregs as an immunomodulatory approach appears to be variable

**Table 1**
Mechanisms of Treg cell-mediated suppression.

| Mechanisms | Description |
|---|---|
| Inhibition by immunoregulatory cytokines | |
| IL-10 | IL-10 inhibits the production of inflammatory cytokines, Th1 responses and Major Histocompatibility Complex Class I (MHC-I) expression in lymphocytes and dendritic cells, increasing the removal of cellular debris at the inflammation site by phagocytic cells. |
| TGFβ | TGFβ inhibits the production of IL-2 and promotes Treg production by inducing FOXP3 expression. It inhibits the activity of macrophages, dendritic cells, and NK cells. It induces the synthesis of indoleamine 2,3-dioxygenase (IDO), an enzyme that inhibits T cell proliferation by dendritic cells (DCs). |
| IL-35 | IL-35 suppresses T helper cell proliferation and favors the conversion of naive T cells into highly suppressive Treg cells. It induces the differentiation of B lymphocytes into B regulatory cells. |
| Inhibition by cytolysis of effector cells | |
| Perforin | Tregs release perforins that form transmembrane pores in T effector cells, through which granzymes enter the target cell. |
| Granzyme B | Granzymes can enter the effector T cells through a pore formed by perforin or by endocytosis to induce apoptosis. |
| Metabolic interruption | |
| High expression of CD25 | Inhibition of proliferative responses of effector T cells by competition for IL-2. |
| Adenosine | CD39 and CD73 are ectoenzymes highly expressed on the surface of Treg cells. CD39 is the cell surface-localized prototypic member of the ecto-nucleoside triphosphate diphosphohydrolase (E-NTPDase) family, and it degrades ATP into AMP. Extracellular AMP is rapidly degraded into adenosine by CD73, known as ecto-5′-nucleotidase. The adenosine resulting from AMP hydrolysis binds to adenosine receptors (A1, A2a, A2b, and A3) present on many cell types, and these regulate multiple physiological responses, including anti-inflammatory effects. |
| Inhibition of dendritic cell maturation and function | |
| CTLA-4 | CTLA-4, also known as CD152, is constitutively expressed in Tregs and induced in conventional T cells following activation. This molecule has a high affinity for dendritic cell-expressed CD80 and CD86; thus, the role of CTLA-4 is to regulate CD28-dependent T cell activation. CTLA-4-CD80/CD86 interaction induces catabolism of tryptophan by means of IDO. Tryptophan catabolism leads to the release of kynurenines, which are potent immune suppressors. |
| Lymphocyte-activation gene 3 (LAG3, CD223) | LAG3 is a cell-surface molecular homologue of the CD4 receptor and is expressed in Tregs. LAG3 binds to MHC-II with higher affinity than CD4. The LAG3-MHC-II union induces a signaling cascade in dendritic cells that decreases the function of antigen capture. |

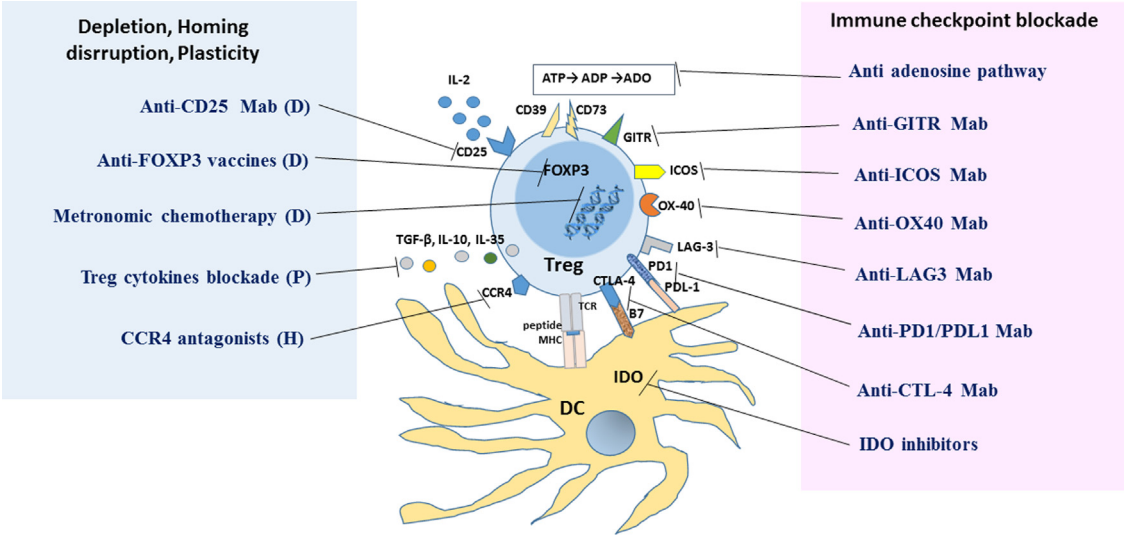


**Fig. 1.** Targets of molecular adjuvants used for Treg depletion/inhibition in vaccines.
Left panel: Representative molecular adjuvants that lead to Treg depletion (D), disruption of Treg homing (H) or Treg differentiation to Th17 cells (plasticity, P). Right panel: checkpoint receptor inhibitors leading to Treg functional inhibition.

[60]. Sevko et al. reported that the myeloid-derived suppressor cells $Gr1^{+}CD11b^{+}$ (MDSCs) are strongly induced in the tumor microenvironment when Tregs are depleted, affecting the desired therapeutic response of low-dose CY. Tregs depletion increases the production of several inflammatory mediators, such as GMCSF, IL-1β, IL-5, IL-10, IFNγ and TNFα, in the tumor microenvironment; all of these factors are involved in MDSC expansion and activation. MDSCs exhibited elevated immunosuppressive activity, including the inhibition of T-cell proliferation and nitric oxide (NO) production. In addition, MDSCs are able to stimulate Tregs, leading to proliferation, survival, and growth of the tumor cells [61].

Other immunosuppressant drugs, including calcineurin inhibitors, cyclosporine (CsA), and tacrolimus (FK506), might also reduce Treg numbers and function depending on the doses utilized. Kawai reported that high-dose CsA abrogates Treg generation, whereas a lower dose (10 mg/kg) of CsA promoted Treg development [62]. On the other hand, mycophenolate mofetil and high doses of rapamycin facilitated the generation of regulatory cells by intratracheal delivery of alloantigens in mice, while low doses of rapamycin and FK506 did not interfere with the generation of Tregs [63]. Paclitaxel is another drug that has been used to improve vaccine immunogenicity. Chen et al. tested metronomic

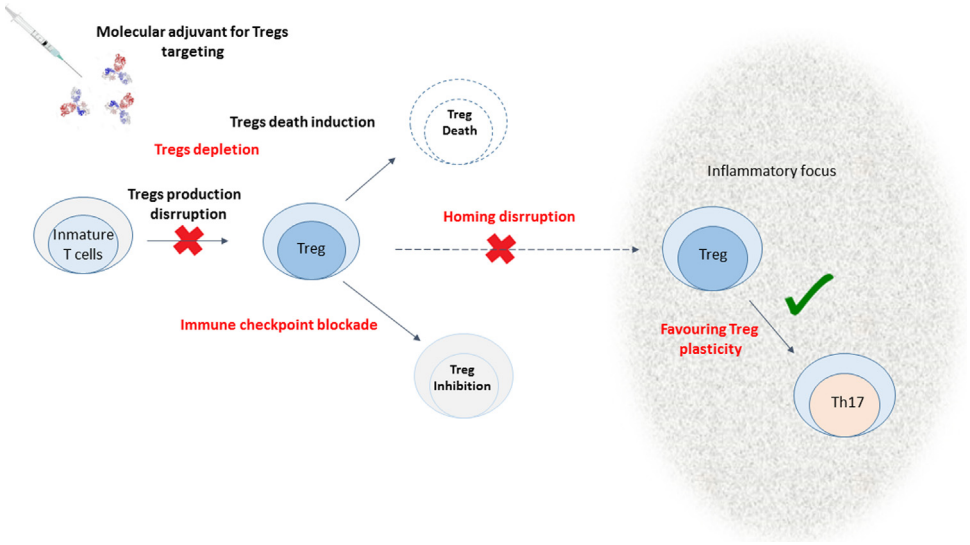


**Fig. 2.** Four defined mechanisms of Treg modulation with molecular adjuvants.
1) Treg depletion by direct killing or inhibition of de novo generation; 2) Inhibition of Treg function by blockade of immune checkpoint receptors; 3) Disrupting homing (migration) of Tregs to inflammatory sites; 4) Favoring Treg conversion into IL-17–secreting T cells (Th17).

low doses of paclitaxel in combination with a chimeric DNA vaccine, CTGF/E7 (connective tissue growth factor linked to the tumor antigen human papillomavirus 16 E7), to treat existing tumors in mice. Paclitaxel enhanced the vaccine's potential to delay tumor growth and decreased metastatic dissemination *in vivo* better than CTGF/E7 DNA vaccination alone. According to the authors, metronomic therapy improved the effects of the vaccine by two mechanisms: depletion of Tregs and inhibition of tumor angiogenesis, rather than direct cytolytic effects [64].

### 4.2. Monoclonal antibody targeting CD25

The human IL-2 receptor exists in three isoforms generated by different combinations of three different proteins; they are often referred to as alpha (IL-2Rα or CD25), beta (IL-2Rβ or CD122), and gamma (IL-2Rγ or CD132). Improved vaccine immunogenicity can be achieved by Treg depletion with systemic anti-CD25 monoclonal antibody administration [65]. A murine model was developed in which C57BL/6 mice were vaccinated with dendritic cells (DCs) pulsed with or without peptides from the syngeneic C1498 myelomonocytic leukemic cell line. In this model, depletion of $CD25^+$ T cells with anti-CD25 monoclonal antibody before DC-based vaccination allowed the generation of long-lived immune responses. Vaccinated mice that underwent Treg depletion were protected from subsequent re-challenge by C1498 cells, with an improved survival in comparison with mice that received vaccination alone [66]. Johnson et al. subsequently evaluated whether anti-CD25 treatment could favor effective antitumor immunity when administered immediately before cell-based vaccination. They observed that anti-CD25 antibodies induced murine neuroblastoma Neuro-2a rejection and elevated survival after challenge with a lethal dose of tumor cells, in association with improved vaccine antitumoral immunogenicity [67].

In another preclinical study, the attenuation of Treg subsets concomitant with BCG vaccination showed a positive but limited impact on the protective capacity of this vaccine against infection with *Mycobacterium tuberculosis* [68]. However, PC61 mAb (anti-murine CD25 rat IgG1) administered in conjunction with CY reduced tumor progression in early-stage but not late-stage mouse models of pancreatic carcinoma immunized with an antitumor vaccine preparation [69]. Similarly, the treatment of mice with established disease in a murine melanoma model using PC61 and DC/tumor fusion enhanced vaccine immunogenicity and significantly reduced the numbers of pulmonary metastases [70].

Daclizumab is a humanized anti-CD25 antibody built by total gene synthesis using oligonucleotides. This antibody is 90% human, retaining 10% of the original murine compartments in critical hypervariable segments for binding specificity. Daclizumab has been used in several human anticancer vaccine trials to enhance immune responses to tumor antigen vaccination. Studies with this product have shown long-lasting depletion of Tregs and enhanced CD8 and CD4 T cell immune responses without the development major autoimmune responses [71,72].

Denileukin diftitox (DAB389IL-2 or ONTAK®) is an immunotoxin that consists of an IL-2 fusion protein and the enzymatically active membrane-translocating domains of diphtheria toxin. After binding to CD25, Denileukin diftitox is internalized, and the released toxin is cytocidal for $CD25^+$ T cells [73]. This compound can deplete Tregs, albeit transiently, with Treg nadirs persisting for less than 3 weeks, although it can also deplete various T subsets, including tumor antigen-specific $CD8^+$ T cells [51]. Litzinger et al. evaluated the effects of denileukin diftitox in combination with a recombinant poxviral vaccine to enhance antigen-specific immune responses. Administration of the immunotoxin one day before vaccination enhanced antigen-specific T cell responses above levels induced by vaccination alone [71,74]. Another report revealed the effects of denileukin diftitox before vaccination with tumor RNA-transfected dendritic cells. A reduction in circulating $CD4^+CD25^+$ T cells with higher specific immune responses to vaccine in comparison with no denileukin diftitox treatment was observed [75].

In a pilot study, fifteen patients with carcinoembryonic antigen (CEA)-expressing malignancies received denileukin diftitox, given as a single dose or via repeated dosing, followed by immunization with DCs modified with the fowlpox vector rF-CEA(6D)-TRICOM. A T-cell response to CEA associated with Treg depletion was observed in the multiple-dose group but not in the single-dose group [76]. In contrast, Curiel et at. Tested the immune and clinical effects of Treg depletion using denileukin diftitox in phase 0/I and phase II epithelial ovarian carcinoma cancer trials. The treatment depleted Tregs without clinical benefit in advanced stages [77]. In another study, denileukin difitox was administered to patients with melanoma on a 5-day schedule. The study revealed a failure in Treg depletion, suggesting that a difference in dose or administration schedule may be important [78]. Recently, a randomized study failed to demon-

strate an improvement in clinical outcomes with a single dose of denileukin diftitox in vaccinated patients suffering advanced melanoma, suggesting that continued investigation with this compound should be discouraged [79].

### 4.3. Monoclonal antibody to CTLA-4

CTLA-4 (cytotoxic T-lymphocyte-associated protein 4), also known as CD152, is a member of the immunoglobulin superfamily constitutively expressed in Tregs and induced in Teffs after activation. CTLA-4 is homologous to the T-cell co-stimulatory protein CD28, and both molecules bind to CD80 (B7-1) and CD86 (B7-2) on antigen-presenting cells. CTLA-4 functions as an immune checkpoint that downregulates the immune responses [80]. The first anti-CTLA-4 antibody was developed by James P. Allisonís group to treat patients with late-stage melanoma, prostate and experimental mammary carcinoma. They demonstrated that *in vivo* antibody-mediated blockade of CTLA-4 enhances antitumor immune responses of experimental vaccines [81–84]. However, anti-CTLA-4 antibody treatment induced autoimmune responses when associated with antitumor vaccines [83–85].

Ipilimumab is a monoclonal antibody that inhibits Tregs by blocking CTLA-4, thus activating the immune system; the Food and Drug Administration (FDA) approved it in 2011 as an adjuvant therapy for the treatment of advanced melanoma [86]. However, ipilimumab treatment causes severe immunological adverse reactions associated with T cell overstimulation in 10–20% of patients, including stomach pain, bloating, constipation or diarrhea, fever, rash, colitis, hypophysitis, hepatitis, pancreatitis, iridocyclitis, lymphadenopathy, neuropathy and other neurologic disorders, and nephritis [87–89].

### 4.4. Anti-GITR

Glucocorticoid-induced TNFR-related protein (GITR), is a member of the tumor-necrosis-factor receptor (TNFR)-family; it is naturally expressed by Tregs but is also detected, at much smaller levels, on $CD4^+$ and $CD8^+$ effector T cells. The constitutive high level of expression of GITR in resting Treg cells unequivocally indicates the important role of this molecule in the modulation of the immune system. [90]. Agonistic antibodies to either GITR or GITR ligand cause both enhanced proliferation of Teffs and the simultaneous suppression of Treg activity [91] as well as protection or tumor regression in experimental models [92,93].

Agonist anti-GITR antibodies have been evaluated as potential tools for vaccine improvement, but autoimmune reactions were concomitantly observed [93–95]. However, dendritic cells transfected with RNA encoding the heavy and light chains of the anti-GITR mAb, to secrete anti-GITR antibodies, are effective adjuvants to DC-based tumor immunotherapy. Vaccination with anti-GITR-secreting DCs and tumor antigen-presenting DCs significantly enhanced antitumor immunity, comparable to levels seen with systemically delivered mAbs along with antigen-presenting DCs but without evidence of autoimmune effects [96,97].

### 4.5. Anti-OX40

OX40 (CD134) is another costimulatory molecule of the TNF receptor family. This immune checkpoint is constitutively expressed on Tregs and is transiently expressed on Teffs. OX40 signaling impairs the suppressive capacity of Tregs, presumably through direct inhibition of Foxp3 expression [98]. OX40 signaling also antagonizes TGFβ and the conversion $CD4^+CD25^-$ naive T Cells to inducible $CD4^+CD25^+$ Tregs [99]. Anti-OX40 antibody treatment has divergent effects on Treg cells (increased and decreased survival, inhibition of Foxp3 expression). Studies using anti-OX40 antibodies suggest that antibody-dependent cell cytotoxicity (ADCC) is an important mechanism for killing cells that express OX40 at their surface, including Tregs [100]. One preclinical study showed that an agonistic anti-OX40 monoclonal antibody was able to inhibit the suppressive activity of Tregs [98]. Another study showed a significant inhibition of tumor growth after intratumoral injection of this antibody, which exhibited a dual role by inhibiting Treg suppression while enhancing Teff functions [101]. A recent critical review analyzed different studies using anti-OX40 antibody in cancer immunotherapy [100].

OX40 ligands have shown adjuvant properties for improving anti-tumor and anti-infectious disease vaccines. OX40 ligand inclusion with BCG vaccination provided enhanced protection against aerosol and intravenous *M. tuberculosis* challenges when compared with BCG vaccination alone in a murine model [102]. More recently, a peptide vaccination strategy against melanoma was developed by combining synthetic peptides with toll-like receptor (TLR) agonists and OX40/CD40 co-stimulation. This vaccination strategy generated significant antitumor effects in a mouse model, and it was efficient in overcoming immune tolerance to a self-tumor-associated antigen [103]. Another study revealed that vaccination with a mixture of synthetic peptides of mouse cytomegalovirus and OX40 agonistic antibodies resulted in relevant antitumor responses mediated by $CD4^+$ and $CD8^+$ T cells. This response was higher when compared to those induced by the virus alone, and it was associated with a reduction in both viral titers and dissemination after viral challenge in mice [104].

### 4.6. Modulation of the programed death-1 pathway

Programmed Cell Death Protein 1, also known as PD-1 or CD279, is a member of the CD28 superfamily. It is expressed by Tregs and other immune cells and acts to downregulate immune responses. This immune checkpoint enhances tolerance by suppressing T cell inflammatory activity through interaction with its ligands PD-L1 and PD-L2, leading to Treg activation [105–107].

PD-1 pathway blockade is a successful strategy for vaccine potentiation. Pembrolizumab and Nivolumab are anti-PD-1 humanized antibodies approved by the FDA for use in cancer immunotherapy against several solid tumors, although several autoimmune reactions have been reported, as seen with other checkpoint inhibitors [108–110]. Despite the encouraging responses seen with the use of these antibodies, the majority of patients do not respond to PD-1/PD-L1 blockade, perhaps owing to the inability of tumors to stimulate a productive T cell response. For this reason, an effective vaccine that stimulates *de novo* high-avidity CD4 and CD8 responses prior to checkpoint blockade could be a valuable alternative strategy for non-responsive patients. Recently, Xue et al. demonstrated that vaccination using a DNA-based strategy to induce high-avidity antitumor specific T cell responses combined with PD-1 blockade resulted in an 85% survival rate in an aggressive B16 melanoma model [111]. In other studies, the effects of antitumor vaccination were evaluated in association with either dual blockade of PD-1/CTLA-4 [112] or a combined CD27 agonist and PD-1 blockade. Combined CTLA-4/PD-1 blockade improved vaccine efficacy, but this combination was much less potent than the CD27 agonist/PD-1 blockade combination [113].

### 4.7. Indoleamine 2,3-dioxygenase inhibitors

Indoleamine 2,3-dioxygenase (IDO) is an endogenous enzyme involved in immune regulation by suppressing T cell function. IDO catalyzes the degradation of tryptophan by the kynurenine pathway and is involved in the generation and activation of Tregs [114,115]. IDO has been associated with reduced immunogenicity following vaccination with MVA85A, a modified vaccinia virus

expressing antigen 85A from *M. tuberculosis* [116]. Blockade of Treg immune-inhibitory signals through inhibition of the IDO pathway is emerging as an important modality for cancer therapy, particularly for the development of antitumor vaccines. Several IDO inhibitors are being investigated in clinical trials with promising initial results, including INCB024360, indoximod, and NLG919 [117–119].

### 4.8. Anti-LAG-3

Lymphocyte Activation Gene-3 (LAG-3) or CD223 is a CD4-related molecule that binds MHC class II and is expressed by nTregs upon activation. LAG-3 plays a role in suppression mediated by nTregs and iTregs. In addition, the expression of LAG-3 on naive $CD4^+$ T cells significantly reduces their proliferative capacity and can confer regulatory functions independent of the FOXP3 pathway [120–122]. Tregs from LAG-$3^{-/-}$ mice exhibit reduced regulatory activity, while antibodies to LAG-3 abolish the suppressive capacity of Tregs [120].

In 1999, Prigent et al. transfected either human LAG-3 or mouse LAG-3 into three types of tumor cells (MCA 205, TS/A and RENCA) to evaluate their capacity to stimulate a tumor-specific immune response *in vivo*. LAG-3-transfected tumors either completely regressed or had their growth markedly reduced. In addition, mice were protected against re-challenge with parental tumor cells. The adjuvant effect of soluble LAG-3 was subsequently evaluated when co-administered with the MCA 205 cell line and the TS/A cell line. LAG-3 enhanced antitumor responses, and significant reductions in tumor growth were observed in both tumor models when compared with animals vaccinated with tumor cells alone [123]. Another study showed that vaccinated mice with particulate hepatitis B surface antigen or soluble ovalbumin (Ova) protein with soluble LAG-3, increased specific CTL responses, including a proliferative response of splenocytes with a greater Th1- but not Th2-type cytokine pattern. Moreover, vaccine formulation with LAG-3 induced higher titers of specific antibodies than in mice immunized without LAG-3 [124]. Other similar approaches using the soluble form of LAG-3 in different models have confirmed its utility as a new type of molecular adjuvant in vaccines [125,126].

### 4.9. CCR4 antagonists

C-C chemokine receptor type 4 (CD194) is a receptor for different chemokines, including both CCL22 and CCL17, chemotactic agents involved in Treg recruitment to inflammatory sites and tumors [127]. More than 85% of Tregs are $CCR4^+$, and CCR4 antagonists can be used as vaccine adjuvants to decrease local recruitment of Tregs at the site of immunization [128,129]. This new generation of molecular adjuvants enhanced specific immune responses against different pathogens and experimental murine tumors when injected in combination with vaccine antigens [128–130]. The half-life of CCR4 antagonists is just 24 h, causing a transitory functional inhibition of Tregs. Their relatively short duration of action reduces the potential for autoimmune reactions when compared with the longer half-life (approximately 2–3 weeks) of monoclonal antibodies that are used to deplete Tregs or to inhibit their functions [131,132]. However, recent clinical studies of Mogamulizumab, a humanized anti-CCR4 monoclonal antibody used for leukemia and lymphoma treatment, showed the development of graft-versus-host disease (GVHD) and other severe adverse drug reactions in some patients. These findings suggest that patients receiving anti-CCR4 therapy should be carefully monitored for adverse events [133,134].

### 4.10. Anti-FOXP3

The FOXP3 transcription factor, the specific marker of Tregs, is regarded as a self-antigen. As FOXP3 is more specific for Tregs than CD25 and other molecules, anti-FOXP3 vaccination does not interfere with vaccine-induced protective immunity. Thus, inducing immune responses against FOXP3 Tregs appears to be a potential approach to decrease the frequency of Tregs and to enhance specific immune responses. However, FOXP3 is not expressed on the cell surface; consequently, it cannot be reached with antibodies *in vivo*.

Vaccination of mice with Foxp3 mRNA-transfected dendritic cells is capable of stimulating specific CTL production and selective depletion of $Foxp3^+$ Tregs. This approach enhances vaccine-induced protective immunity similar to that of αCD25 antibody administration [135]. Mousavi et al. used a chimeric FOXP3-Fc(IgG) fusion construct/protein to effectively stimulate immune responses against Tregs. Their study revealed the efficiency of a Foxp3 DNA-prime protein-boost strategy to decrease Tregs with significant superiority when compared to the Fc(IgG) fusion strategy. Treg depletion was also associated with higher FOXP3-specific CTL and Th1 responses in FOXP3-Fc vaccinated mice [136]. Gene silencing was used to block the expression of either Foxp3 or CTLA-4. This strategy improved the antitumor efficacy of a GM-CSF genetically modified tumor cell vaccine against B16 melanoma. Interestingly, the characteristic adverse effects of treatment with anti-CTLA-4 antibodies, i.e., depigmentation, diarrhea, or weight loss, were not observed [137]. However, a vaccine designed to eliminate polyclonal Treg cells in a permanent way could theoretically cause severe medium- or long-term immunological disturbances. Thus, further studies are necessary for definitive conclusions regarding the safety of these molecules as vaccine adjuvants.

Finally, a 15-mer synthetic peptide (P60) with the capacity to inhibit FOXP3 function was identified using phage-displayed peptide libraries. P60 enters the cells and reduces the ability of FOXP3 to suppress the transcription factors NF-kB and NFAT. *In vitro*, P60 inhibited murine and human-derived Tregs and elicited Teff stimulation. When evaluated *in vivo*, P60 improved the antiviral efficacy of a recombinant adenovirus vaccine, expressing the NS3 protein from hepatitis C virus. Moreover, protection was induced against CT26 tumor cell implantation in mice immunized with the cytotoxic T cell epitope AH1 derived from these cells. P60 did not cause toxic effects in adult mice, although in newborn mice, it induced a lymphoproliferative autoimmune syndrome similar to that observed in Scurfy mice [138]. Another peptide (FOXP3 393–403) generated by the same group also exhibited similar properties as P60 and showed antitumor efficacy in mice bearing Hepa129 or TC1 tumor cells when combined with sorafenib (a kinase inhibitor anticancer drug) or with an antitumor vaccine, respectively [139]. More recently, P60 was conjugated with a CD28-targeting aptamer to deliver the peptide to CD28-expressing cells. The efficacy and biodistribution of P60 was improved with this approach [140].

These findings suggest that targeting FOXP3 with inhibitory peptides can provide a specific and simple protocol to enhance vaccination efficacy with reduced risk of autoimmunity, but the clinical applicability of these strategies still needs to be demonstrated.

### 4.11. Blockade of TGFβ

P17 is a short synthetic peptide that inhibits TGFβ1 and TGFβ2 and was created by the same group that developed the FOXP3 peptide inhibitors. This peptide inhibited the TGFβ1-dependent expression of collagen type I mRNA in the livers of mice orally challenged with carbon tetrachloride and blocked the inhibitory activity of TGFβ1 on the growth of Mv-1-Lu cells *in vitro* [141].

Moreover, P17 inhibited Treg cells *in vitro* and improved the efficacy of vaccination *in vivo*. P17 inhibited tumor growth, reduced the number of $CD4^+FOXP3^+$ T cells and augmented the proliferation of $CD4^+$ T cells purified from OT-II transgenic mice adoptively transferred into C57BL/6 mice bearing EG.7-OVA tumors. Moreover, P17 blocked the induction of Ag-specific oral tolerance induced *in vivo* after intragastric supplementation of the OVA-secreting *Lactococcus lactis* (LL-OVA) in DO11.10 mice [142]. Another strategy for blockade of TGFβ is antibody-mediated inhibition of TGFβ, which enhanced tumor vaccine efficacy and protection mediated by CD8 + T cells, although the adjuvant effects appear to be independent of Treg targeting [143].

### 4.12. Listeriolysin O

In 2007, Nitcheu-Tefit et al. provided the first evidence that listeriolysin O (LLO) affects Treg function [144]. A remarkable level of protection against ovalbumin (OVA)-expressing tumor cells was observed in mice vaccinated with *Escherichia coli* expressing LLO and OVA (*E. coli* LLO/OVA) in comparison with mice vaccinated with *E. coli* expressing OVA alone (*E. coli*/OVA). In addition, Tregs from *E. coli* LLO/OVA-vaccinated animals were unable to suppress Teff proliferation. Subsequent studies showed that immunization with an *E. coli*-LLO vaccine carrying a cancer-relevant antigen (Wilms tumor antigen-1) inhibited Treg expansion and enhanced antitumor responses [145,146]. The underlying mechanism related to the Treg inhibition by LLO remains to be elucidated.

### 4.13. Blockade of adenosine-mediated immune suppression

Extracellular ectonucleotidases CD39 and CD73 of Tregs produce adenosine (ADO) via catabolism of adenine nucleotides (ATP, ADP and AMP) [147]. ADO is a major mediator involved in Treg function due to its direct immunosuppressive effects via reduction of T-cell and natural killer (NK)-cell responses. ADO also contributes to reduced transendothelial migration of Teffs into tumors [148,149]. Inhibition of CD39 with enzymatic inhibitors and adenosine receptor antagonists blocks Treg function. Different molecular structures, some of which have already been used in clinical studies for diverse indications, are being evaluated to block adenosine-mediated immune suppression as a therapeutic alternative for different diseases and as a vaccine adjuvant [148,150]. However, even though CD39, CD73, adenosine, and adenosine receptors act in synergy, they are not equal, and each represents a unique target with a singular profile. Thus, a thorough investigation of the expression and the role of each molecule is required for future clinical use in vaccines [149,150].

### 4.14. Anti-angiogenic molecules

Tregs make significant direct contributions to tumor angiogenesis [151]. Various studies have shown that angiogenesis inhibitors also inhibit various Treg-immunosuppressive networks [152]. Sunitinib, an inhibitor of tyrosine kinases involved in angiogenesis, causes depletion of Tregs, especially after the second cycle of therapy. Other tyrosine kinase inhibitors, such as imatinib mesylate, dasatinib, and temozolomide, can also deplete Tregs in association with an inhibitory effect on their function. Potential uses of these molecules as immunological adjuvants requires further study [52].

### 4.15. Anti-folate receptor 4 antibodies

Anti-Folate Receptor 4 (FR4) was identified by Spiegelstein et al. in mice. This molecule is homologous to human FR-δ [153]. FR4 is highly expressed in nTregs, and it plays an important role in the maintenance of Treg phenotypes [154,155]. Several studies in murine tumor models have demonstrated the beneficial effects of modulating Treg function by anti-FR4 antibodies. Partial rejection of MethA and MCA fibrosarcomas, CT26 colon carcinomas, and RENCA renal carcinoma were observed after direct anti-FR4 antibody administration [154,156]. Moreover, an enhanced antitumor response associated with modest protection against autologous B16m5T4 melanoma challenge was observed when anti-FR4 antibodies were administered after immunization with 5T4 oncofetal glycoprotein [157]. As anti-FR4 antibody treatment as a monotherapy can result in modest rejection of tumors, Liang et al., depleted Tregs as part of a combination therapy using an anti-FR4 antibody and GM-CSF-secreting tumor cell immunotherapy (GVAX) for treatment of melanoma-bearing animals. With this strategy, they showed that these two agents could function cooperatively to enhance the median survival time in this tumor model. In addition, triple combination of a blocking CTLA-4 antibody with GVAX and anti-FR4 treatment further enhanced overall survival and reduced growth of well-established melanomas [158].

### 4.16. Nicotinamide adenine dinucleotide

Functional levels of nicotinamide adenine dinucleotide ($NAD^+$) are released during inflammation. This mediator participates in the regulation of T cell homeostasis, causing T cell death, principally of Tregs, a process that is known as NAD-induced cell death (NICD) [159,160]. Endogenous $NAD^+$ influences survival, phenotype, and function of Tregs by an ART2/P2 × 7-dependent process [161]. Systemic administration of $NAD^+$ reduced Treg numbers and provoked significant anti-tumor immunity [161]. Recently, it was demonstrated that in addition to depletion of murine $CD4^+CD25^{high}FOXP3^+$ Tregs, $NAD^+$ favors the conversion of Tregs into IL-17A-producing cells and promotes allograft survival by Treg-independent systemic IL-10 production [162].

### 4.17. Toll-like receptor (TLR) modulation

TLR agonists have been widely used as adjuvants in co-administration with different types of antigens. In addition to their relevant role in activating the innate immune response, studies of the basic biology of TLR agonists suggest a role in modifying Treg functions. [163]. Exposure of Tregs to poli-G10, a TLR8 ligand, elicited improved antitumor immunity by abolishing their suppressive action on $CD8^+$ T cells [164]. Another report showed that the synthetic bacterial lipoprotein BLP, a TLR1/TLR2 agonist, led to dose-dependent tumor regression and a long-lasting protective response against tumor challenge. This effect was associated with a reduced suppressive function of Tregs and enhanced cytotoxicity of tumor-specific CTLs *in vitro* and *in vivo* [165]. A recent study demonstrated the effects of the high mobility group box-1 protein 1 (HMGB1) and lipopolysaccharide (LPS) on TLR4 signaling in Tregs. Tregs treated with HMGB1 directly increased the expression of the pro-inflammatory cytokine IFNγ and inhibited its suppressive function, whereas LPS treatment upregulated the expression of IL-1β and IL-10 and promoted their suppressive capacity. In both cases, the activation of the NF-κB pathway was involved [166]. These findings suggest that immunomodulation through TLRs can induce either activation or reduction of Treg responses. Further studies are necessary to define the critical factors leading to each of these effects.

### 4.18. Anti-co-stimulatory receptor (ICOS) antibody

Inducible co-stimulator (ICOS) or CD278 is a member of the CD28/Cytotoxic T-lymphocyte Antigen-t4 (CTLA-4) family broadly and is expressed in activated CD4 + T cells and iTregs. ICOS is implicated in important Tregs functions, as $ICOS^+$ Tregs exhibit

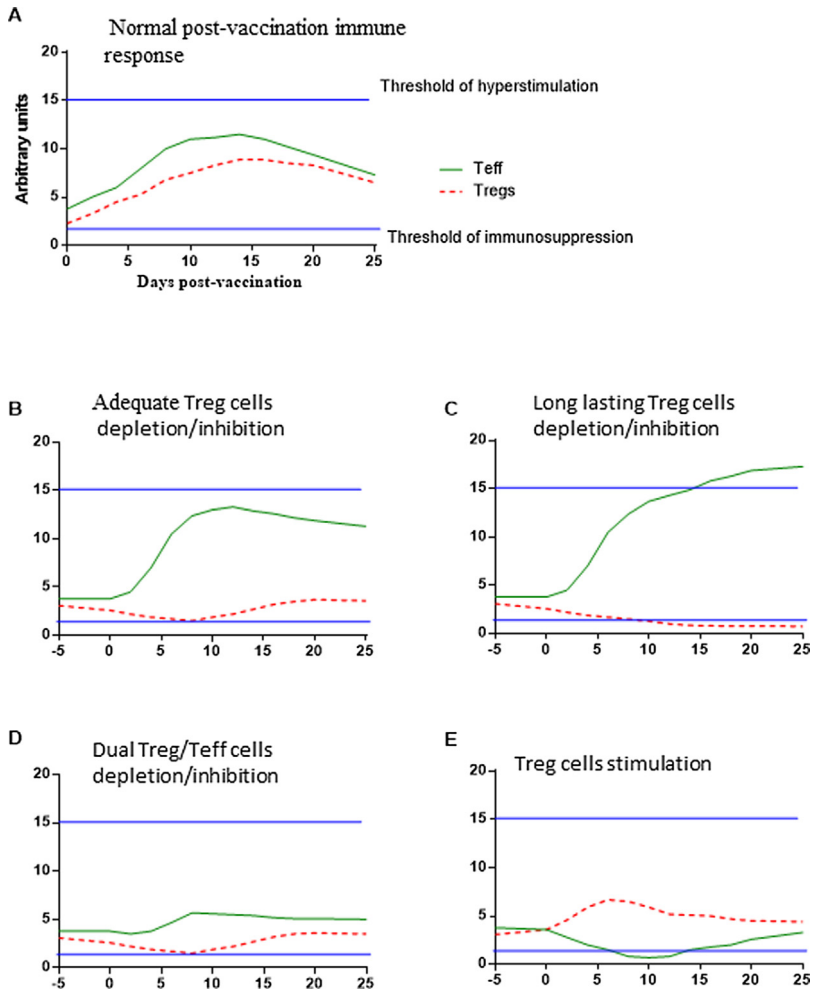


**Fig. 3.** Possible effects of Treg modulation on immune responses.
A. Normal post-vaccination immune response without Treg modulation. B. Transitory depletion/inhibition of Tregs elicits a strong immune response. C) A longer depletion/inhibition of Tregs can cause excessive and uncontrolled immune responses with a risk of developing autoimmune processes. D. Nonspecific Treg depletion/inhibition can induce functional or physical reductions in Teff cells. F. Undesired Treg overstimulation can elicit a reduction in Teff function with a risk of immunosuppression. The values shown in the figure are for illustrative purposes only.

stronger suppressive activity and increased survival and proliferation than ICOS$^-$ Tregs [167,168]. Recently, Mo et al. evaluated the anti-tumor effects of a therapeutic cell vaccine against prostate cancer in combination with an anti-ICOS monoclonal antibody in a mouse model. They demonstrated that ICOS inhibition depleted the tumor-infiltrated ICOS$^+$ Tregs and resulted in improved antitumor immune responses compared with vaccination alone [169].

## 5. Risks versus benefits of Treg manipulation

Enhancement of adaptive immune responses by Treg modulation does not always correlate with improved disease outcomes and is often associated with undesired effects. Treg regulation involves highly complex mechanisms that are not yet well-defined [170]. Hence, all attempts of Treg modulation for disease treatment and prevention should be carefully evaluated. Fig. 3 depicts an overview of possible consequences of Treg manipulation. In the case of Treg targeting for vaccine improvement, the ideal response should be a transient Treg inhibition during the active phase of immunological induction to avoid overstimulation. A delay in Treg recovery by excessive or long-lasting depletion could disrupt immune tolerance, inducing inflammatory disorders and potential autoimmune processes [131]. This risk has been observed in mice as a consequence of Treg depletion, resulting in the appearance of localized autoimmune disease, such as skin depigmentation associated with tyrosinase-related protein 2 (180–188)-specific CTLs [83]. The incidence and spectrum of autoimmune diseases induced by Tregs depletion depends in part on the degree and duration of Treg depletion, the age of the host (the risk is greater in neonatal mice than in adult mice), the route of administration (systemic or local), and the genetic background of the host [171,172].

On the other hand, improper modulation inducing the opposite effect (Treg stimulation) can lead to the failure of vaccination and may cause unwanted effects due to immunosuppression. This potential effect can occur when the same receptors are used to inhibit or stimulate Tregs or with a nonspecific depletion by targeting common pathways, affecting both Tregs and Teffs. Certainly, Tregs and activated T cells share several features and receptors, which explains the weak selectivity of different immunomodulators for specific Treg inhibition [52,171]. New strategies are currently being designed to inhibit Treg function rather than their deletion using combined checkpoint inhibition to improve vaccine efficacy and safety [52,171,173].

## 6. Conclusions

Although Treg responses have rarely been evaluated during pharmacological studies of vaccine candidates, several studies in

different models have provided convincing evidence that these cells can constrain the induction of effector T-cell responses induced by vaccination. In fact, depletion or inhibition of Tregs by means of molecular adjuvants is becoming a new tool for vaccine improvement. Many reports, several of which are analyzed in this work, demonstrate that this approach can be potentially useful for different types of vaccines. However, the vast majority of studies on the inhibition of Tregs during vaccination have been performed with antitumor vaccines. Very few studies have been conducted with vaccines targeting infectious pathogens. Accordingly, more studies need to be conducted to evaluate the utility of Treg manipulation for anti-infectious disease approaches, and these approaches should be tailored on a *case by case* basis. One question that remains to be answered is the role of Tregs in the inducement of memory and/or effector immune responses, two important aspects for rational vaccine design [170].

Another important aspect to consider is the type of vaccine being designed (prophylactic or therapeutic). The current attitude regarding the risks and benefits of vaccination favors safety over efficacy when a prophylactic vaccine is given to a healthy population, especially in children. However, for therapeutic vaccines, including for patients with cancer and chronic infectious diseases, a certain level of toxicity may be acceptable if the benefit of the vaccine is substantial [174]. Treg modulation could potentially cause immunological disturbances with dire consequences that cannot be predicted in advance. Therefore, any approach that targets Tregs for vaccination purposes should be confined to a short period, thus guaranteeing the necessary regulatory function to avoid long-term deleterious effects, including autoimmune reactions [15,175]. In this way, differences in Tregs patterns between rodents and humans must be carefully analyzed to extrapolate experimental results from laboratory animals to humans (Fig. 3).

Ultimately, the success of the new generation of molecular adjuvants targeting Tregs will depend on their ability to achieve potent but controlled immune responses. Subsequent studies should be aimed at achieving inhibition by means of antigen-specific Treg modulation. Thus, a deep understanding of Treg biology as part of the pathogenic mechanisms of disease is critical to support the increasing demand for potent and safe vaccines in the future.

## Conflict of interest

The authors declare no conflict of interest.

## Acknowledgment

This work was supported by Fundacão de Amparo à Pesquisa do Estado de São Paulo (FAPESP, grant No 2016/04250-0).

## References

[1] V.E. Schijns, E.C. Lavelle, Trends in vaccine adjuvants, Expert Rev. Vaccines 10 (4) (2011) 539–550, http://dx.doi.org/10.1586/erv.11.21.
[2] N. Garçon, A. Di Pasquale, From discovery to licensure, the Adjuvant System story, Hum. Vaccines Immunother. 13 (1) (2017) 19–33, http://dx.doi.org/10.1080/21645515.2016.1225635.
[3] O. Pérez, A. Batista-Duharte, E. González, C. Zayas, J. Balboa, M. Cuello, O. Cabrera, M. Lastre, V.E. Schijns, Human prophylactic vaccine adjuvants and their determinant role in new vaccine formulations, Braz. J. Med. Biol. Res. 45 (8) (2012) 681–692, http://dx.doi.org/10.1590/S0100-879x2012007500067.
[4] A. Batista-Duharte, E.B. Lindblad, E. Oviedo-Orta, Progress in understanding adjuvant immunotoxicity mechanisms, Toxicol. Lett. 10 (2) (2011) 97–105, http://dx.doi.org/10.1016/j.toxlet.2011.03.001.
[5] S.R. Bonam, C.D. Partidos, S.K.M. Halmuthur, S. Muller, An overview of novel adjuvants designed for improving vaccine efficacy, Trends Pharmacol. Sci. (2017), http://dx.doi.org/10.1016/j.tips.2017.06.002, pii: S0165-6147(17)30120-7.
[6] M.N. Davies, H. Pere, I. Bosschem, F. Haesebrouck, B. Flahou, E. Tartour, D.R. Flower, D.F. Tough, J. Bayry, In silico adjuvant design and validation, Methods Mol. Biol. 1494 (2017) 107–125, http://dx.doi.org/10.1007/978-1-4939-6445-1_8.
[7] S. Sakaguchi, M. Miyara, C.M. Costantino, D.A. Hafler, FOXP3 + regulatory T cells in the human immune system, Nat. Rev. Immunol. 10 (7) (2010) 490–500, http://dx.doi.org/10.1038/nri2785.
[8] N. Rakebrandt, K. Littringer, N. Joller, Regulatory T cells: balancing protection versus pathology, Swiss Med. Wkly. 146 (2016) w14343, http://dx.doi.org/10.4414/smw.2016.14343.
[9] D.J. Campbell, M.A. Koch, Phenotypical and functional specialization of FOXP3+ regulatory T cells, Nat. Rev. Immunol. 11 (2) (2011) 119–130, http://dx.doi.org/10.1038/nri2916.
[10] S. Hori, Lineage stability and phenotypic plasticity of Foxp3(+) regulatory T cells, Immunol. Rev. 259 (1) (2014) 159–172, http://dx.doi.org/10.1111/imr.12175.
[11] B.D. Singer, L.S. King, F.R. D'Alessio, Regulatory T cells as immunotherapy, Front. Immunol. 5 (2014) 46, http://dx.doi.org/10.3389/fimmu.2014.00046.
[12] A. Schmidt, N. Oberle, P.H. Krammer, Molecular mechanisms of treg mediated T cell suppression, Front. Immunol. 3 (2012) 51, http://dx.doi.org/10.3389/fimmu.2012.00051.
[13] I.R. Cohen, F.J. Quintana, A. Mimran, Tregs in T cell vaccination: exploring the regulation of regulation, J. Clin. Invest. 114 (9) (2004) 1227–1232, http://dx.doi.org/10.1172/JCI23396.
[14] J. Ndure, K.L. Flanagan, Targeting regulatory T cells to improve vaccine immunogenicity in early life, Front. Microbiol. 5 (2014) 477, http://dx.doi.org/10.3389/fmicb.2014.00477.
[15] V. Brezar, V. Godot, L. Cheng, L. Su, Y. Lévy, N.T. Seddiki, Conflict of interest pay attention and do not neglect them: lessons from HIV and cancer vaccine trials, Vaccines (Basel) 4 (3) (2016), http://dx.doi.org/10.3390/vaccines4030030 (pii: E30).
[16] R.K. Gershon, K. Kondo, Cell interactions in the induction of tolerance: the role of thymic lymphocytes, Immunology 18 (5) (1970) 723–737 (PMCID: PMC1455602).
[17] S. Sakaguchi, N. Sakaguchi, M. Asano, M. Itoh, M. Toda, Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases, J. Immunol. 155 (3) (1995) 1151–1164 (PMID: 7636184).
[18] C. Baecher-Allan, J.A. Brown, G.J. Freeman, D.A. Hafler, CD4 + CD25high regulatory cells in human peripheral blood, J. Immunol. 167 (3) (2001) 1245–1253 (PMID: 11466340).
[19] D. Dieckmann, H. Plottner, S. Berchtold, T. Berger, G. Schuler, Ex vivo isolation and characterization of CD4(+)CD25(+) T cells with regulatory properties from human blood, J. Exp. Med. 193 (11) (2001) 1303–1310 (PMCID: PMC2193384).
[20] H. Jonuleit, E. Schmitt, M. Stassen, A. Tuettenberg, J. Knop, A.H. Enk, Identification and functional characterization of human CD4(+)CD25(+) T cells with regulatory properties isolated from peripheral blood, Exp Med. 193 (11) (2001) 1285–1294 (PMCID: PMC2193380).
[21] L.A. Stephens, C. Mottet, D. Mason, F. Powrie, Human CD4(+)CD25(+) thymocytes and peripheral T cells have immune suppressive activity in vitro, Eur. J. Immunol. 31 (4) (2001) 1247–1254 (PMID:11298351).
[22] L.S. Taams, J. Smith, M.H. Rustin, M. Salmon, L.W. Poulter, A.N. Akbar, Human anergic/suppressive CD4(+)CD25(+) T cells: a highly differentiated and apoptosis-prone population, Eur. J. Immunol. 31 (4) (2001) 1122–1131 (PMID: 11298337).
[23] M.E. Brunkow, E.W. Jeffery, K.A. Hjerrild, B. Paeper, L.B. Clark, S.A. Yasayko, J.E. Wilkinson, D. Galas, S.F. Ziegler, F. Ramsdell, Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferativedisorder of the scurfy mouse, Nat. Genet. 27 (1) (2001) 68–73, http://dx.doi.org/10.1038/83784.
[24] T.A. Chatila, F. Blaeser, N. Ho, H.M. Lederman, C. Voulgaropoulos, C. Helms, A.M. Bowcock, JM2, encoding a fork head-related protein, is mutated in X-linked autoimmunity-allergic disregulation syndrome, J. Clin. Invest. 106 (2000) R75–R81, http://dx.doi.org/10.1172/JCI11679.
[25] C.L. Bennett, J. Christie, F. Ramsdell, M.E. Brunkow, P.J. Ferguson, L. Whitesell, T.E. Kelly, F.T. Saulsbury, P.F. Chance, H.D. Ochs, The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3, Nat. Genet. 27 (1) (2001) 20–21, http://dx.doi.org/10.1038/83713.
[26] R.S. Wildin, F. Ramsdell, J. Peake, F. Faravelli, J.L. Casanova, N. Buist, E. Levy-Lahad, M. Mazzella, O. Goulet, L. Perroni, F.D. Bricarelli, G. Byrne, M. McEuen, S. Proll, M. Appleby, M.E. Brunkow, X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the humanequivalent of mouse scurfy, Nat. Genet. 27 (1) (2001) 18–20, http://dx.doi.org/10.1038/83707.
[27] S. Hori, T. Nomura, S. Sakaguchi, Control of regulatory T cell development by the transcription factor Foxp3, Science 299 (2003) 1057–1061, http://dx.doi.org/10.1126/science.1079490.
[28] J.D. Fontenot, M.A.A.Y. Gavin Rudensky, Foxp3 programs the development and function of CD4 + CD25+ regulatory T cells, Nat. Immunol. 4 (2003) 330–336, http://dx.doi.org/10.1038/ni904.
[29] R. Khattri, T. Cox, S.A. Yasayko, F. Ramsdell, An essential role for scurfin in CD4 + CD25+ T regulatory cells, Nat. Immunol. 4 (2003) 337–342, http://dx.doi.org/10.1038/ni909.
[30] H. Yagi, T. Nomura, K. Nakamura, S. Yamazaki, T. Kitawaki, S. Hori, M. Maeda, M. Onodera, T. Uchiyama, S. Fujii, S. Sakaguchi, Crucial role of FOXP3

in the development and function of human CD25 + CD4+ regulatory T cells, Int. Immunol. 16 (11) (2004) 1643–1656, http://dx.doi.org/10.1093/intimm/dxh165.
[31] G. Roncador, P.J. Brown, L. Maestre, S. Hue, J.L. Martínez-Torrecuadrada, K.L. Ling, S. Pratap, C. Toms, B.C. Fox, V. Cerundolo, F. Powrie, A.H. Banham, Analysis of FOXP3 protein expression in human CD4 + CD25+ regulatory T cells at the single-cell level, Eur. J. Immunol. 35 (6) (2005) 1681–1691, http://dx.doi.org/10.1002/eji.200526189.
[32] V. Pillai, S.B. Ortega, C.K. Wang, N.J. Karandikar, Transient regulatory T-cells: a state attained by all activated human T-cells, Clin. Immunol. 123 (1) (2007) 18–29, http://dx.doi.org/10.1016/j.clim.2006.10.014.
[33] N. Yu, X. Li, W. Song, D. Li, D. Yu, X. Zeng, M. Li, X. Leng, X. Li, CD4(+)CD25 (+)CD127 (low/-) T cells: a more specific Treg population in human peripheral blood, Inflammation 35 (6) (2012) 1773–1780, http://dx.doi.org/10.1007/s10753-012-9496-8.
[34] M.A. Curotto de Lafaille, J.J. Lafaille, Natural and adaptive Foxp3 + regulatory Tcells: more of the same or a division of labor? Immunity 30 (2009) 626–635, http://dx.doi.org/10.1016/j.immuni.2009.05.002.
[35] K. Singh, M. Hjort, L. Thorvaldson, S. Sandler, Concomitant analysis of Helios and Neuropilin-1 as a marker to detect thymic derived regulatory T cells in naïve mice, Sci. Rep. 5 (2015) 7767, http://dx.doi.org/10.1038/srep07767.
[36] E. Szurek, A. Cebula, L. Wojciech, M. Pietrzak, G. Rempala, P. Kisielow, L. Ignatowicz, Differences in expression level of helios and neuropilin-1 do not distinguish thymus-derived from extrathymically-induced CD4 + foxp3 + regulatory T cells, PLoS One 10 (10) (2015) e0141161, http://dx.doi.org/10.1371/journal.pone.0141161.
[37] B.M. Hall, T cells: soldiers and spies–the surveillance and control of effector t cells by regulatory T cells, Clin. J. Am. Soc. Nephrol. 10 (11) (2015) 2050–2064, http://dx.doi.org/10.2215/CJN.06620714.
[38] I. Wollenberg, A. Agua-Doce, A. Hernández, C. Almeida, V.G. Oliveira, J. Faro, L. Graca, Regulation of the germinal center reaction by Foxp3+ follicular regulatory T cells, J. Immunol. 187 (9) (2011) 4553–4560, http://dx.doi.org/10.2215/CJN.06620714.
[39] M. Gol-Ara, F. Jadidi-Niaragh, R. Sadria, G. Azizi, A. Mirshafiey, The role of different subsets of regulatory T cells in immunopathogenesis of rheumatoid arthritis, Arthritis 2012 (2012) 805875, http://dx.doi.org/10.1155/2012/805875.
[40] A. Arce-Sillas, D.D. Álvarez-Luquín, B. Tamaya-Domínguez, S. Gomez-Fuentes, A. Trejo-García, M. Melo-Salas, G. Cárdenas, J. Rodríguez-Ramírez, L. Adalid-Peralta, Regulatory T cells: molecular actions on effector cells in immune regulation, J. Immunol. Res. 2016 (2016) 1720827, http://dx.doi.org/10.1155/2012/805875.
[41] K. Klages, C.T. Mayer, K. Lahl, C. Loddenkemper, M.W. Teng, S.F. Ngiow, M.J. Smyth, A. Hamann, J. Huehn, T. Sparwasser, Selective depletion of Foxp3+ regulatory T cells improves effective therapeutic vaccination against established melanoma, Cancer Res. 70 (20) (2010) 7788–7799, http://dx.doi.org/10.1158/0008-5472.CAN-10-1736.
[42] S.R. Mattarollo, K. Steegh, M. Li, H. Duret, S. Foong Ngiow, M.J. Smyth, Transient Foxp3(+) regulatory Tcell depletion enhances therapeutic anticancer vaccinationtargeting the immune-stimulatory properties of NKT cells, Immunol. Cell Biol. 91 (1) (2013) 105–114.
[43] M.R. Espinoza Mora, C. Steeg, S. Tartz, V. Heussler, T. Sparwasser, A. Link, B. Fleischer, T. Jacobs, Depletion of regulatory T cells augments a vaccine-induced T effector cell response against the liver-stage of malaria but fails to increase memory, PLoS One 9 (8) (2014) e104627, http://dx.doi.org/10.1371/journal.pone.0104627.
[44] S.A. Fisher, W.J. Aston, J. Chee, A. Khong, A.L. Cleaver, J.N. Solin, S. Ma, W.J. Lesterhuis, I. Dick, R.A. Holt, J. Creaney, L. Boon, B. Robinson, R.A. Lake, Transient Treg depletion enhances therapeutic anti-cancer vaccination, Immun. Inflamm. Dis. 5 (1) (2016) 16–28, http://dx.doi.org/10.1002/iid3.136.
[45] T. Knuschke, O. Rotan, W. Bayer, V. Sokolova, W. Hansen, T. Sparwasser, U. Dittmer, M. Epple, J. Buer, A.M. Westendorf, Combination of nanoparticle-based therapeutic vaccination and transient ablation of regulatory T cells enhances anti-viral immunity during chronic retroviral infection, Retrovirology 13 (2016) 24, http://dx.doi.org/10.1186/s12977-016-0258-9.
[46] K. Lahl, C. Loddenkemper, C. Drouin, J. Freyer, J. Arnason, G. Eberl, A. Hamann, H. Wagner, J. Huehn, T. Sparwasser, Selective depletion of Foxp3+ regulatory T cells induces a scurfy-like disease, J. Exp. Med. 204 (2007) 57–63, http://dx.doi.org/10.1084/jem.20061852.
[47] N. Komatsu, M.E. Mariotti-Ferrandiz, Y. Wang, B. Malissen, H. Waldmann, S. Hori, Heterogeneity of natural Foxp3+ T cells: a committed regulatory T-cell lineage and an uncommitted minor population retaining plasticity, Proc. Natl. Acad. Sci. U. S. A. 106 (6) (2009) 1903–1908, http://dx.doi.org/10.1073/pnas.0811556106.
[48] A. Tanaka, S. Sakaguchi, Regulatory T cells in cancer immunotherapy, Cell Res. 27 (1) (2017) 109–118, http://dx.doi.org/10.1038/cr.2016.151.
[49] L. Moise, F. Terry, A.H. Gutierrez, R. Tassone, P. Losikoff, S.H. Gregory, C. Bailey-Kellogg, W.D. Martin, A.S. De Groot, Smarter vaccine design will circumvent regulatory T cell-mediated evasion in chronic HIV and HCV infection, Front. Microbiol. 5 (2014) 502, http://dx.doi.org/10.3389/fmicb.2014.00502.
[50] M.C. Boer, S.A. Joosten, T.H. Ottenhoff, Regulatory T-cells at the interface between human host and pathogens in infectious diseases and vaccination, Front. Immunol. 6 (2015) 217, http://dx.doi.org/10.3389/fimmu.2015.00217.
[51] W.L. Byrne, K.H. Mills, J.A. Lederer, G.C. O'Sullivan, Targeting regulatory T cells in cancer, Cancer Res. 71 (22) (2011) 6915–6920, http://dx.doi.org/10.1158/0008-5472.CAN-11-1156.
[52] H. Pere, C. Tanchot, J. Bayry, M. Terme, J. Taieb, C. Badoual, O. Adotevi, N. Merillon, E. Marcheteau, V.R. Quillien, C. Banissi, A. Carpentier, F. Sandoval, M. Nizard, F. Quintin-Colonna, G. Kroemer, W.H. Fridman, L. Zitvogel, S.P. Oudard, E. Tartour, Comprehensive analysis of current approaches to inhibit regulatory T cells in cancer, Oncoimmunology 1 (3) (2012) 326–333, http://dx.doi.org/10.4161/onci.18852.
[53] C.M. Barbon, M. Yang, G.D. Wands, R. Ramesh, B.S. Slusher, M.L. Hedley, T.M. Luby, Consecutive low doses of cyclophosphamide preferentially target Tregs and potentiate T cell responses induced by DNA PLG microparticle immunization, Cell. Immunol. 262 (2) (2010) 150–161, http://dx.doi.org/10.1016/j.cellimm.2010.02.007.
[54] D.T. Le, E.M. Jaffee, Regulatory T-cell modulation using cyclophosphamide in vaccine approaches: a current perspective, Cancer Res. 72 (14) (2012) 3439–3444, http://dx.doi.org/10.1158/0008-5472.CAN-11-3912.
[55] L. Polak, J.L. Turk, Reversal of immunological tolerance by cyclophosphamide through inhibition of suppressor cell activity, Nature (Lond.) 249 (1974) 654 (PMID:4275846).
[56] D. Berd, M.J. Mastrangelo, P.F. Engstrom, A. Paul, H. Maguire, Augmentation of the human immune response by cyclophosphamide, Cancer Res. 42 (11) (1982) 4862–4866 (PMID: 7127320).
[57] D. Berd, H.C. Maguire Jr., M.J. Mastrangelo, Induction of cell mediated immunity to autologous melanoma cells and regression of metastases after treatment with a melanoma cell vaccine preceded by cyclophosphamide, Cancer Res. 46 (1986) 2572–2577 (PMID: 3697996).
[58] M.E. Lutsiak, R.T. Semnani, R. De Pascalis, S.V. Kashmiri, J. Schlom, H. Sabzevari, Inhibition of CD4(+)25(+) T regulatory cell function implicated in enhanced immune response by low-dose cyclophosphamide, Blood 105 (2005) 2862–2868, http://dx.doi.org/10.1182/blood-2004-06-2410.
[59] D. Heylmann, M. Bauer, H. Becker, S. van Gool, N. Bacher, K. Steinbrink, B. Kaina, Human CD4 + CD25 + regulatory T cells are sensitive to low dose cyclophosphamide: implications for the immune response, PLoS One. 8 (12) (2013) e83384, http://dx.doi.org/10.1371/journal.pone.0083384.
[60] J.C. Becker, D. Schrama, The dark side of cyclophosphamide: cyclophosphamide-mediated ablation of regulatory T cells, J. Invest. Dermatol. 133 (6) (2013) 1462–1465, http://dx.doi.org/10.1038/jid.2013.67.
[61] A. Sevko, M. Sade-Feldman, J. Kanterman, T. Michels, C.S. Falk, L. Umansky, M. Ramacher, M. Kato, D. Schadendorf, M. Baniyash, V. Umansky, Cyclophosphamide promotes chronic inflammation-dependent immunosuppression and prevents antitumor response in melanoma, Invest. Dermatol. 133 (6) (2013) 1610–1619, http://dx.doi.org/10.1038/jid.2012.444.
[62] M. Kawai, H. Kitade, C. Mathieu, M. Waer, J. Pirenne, Inhibitory and stimulatory effects of cyclosporine A on the development of regulatory T cells in vivo, Transplantation 79 (9) (2005) 1073–1077 (PMID: 15880046).
[63] S. Shibutani, F. Inoue, O. Aramaki, Y. Akiyama, K. Matsumoto, M. Shimazu, M. Kitajima, Y. Ikeda, N. Shirasugi, M. Niimi, Effects of immunosuppressants on induction of regulatory cells after intratracheal delivery of alloantigen, Transplantation 79 (8) (2005) 904–913 (PMID: 15849542).
[64] C.A. Chen, C.M. Ho, M.C. Chang, W.Z. Sun, Y.L. Chen, Y.C. Chiang, M.H. Syu, C.Y. Hsieh, W.F. Cheng, Metronomic chemotherapy enhances antitumor effects of cancer vaccine by depleting regulatoryT lymphocytes and inhibiting tumor angiogenesis, Mol. Ther. 18 (6) (2010) 1233–1243, http://dx.doi.org/10.1038/mt.2010.34.
[65] A.C. Moore, A. Gallimore, S.J. Draper, K.R. Watkins, S.C. Gilbert, A.V. Hill, Anti-CD25 antibody enhancement of vaccine-induced immunogenicity: increased durable cellular immunity with reduced immunodominance, J. Immunol. 175 (11) (2005) 7264–7273 (PMID: 16301631).
[66] S. Delluc, A. Gaston, C. Marchiol-Foumigault, D. Fradelizi, A. Regnault, K.H.L.Q. Sang, L. Tourneur, G. Chiocchia, A. Buzyn, Depletion of regulatory T cells dramatically improves DC-based immunization against acute myeloid leukemia, Blood (2006) 1055a.
[67] B.D. Johnson, W. Jing, R.J. Orentas, CD25+ regulatory T cell inhibition enhances vaccine-induced immunity to neuroblastoma, J. Immunother. 30 (2) (2007) 203–214, http://dx.doi.org/10.1097/01.cji.0000211336.91513.dd.
[68] B. Jaron, E. Maranghi, C. Leclerc, L. Majlessi, Effect of attenuation of Treg during BCG immunization on anti-mycobacterial Th1 responses and protection against Mycobacterium tuberculosis, PLoS One 3 (7) (2008) e2833, http://dx.doi.org/10.1371/journal.pone.0002833.
[69] B.P. Keenan, Y. Saenger, M.I. Kafrouni, A. Leubner, P. Lauer, A. Maitra, A.A. Rucki, A.J. Gunderson, L.M. Coussens, D.G. Brockstedt, T.W. Dubensky Jr., R. Hassan, T.D. Armstrong, E.M. Jaffee, A Listeria vaccine and depletion of T-regulatory cells activate immunity against early stage pancreatic intraepithelial neoplasms and prolong survival of mice, Gastroenterology 146 (7) (2014) 1784–1794, http://dx.doi.org/10.1053/j.gastro.2014.02.055 (e6).
[70] C. Tan, V. Reddy, J. Dannull, E. Ding, S.K. Nair, D.S. Tyler, S.K. Pruitt, W.T. Lee, Impact of anti-CD25 monoclonal antibody on dendritic cell-tumor fusion vaccine efficacy in a murinemelanoma model, J. Transl. Med. 11 (2013) 148, http://dx.doi.org/10.1186/1479-5876-11-148.
[71] A.J. Rech, R.H. Vonderheide, Clinical use of anti-CD25 antibody daclizumab to enhance immune responses to tumor antigen vaccination by targeting regulatory T cells, Ann. N. Y. Acad. Sci. 1174 (2009) 99–106, http://dx.doi.org/10.1111/j.1749-6632.2009.04939.x.

[72] A.J. Rech, R. Mick, S. Martin, A. Recio, N.A. Aqui, D.J. Powell Jr., T.A. Colligon, J.A. Trosko, L.I. Leinbach, C.H. Pletcher, C.K. Tweed, A. DeMichele, K.R. Fox, S.M. Domchek, J.L. Riley, R.H. Vonderheide, CD25 blockade depletes and selectively reprograms regulatory T cells in concert with immunotherapy in cancer patients, Sci. Transl. Med. 4 (134) (2012), http://dx.doi.org/10.1126/scitranslmed.3003330 (134ra62).
[73] F.M. Foss, DAB(389)IL-2 (denileukin diftitox, ONTAK): a new fusion protein technology, Clin. Lymphoma 1 (Suppl. 1) (2000) S27–31 (PMID:11707860).
[74] M.T. Litzinger, R. Fernando, T.J. Curiel, D.W. Grosenbach, J. Schlom, C. Palena, IL-2 immunotoxin denileukin diftitox reduces regulatory T cells and enhances vaccine-mediatedT-cell immunity, Blood 110 (November (9)) (2007) 3192–3201, http://dx.doi.org/10.1182/blood-2007-06-094615.
[75] J. Dannull, Z. Su, D. Rizzieri, B.K. Yang, D. Coleman, D. Yancey, A. Zhang, P. Dahm, N. Chao, E. Gilboa, J. Vieweg, Enhancement of vaccine-mediated antitumor immunity in cancer patients after depletion of regulatory T cells, J. Clin. Invest. 115 (2005) 3623–3633, http://dx.doi.org/10.1172/JCI25947.
[76] M.A. Morse, A.C. Hobeika, T. Osada, D. Serra, D. Niedzwiecki, H.K. Lyerly, T.M. Clay, Depletion of human regulatory T cells specifically enhances antigen-specific immune responses to cancer vaccines, Blood 112 (3) (2008) 610–618, http://dx.doi.org/10.1182/blood-2008-01-135319.
[77] T. Curiel, S. Thibodeaux, S. Wall, S.L. Pandeswara, B. Daniel, J. Drerup, K. Murthy, I. Kryczek, W. Zou, B. Barnett, Denileukin diftitox depletes regulatory T cells without clinical benefit in advanced stage epithelial ovarian carcinoma (VAC3P. 945), J. Immunol. 192 (Supplement 1) (2014) 73–77.
[78] P. Attia, A.V. Maker, L.R. Haworth, L. Rogers-Freezer, S.A. Rosenberg, Inability of a fusion protein of IL-2 and diphtheria toxin (Denileukin Diftitox, DAB389IL-2, ONTAK) to eliminate regulatory T lymphocytes in patients with melanoma, J. Immunother. 28 (2005) 582–592 (PMID:16224276).
[79] J.J. Luke, Y. Zha, K. Matijevich, T.F. Gajewski, Single dose denileukin diftitox does not enhance vaccine-induced T cell responses or effectively deplete Tregs in advanced melanoma: immune monitoring and clinical results of a randomized phase II trial, J. ImmunoTher. Cancer 4 (1) (2016) 35, http://dx.doi.org/10.1186/s40425-016-0140-2.
[80] K.M. Lee, E. Chuang, M. Griffin, R. Khattri, D.K. Hong, W. Zhang, D. Straus, L.E. Samelson, C.B. Thompson, J.A. Bluestone, Molecular basis of T cell inactivation by CTLA-4, Science 282 (5397) (1998) 2263–2266, http://dx.doi.org/10.1126/science.282.5397.2263.
[81] E.D. Kwon, A.A. Hurwitz, B.A. Foster, C. Madias, A.L. Feldhaus, N.M. Greenberg, M.B. Burg, J.P. Allison, Manipulation of T cell costimulatory and inhibitory signals for immunotherapy of prostate cancer, Proc. Natl. Acad. Sci. U. S. A. 94 (15) (1997) 8099–8103 (PMID:9223321).
[82] A.A. Hurwitz, T.F. Yu, D.R. Leach, J.P. Allison, CTLA-4 blockade synergizes with tumor-derived granulocyte-macrophage colony-stimulating factor for treatment of an experimental mammary carcinoma, Proc. Natl. Acad. Sci. U. S. A. 18 (17) (1998) 10067–10071 (PMID:9707601).
[83] A. van Elsas, R.P. Sutmuller, A.A. Hurwitz, J. Ziskin, J. Villasenor, J.P. Medema, W.W. Overwijk, N.P. Restifo, C.J. Melief, R. Offringa, J.P. Allison, Elucidating the autoimmune and antitumor effector mechanisms of a treatment based on cytotoxic T lymphocyte antigen-4 blockade in combination with a B16 melanoma vaccine: comparison of prophylaxis and therapy, J. Exp. Med. 194 (4) (2001) 481–489 (PMID: 11514604).
[84] G.Q. Phan, J.C. Yang, R.M. Sherry, P. Hwu, S.L. Topalian, D.J. Schwartzentruber, N.P. Restifo, L.R. Haworth, C.A. Seipp, L.J. Freezer, K.E. Morton, S.A. Mavroukakis, P.H. Duray, S.M. Steinberg, J.P. Allison, T.A. Davis, S.A. Rosenberg, Cancer regression and autoimmunity induced by cytotoxic T lymphocyte-associated antigen 4 blockade in patients with metastatic melanoma, Proc. Natl. Acad. Sci. U. S. A. 100 (14) (2003) 8372–8377, http://dx.doi.org/10.1073/pnas.1533209100.
[85] P.D. Gregor, J.D. Wolchok, C.R. Ferrone, H. Buchinshky, J.A. Guevara-Patiño, M.A. Perales, F. Mortazavi, D. Bacich, W. Heston, J.B. Latouche, M. Sadelain, J.P. Allison, H.I. Scher, A.N. Houghton, CTLA-4 blockade in combination with xenogeneic DNA vaccines enhances T-cell responses, tumor immunity and autoimmunity to self antigens in animal and cellular model systems, Vaccine 22 (13–14) (2004) 1700–1708, http://dx.doi.org/10.1016/j.vaccine.2003.10.048.
[86] M. Goozner, Drug approvals 2011: focus on companion diagnostics, J. Natl. Cancer Inst. 104 (2) (2012) 84–86, http://dx.doi.org/10.1093/jnci/djr552.
[87] J. Weber, Ipilimumab: controversies in its development, utility and autoimmune adverse events, Cancer Immunol. Immunother. 58 (5) (2009) 823–830, http://dx.doi.org/10.1007/s00262-008-0653-8.
[88] J.S. Weber, K.C. Kahler, A. Hauschild, Management of immune-related adverse events and kinetics of response with ipilimumab, J. Clin. Oncol. 30 (2012) 2691–2697, http://dx.doi.org/10.1200/JCO.2012.41.6750.
[89] J.B. Korman, D.E. Fisher, Developing melanoma therapeutics: overview and update, Wiley Interdiscip. Rev. Syst. Biol. Med. 5 (3) (2013) 257–271, http://dx.doi.org/10.1002/wsbm.1210.
[90] R.S. McHugh, M.J. Whitters, C.A. Piccirillo, D.A. Young, E.M. Shevach, M. Collins, M.C. Byrne, CD4 + CD25+ immunoregulatory T cells: gene expression analysis reveals a functional role for the glucocorticoid-induced TNF receptor, Immunity 16 (2002) 311–323, http://dx.doi.org/10.1016/S1074-7613(02)00280-7.
[91] E.M. Shevach, G.L. Stephens, The GITR–GITRL interaction: co-stimulation or contrasuppression of regulatory activity? Nat. Rev. Immunol. 6 (2006) 613–618, http://dx.doi.org/10.1038/nri1867.
[92] M.J. Turk, J.A. Guevara-Patiño, G.A. Rizzuto, M.E. Engelhorn, S. Sakaguchi, A.N. Houghton, Concomitant tumor immunity to a poorly immunogenic melanoma is prevented by regulatory T cells, J. Exp. Med. 200 (6) (2004) 771–782, http://dx.doi.org/10.1084/jem.20041130.
[93] J. Shimizu, S. Yamazaki, T. Takahashi, Y. Ishida, S. Sakaguchi, Stimulation of CD25 + CD4+ regulatory T cells through GITR breaks immunological self tolerance, Nat. Immunol. 3 (2002) 135–142, http://dx.doi.org/10.1038/ni759.
[94] A.P. Kohm, J.S. Williams, S.D. Miller, Cutting edge: ligation of the glucocorticoid-induced TNF receptor enhances autoreactive CD4+ T cell activation and experimental autoimmune encephalomyelitis, J. Immunol. 172 (2004) 4686–4690 (PMID:15067043).
[95] A.D. Cohen, A. Diab, M.A. Perales, J.D. Wolchok, G. Rizzuto, T. Merghoub, D. Huggins, C. Liu, M.J. Turk, N.P. Restifo, S. Sakaguchi, A.N. Houghton, Agonist anti-GITR antibody enhances vaccine-induced CD8(+) T-cell responses and tumor immunity, Cancer Res. 66 (9) (2006) 4904–4912, http://dx.doi.org/10.1158/0008-5472.CAN-05-2813.
[96] D. Boczkowski, J. Lee, S. Pruitt, S. Nair, Dendritic cells engineered to secrete anti GITR antibodies are effective adjuvants to dendritic cell-based immunotherapy, Cancer Gene Ther. 16 (12) (2009) 900–911, http://dx.doi.org/10.1038/cgt.2009.39.
[97] S.K. Pruitt, D. Boczkowski, N. de Rosa, N.R. Haley, M.A. Morse, D.S. Tyler, J. Dannull, S. Nair, Enhancement of antitumor immunity through local modulation of CTLA-4 and GITR by dendritic cells, Eur. J. Immunol. 41 (12) (2011) 3553–3563, http://dx.doi.org/10.1002/eji.201141383.
[98] B. Valzasina, C. Guiducci, H. Dislich, N. Killeen, A.D. Weinberg, M.P. Colombo, Triggering of OX40 (CD134) on CD4(+)CD25+ T cells blocks their inhibitory activity: a novel regulatory role for OX40 and its comparison with GITR, Blood 105 (2005) 2845–2851, http://dx.doi.org/10.1182/blood-2004-07-2959.
[99] M. Croft, T. So, W. Duan, P. Soroosh, The significance of OX40 and OX40L to t cell biology and immune disease, Immunol. Rev. 229 (1) (2009) 173–191, http://dx.doi.org/10.1111/j.1600-065X.2009.00766.x.
[100] S. Aspeslagh, S. Postel-Vinay, S. Rusakiewicz, J.C. Soria, L. Zitvogel, A. Marabelle, Rationale for anti-OX40 cancer immunotherapy, Eur. J. Cancer 52 (2016) 50–66, http://dx.doi.org/10.1016/j.ejca.2015.08.021.
[101] S. Piconese, B. Valzasina, M.P. Colombo, OX40 triggering blocks suppression by regulatory T cells and facilitates tumor rejection, J. Exp. Med. 205 (4) (2008) 825–839, http://dx.doi.org/10.1084/jem.20071341.
[102] R.J. Snelgrove, M.M. Cornere, L. Edwards, B. Dagg, J. Keeble, A. Rodgers, D.E. Lyonga, G.R. Stewart, D.B. Young, B. Walker, T. Hussell, OX40 ligand fusion protein delivered simultaneously with the BCG vaccine provides superior protection against murine *Mycobacterium tuberculosis* infection, J. Infect. Dis. 205 (6) (2012) 975–983, http://dx.doi.org/10.1093/infdis/jir868.
[103] T. Kumai, S. Lee, H.I. Cho, H. Sultan, H. Kobayashi, Y. Harabuchi, E. Celis, Optimization of peptide vaccines to induce robust antitumor CD4 T-cell responses, Cancer Immunol. Res. 5 (1) (2017) 72–83, http://dx.doi.org/10.1158/2326-6066.CIR-16-0194.
[104] E. Panagioti, L. Boon, R. Arens, S.H. van der Burg, Enforced OX40 stimulation empowers booster vaccines to induce effective CD4+ and CD8+ T Cell responses against mouse Cytomegalovirus infection, Front. Immunol. 8 (2017) 144, http://dx.doi.org/10.3389/fimmu.2017.00144.
[105] S. Yao, L. Chen, PD-1 as an immune modulatory receptor, Cancer J. 20 (4) (2014) 262–264, http://dx.doi.org/10.1097/PPO.0000000000000060.
[106] S.S. Hassan, M. Akram, E.C. King, H.M. Dockrell, J.M. Cliff, PD-1, PD-L1 and PD-L2 gene expression on T-cells and natural killer cells declines in conjunction with a reduction in PD-1 protein during the intensive phase of tuberculosis treatment, PLoS One 10 (9) (2015), http://dx.doi.org/10.1371/journal.pone.0137646 (e0137646).
[107] A. Taylor, J. Verhagen, K. Blaser, M. Akdis, C.A. Akdis, Mechanisms of immune suppression by interleukin-10 and transforming growth factor-beta: the role of T regulatory cells, Immunology 117 (4) (2006) 433–442, http://dx.doi.org/10.1111/j.1365-2567.2006.02321.x.
[108] Y.K. Chae, L. Chiec, N. Mohindra, R. Gentzler, J. Patel, F. Giles, A case of pembrolizumab-induced type-1 diabetes mellitus and discussion of immune checkpoint inhibitor-induced type 1 diabetes, Cancer Immunol. Immunother. 66 (1) (2017) 25–32, http://dx.doi.org/10.1007/s00262-016-1913-7.
[109] A.R. Palla, D. Kennedy, H. Mosharraf, D. Doll, Autoimmune hemolytic anemia as a complication of Nivolumab therapy, Case Rep. Oncol. 9 (3) (2016) 691–697, http://dx.doi.org/10.1159/000452296.
[110] K.M. Mahoney, G.J. Freeman, D.F. McDermott, The next immune-checkpoint inhibitors: PD-1/PD-L1 blockade in melanoma, Clin. Ther. 37 (April (4)) (2015) 764–782, http://dx.doi.org/10.1016/j.clinthera.2015.02.018.
[111] W. Xue, V.A. Brentville, P. Symonds, K.W. Cook, H. Yagita, R.L. Metheringham, L.G. Durrant, SCIB1, a huIgG1 antibody DNA vaccination, combined with PD-1 blockade induced efficient therapy of poorly immunogenic tumors, Oncotarget 7 (50) (2016) 83088–83100, http://dx.doi.org/10.18632/oncotarget.13070.
[112] J. Duraiswamy, K.M. Kaluza, G.J. Freeman, G. Coukos, Dual blockade of PD-1 and CTLA-4 combined with tumor vaccine effectively restores T-cell rejection function in tumors, Cancer Res. 73 (12) (2013) 3591–3603, http://dx.doi.org/10.1158/0008-5472.CAN-12-4100.
[113] T. Ahrends, N. Bąbała, Y. Xiao, H. Yagita, H. van Eenennaam, J. Borst, CD27 Agonism plus PD-1 blockade recapitulates CD4+ T-cell help in therapeutic

anticancer vaccination, Cancer Res. 76 (10) (2016) 2921–2931, http://dx.doi.org/10.1158/0008-5472.CAN-15-3130.
[114] D.H. Munn, Indoleamine 2,3-dioxygenase, tregs and cancer, Curr. Med. Chem. 18 (15) (2011) 2240–2246 (0929-8673/11 $58.00+.00).
[115] W. Chen, X. Liang, A.J. Peterson, D.H. Munn, B.R. Blazar, The IDO pathway is essential for human plasmacytoid dendritic cell-induced adaptive T regulatory cell generation, J. Immunol. 181 (2008) 5396–5404 (PMCID:PMC2614675).
[116] R. Tanner, K. Kakalacheva, E. Miller, A.A. Pathan, R. Chalk, C.R. Sander, T. Scriba, M. Tameris, T. Hawkridge, H. Mahomed, G. Hussey, W. Hanekom, A. Checkley, H. McShane, H.A. Fletcher, Serum indoleamine 2,3-dioxygenase activity is associated with reduced immunogenicity following vaccination with MVA85A, BMC Infect. Dis. 14 (2014) 660, http://dx.doi.org/10.1186/s12879-014-0660-7.
[117] Y.W. Moon, J. Hajjar, P. Hwu, A. Naing, Targeting the indoleamine 2,3-dioxygenase pathway in cancer, J. ImmunoTher. Cancer 3 (2015) 51, http://dx.doi.org/10.1186/s40425-015-0094-9.
[118] J. Bjoern, T.Z. Iversen, N.J. Nitschke, M.H. Andersen, I.M. Svane, Safety, immune and clinical responses in metastatic melanoma patients vaccinated with a long peptide derived from indoleamine 2,3-dioxygenase in combination with ipilimumab, Cytotherapy 18 (8) (2016) 1043–1055, http://dx.doi.org/10.1016/j.jcyt.2016.05.010.
[119] M.H. Andersen, I.M. Svane, Indoleamine 2, 3-dioxygenase vaccination, Oncoimmunology 4 (1) (2015), http://dx.doi.org/10.4161/2162402X.2014.983770 (e983770).
[120] C.T. Huang, C.J. Workman, D. Flies, X. Pan, A.L. Marson, G. Zhou, E.L. Hipkiss, S. Ravi, J. Kowalski, H.I. Levitsky, J.D. Powell, D.M. Pardoll, C.G. Drake, D.A. Vignali, Role of LAG-3 in regulatory T cells, Immunity 21 (4) (2004) 503–513, http://dx.doi.org/10.1016/j.immuni.2004.08.010.
[121] S. Fougeray, C. Brignone, F. Triebel, A soluble LAG-3 protein as an immunopotentiator for therapeutic vaccines: preclinical evaluation of IMP321, Vaccine 24 (26) (2006) 5426–5433, http://dx.doi.org/10.1016/j.vaccine.2006.03.050.
[122] S. Sierro, P. Romero, D.E. Speiser, The CD4-like molecule LAG-3, biology and therapeutic applications, Expert Opin. Ther. Targets 15 (1) (2011) 91–101, http://dx.doi.org/10.1517/14712598.2011.540563.
[123] P.E. Prigent, S. Mir, M. Dréano, F. Triebel, Lymphocyte activation gene-3 induces tumor regression and antitumor immune responses, Eur. J. Immunol. 29 (12) (1999) 3867–3876 (PMID: 10601994).
[124] S. El Mir, F. Triebel, A soluble lymphocyte activation gene-3 molecule used as a vaccine adjuvant elicits greater humoral and cellular immune responses to both particulate and soluble antigens, J. Immunol. 164 (2000) 5583–5589 (PMID:10820232).
[125] P. Cappello, F. Triebel, M. Iezzi, C. Caorsi, E. Quaglino, P.L. Lollini, A. Amici, E. Di Carlo, P. Musiani, M. Giovarelli, G. Forni, LAG-3 enables DNA vaccination to persistently prevent mammary carcinogenesis in HER-2/neu transgenic BALB/c mice, Cancer Res. 63 (10) (2003) 2518–2525 (PMID: 12750275).
[126] C. Brignone, C. Grygar, M. Marcu, G. Perrin, F. Triebel, IMP321 (sLAG-3), an immunopotentiator for T cell responses against a HBsAg antigen in healthy adults: a single blind randomised controlled phase I study, J. Immune Based Ther. Vaccines 5 (2007) 5, http://dx.doi.org/10.1186/1476-8518-5-5.
[127] A.W. Mailloux, M.R. Young, Regulatory T-cell trafficking: from thymic development to tumor-induced immune suppression, Crit. Rev. Immunol. 30 (5) (2010) 435–447 (PMCID: PMC3196532).
[128] J. Bayry, E.Z. Tchilian, M.N. Davies, E.K. Forbes, S.J. Draper, S.V. Kaveri, A.V. Hill, M.D. Kazatchkine, P.C. Beverley, D.R. Flower, D.F. Tough, In silico identified CCR4 antagonists target regulatory T cells and exert adjuvant activity in vaccination, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 10221–10226, http://dx.doi.org/10.1073/pnas.0803453105.
[129] M.N. Davies, J. Bayry, E.Z. Tchilian, J. Vani, M.S. Shaila, E.K. Forbes, S.J. Draper, P.C. Beverley, D.F. Tough, D.R. Flower, Toward the discovery of vaccine adjuvants: coupling in silico screening and in vitro analysis of antagonist binding to human and mouse CCR4 receptors, PLoS One 4 (2009) e8084, http://dx.doi.org/10.1371/journal.pone.0008084.
[130] H. Pere, Y. Montier, J. Bayry, F. Quintin-Colonna, N. Merillon, E. Dransbayart, C. Badoual, A. Gey, P. Ravel, E. Marcheteau, F. Batteux, F. Sandoval, O. Adotevi, C. Chiu, S. Garcia, C. Tanchot, Y.C. Lone, L.C. Ferreira, B.H. Nelson, D. Hanahan, W.H. Fridman, L. Johannes, E. Tartour, A CCR4 antagonist combined with vaccines induces antigen specific CD8 + T cells and tumor immunity against self antigens, Blood 118 (18) (2011) 4853–4862, http://dx.doi.org/10.1182/blood-2011-01-329656.
[131] J. Bayry, Regulatory T cells as adjuvant target for enhancing the viral disease vaccine efficacy, Virusdisease 25 (1) (2014) 18–25, http://dx.doi.org/10.1007/s13337-013-0187-3.
[132] D.1 Sugiyama, H. Nishikawa, Y. Maeda, M. Nishioka, A. Tanemura, I. Katayama, S. Ezoe, Y. Kanakura, E. Sato, Y. Fukumori, J. Karbach, E. Jäger, S. Sakaguchi, Anti-CCR4 mAb selectively depletes effector-type FoxP3 + CD4 + regulatory T cells, evoking antitumor immune responses in humans, Proc. Natl. Acad. Sci. U. S. A. 110 (44) (2013) 17945–17950, http://dx.doi.org/10.1073/pnas.1316796110.
[133] S. Iyama, T. Sato, H. Ohnishi, Y. Kanisawa, S. Ohta, T. Kondo, A. Mori, Y. Tsutsumi, H. Kuroda, Y. Kakinoki, S. Yamamoto, T. Takahashi, M. Shindo, Y. Torimoto, K. Sato, H. Iwasaki, Y. Haseyama, K. Kohda, Y. Nagamachi, Y. Hirayama, H. Sakai, Y. Hirata, T. Fukuhara, H. Ikeda, M. Kobune, J. Kato, M. Kurosawa, A Multicenter retrospective study of mogamulizumab efficacy in adult T-cell leukemia/lymphoma, Clin. Lymphoma Myeloma Leuk. 17 (1) (2017) 23–30, http://dx.doi.org/10.1016/j.clml.2016.09.009 (e2).
[134] K. Ishitsuka, S. Yurimoto, K. Kawamura, Y. Tsuji, M. Iwabuchi, T. Takahashi, K. Tobinai, Safety and efficacy of mogamulizumab in patients with adult T-cell leukemia-lymphoma in Japan: interim results of postmarketing all-case surveillance, Int. J. Hematol. 9 (June) (2017), http://dx.doi.org/10.1007/s12185-017-2270-9.
[135] S. Nair, D. Boczkowski, M. Fassnacht, D. Pisetsky, E. Gilboa, Vaccination against the forkhead family transcription factor Foxp3 enhances tumor immunity, Cancer Res. 67 (1) (2007) 371–380, http://dx.doi.org/10.1158/0008-5472.CAN-06-2903.
[136] N. Mousavi Niri, A. Memarnejadian, Y. Pilehvar-Soltanahmadi, M. Agha Sadeghi, M. Mahdavi, N. Kheshtchin, S. Arab, A. Namdar, F. Jadidi, N. Zarghami, J. Hajati, Improved Anti-Treg vaccination targeting Foxp3 efficiently decreases regulatory T cells in mice, J. Immunother. 39 (7) (2016) 269–275, http://dx.doi.org/10.1097/CJI.0000000000000133.
[137] A. Miguel, L. Sendra, V. Noé, C.J. Ciudad, F. Dasí, D. Hervas, M.J. Herrero, S.F. Aliño, Silencing of Foxp3 enhances the antitumor efficacy of GM −CSF genetically modified tumor cell vaccine against B16 melanoma, Onco Targets Ther. 10 (2017) 503–514, http://dx.doi.org/10.2147/OTT.S104393.
[138] N. Casares, F. Rudilla, L. Arribillaga, D. Llopiz, J.I. Riezu-Boj, T. Lozano, J. López-Sagaseta, L. Guembe, P. Sarobe, J. Prieto, F. Borrás-Cuesta, J.J. Lasarte, A peptide inhibitor of FOXP3 impairs regulatory T cell activity and improves vaccine efficacy in mice, J. Immunol. 185 (9) (2010) 5150–5159, http://dx.doi.org/10.4049/jimmunol.1001114.
[139] T. Lozano, L. Villanueva, M. Durántez, M. Gorraiz, M. Ruiz, V. Belsúe, J.I. Riezu-Boj, S. Hervás-Stubbs, J. Oyarzábal, H. Bandukwala, A.R. Lourenço, P.J. Coffer, P. Sarobe, J. Prieto, N. Casares, J.J. Lasarte, Inhibition of FOXP3/NFAT interaction enhances t cell function after TCR stimulation, J. Immunol. 195 (7) (2015) 3180–3189, http://dx.doi.org/10.4049/jimmunol.1402997.
[140] T. Lozano, M.M. Soldevilla, N. Casares, H. Villanueva, M. Bendandi, J.J. Lasarte, F. Pastor, Targeting inhibition of Foxp3 by a CD28 2'-Fluro oligonucleotide aptamer conjugated to P60-peptide enhances active cancer immunotherapy, Biomaterials 91 (2016) 73–80, http://dx.doi.org/10.1016/j.biomaterials.2016.03.007.
[141] J. Dotor, A.B. López-Vázquez, J.J. Lasarte, P. Sarobe, M. García-Granero, J.I. Riezu-Boj, A. Martínez, E. Feijoó, J. López-Sagaseta, J. Hermida, J. Prieto, F. Borrás-Cuesta, Identification of peptide inhibitors of transforming growth factor beta 1 using a phage-displayedpeptide library, Cytokine 39 (2) (2007) 106–115.
[142] L. Gil-Guerrero, J. Dotor, I.L. Huibregtse, N. Casares, A.B. López-Vázquez, F. Rudilla, J.I. Riezu-Boj, J. López-Sagaseta, J. Hermida, S. Van Deventer, J. Bezunartea, D. Llopiz, P. Sarobe, J. Prieto, F. Borrás-Cuesta, J.J. Lasarte, In vitro and in vivo down regulation of regulatory T cell activity with a peptide inhibitor of TGF-beta1, J. Immunol. 181 (1) (2008) 126–135, http://dx.doi.org/10.1016/j.cyto.2007.06.004.
[143] S. Takaku, M. Terabe, E. Ambrosino, J. Peng, S. Lonning, J.M. McPherson, J.A. Berzofsky, Blockade of TGFβ enhances tumor vaccine efficacy mediated by CD8 + T cells, Int. J. Cancer 126 (7) (2010) 1666, http://dx.doi.org/10.1002/ijc.24961.
[144] J. Nitcheu-Tefit, M.S. Dai, R.J. Critchley-Thorne, F. Ramirez-Jimenez, M. Xu, S. Conchon, N. Ferry, H.J. Stauss, G. Vassaux, Listeriolysin O expressed in a bacterial vaccine suppresses CD4 + CD25high regulatory T cell function in vivo, J. Immunol. 179 (3) (2007) 1532–1541 (PMID:17641019).
[145] M. Xu, M.S. Dai, C. Mi, Expression of cytokines in murine BMDCs induced by recombinant E.coli LLO/OVA via TLR4 and NOD1 receptors, Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi 25 (1) (2009) 9–12 (PMID:19126379).
[146] M.S. Dai, J. Nitcheu-Tefit, S. Alcock, F. Ramirez-Jimenez, T.Y. Chao, P. Baril, M. Rocha, S.J. Brett, H.J. Stauss, G. Vassaux, Development of an Escherichia coli expressing listeriolysin-O vaccine against Wilms tumor gene 1-expressing tumors, J. Immunother. 32 (8) (2009) 845–855, http://dx.doi.org/10.1097/CJI.0b013e3181aee259.
[147] A. Ohta, M. Sitkovsky, Extracellular adenosine-mediated modulation of regulatory T cells, Front. Immunol. 5 (2014) 304, http://dx.doi.org/10.3389/fimmu.2014.00304.
[148] M.R. Blackburn, C.O. Vance, E. Morschl, C.N. Wilson, Adenosine receptors and inflammation, Handb. Exp. Pharmacol. (2009) 215–269, http://dx.doi.org/10.1007/978-3-540-89615-9_8.
[149] J. Bastid, A. Regairaz, N. Bonnefoy, C. Déjou, J. Giustiniani, C. Laheurte, S. Cochaud, E. Laprevotte, E.6 Funck-Brentano, P. Hemon, L. Gros, N. Bec, C. Larroque, G. Alberici, A. Bensussan, J.F. Eliaou, Inhibition of CD39 enzymatic function at the surface of tumor cells alleviates their immunosuppressive activity, Cancer Immunol. Res. 3 (3) (2015) 254–265, http://dx.doi.org/10.1158/2326-6066.CIR-14-0018.
[150] H. Zhao, C. Bo, Y. Kang, H. Li, What else can CD39 tell us? Front. Immunol. 8 (2017) 727, http://dx.doi.org/10.3389/fimmu.2017.00727.
[151] A.1 Facciabene, G.T. Motz, G. Coukos, T-regulatory cells: key players in tumor immune escape and angiogenesis, Cancer Res. 72 (9) (2012) 2162–2171, http://dx.doi.org/10.1158/0008-5472.CAN-11-3687.
[152] E. Tartour, H. Pere, B. Maillere, M. Terme, N. Merillon, J. Taieb, F. Sandoval, F. Quintin-Colonna, K. Lacerda, A. Karadimou, C. Badoual, A. Tedgui, W.H. Fridman, S. Oudard, Angiogenesis and immunity: a bidirectional link potentially relevant for the monitoring of antiangiogenic therapy and the development of novel therapeutic combination with Immunotherapy, Cancer Metastasis Rev. 30 (1) (2011) 83–95, http://dx.doi.org/10.1007/s10555-011-9281-4.

[153] O. Spiegelstein, J.D. Eudy, R.H. Finnell, Identification of two putative novel folate receptor genes in humans and mouse, Gene 258 (1–2) (2000) 117–125 (PMID:11111049).
[154] T. Yamaguchi, K. Hirota, K. Nagahama, K. Ohkawa, T. Takahashi, T. Nomura, S. Sakaguchi, Control of immune responses by antigen-specific regulatory T cells expressing the folate receptor, Immunity 27 (1) (2007) 145–159, http://dx.doi.org/10.1016/j.immuni.2007.04.017.
[155] Y. Tian, G. Wu, J.C. Xing, J. Tang, Y. Zhang, Z.M. Huang, Z.C. Jia, R. Zhao, Z.Q. Tian, S.F. Wang, X.L. Chen, L. Wang, Y.Z. Wu, B. Ni, A novel splice variant of folate receptor 4 predominantly expressed in regulatory T cells, BMC Immunol. 13 (2012) 30, http://dx.doi.org/10.1186/1471-2172-13-30.
[156] N. Matsushita, S.A. Pilon-Thomas, L.M. Martin, A.I. Riker, Comparative methodologies of regulatory T cell depletion in a murine melanoma model, J. Immunol. Methods 333 (1-2) (2008) 167–179, http://dx.doi.org/10.1016/j.jim.2008.01.012.
[157] F.V. Castro, M. Al-Muftah, K. Mulryan, H.R. Jiang, J.W. Drijfhout, S. Ali, A.J. Rutkowski, M. Kalaitsidou, D.E. Gilham, P.L. Stern, Regulation of autologous immunity to the mouse 5T4 oncofoetal antigen: implications for immunotherapy, Cancer Immunol. Immunother. 61 (7) (2012) 1005–1018, http://dx.doi.org/10.1007/s00262-011-1167-3.
[158] S.C. Liang, M. Moskalenko, M. Van Roey, K. Jooss, Depletion of regulatory T cells by targeting folate receptor 4 enhances the potency of a GM-CSF-secreting tumor cell immunotherapy, Clin. Immunol. 148 (2) (2013) 287–298, http://dx.doi.org/10.1016/j.clim.2013.05.011.
[159] S. Adriouch, F. Haag, O. Boyer, M. Seman, F. Koch-Nolte, Extracellular NAD(+): a danger signal hindering regulatory T cells, Microbes Infect. 14 (14) (2012) 1284–1292, http://dx.doi.org/10.1016/j.micinf.2012.05.011.
[160] F. Aswad, H. Kawamura, G. Dennert, High sensitivity of CD4 + CD25+ regulatory T cells to extracellular metabolites nicotinamide adenine dinucleotide and ATP: a role for P2 × 7 receptors, J. Immunol. 175 (2005) 3075–3083 (PMID: 16116196).
[161] S. Hubert, B. Rissiek, K. Klages, J. Huehn, T. Sparwasser, F. Haag, F. Koch-Nolte, O. Boyer, M. Seman, S. Adriouch, Extracellular NAD + shapes the Foxp3 + regulatory t cell compartment through the ART2-P2 × 7pathway, J. Exp. Med. 207 (12) (2010) 2561–2568, http://dx.doi.org/10.1084/jem.20091154.
[162] A. Elkhal, H. Rodriguez Cetina Biefer, T. Heinbokel, H. Uehara, M. Quante, M. Seyda, J.M. Schuitenmaker, F. Krenzien, V. Camacho, M.A. de la Fuente, I. Ghiran, S.G. Tullius, NAD(+) regulates Treg cell fate and promotes allograft survival via a systemic IL-10 production that is CD4(+) CD25(+) Foxp3(+) T cells independent, Sci. Rep. 6 (2016) 22325, http://dx.doi.org/10.1038/srep22325.
[163] H. Conroy, N.A. Marshall, K.H. Mills, TLR ligand suppression or enhancement of Treg cells? A double-edged sword in immunity to tumours, Oncogene 27 (2) (2008) 168–180, http://dx.doi.org/10.1038/sj.onc.1210910.
[164] G. Peng, Z. Guo, Y. Kiniwa, K.S. Voo, W. Peng, T. Fu, D.Y. Wang, Y. Li, H.Y. Wang, R.F. Wang, Toll-like receptor 8-mediated reversal of CD4 + regulatory T cell function, Science 309 (5739) (2005) 1380–1384.
[165] Y. Zhang, F. Luo, Y. Cai, N. Liu, L. Wang, D. Xu, Y. Chu, TLR1/TLR2 agonist induces tumor regression by reciprocal modulation of effector and regulator T cells, J. Immunol. 186 (4) (2011) 1963–1969, http://dx.doi.org/10.4049/jimmunol.1002320.
[166] C. Luo, H. Liu, H. Wang, J. Wang, Toll-like receptor 4 signaling in high mobility group box-1 protein 1 mediated the suppression of regulatory T-Cells, Med. Sci. Monit. 23 (2017) 300–308, http://dx.doi.org/10.12659/MSM.902081.
[167] T. Ito, S. Hanabuchi, Y.H. Wang, W.R. Park, K. Arima, L. Bover, F.X. Qin, M. Gilliet, Y.J. Liu, Two functional subsets of FOXP3 + regulatory T cells in human thymus and periphery, Immunity 28 (6) (2008) 870–880, http://dx.doi.org/10.1016/j.immuni.2008.03.018.
[168] J. Zheng, P.L. Chan, Y. Liu, G. Qin, Z. Xiang, K.T. Lam, D.B. Lewis, Y.L. Lau, W. Tu, ICOS regulates the generation and function of human CD4 + Treg in a CTLA-4 dependent manner, PLoS One 8 (12) (2013) e82203, http://dx.doi.org/10.1371/journal.pone.0082203.
[169] L. Mo, Q. Chen, X. Zhang, X. Shi, L. Wei, D. Zheng, H. Li, J. Gao, J. Li, Z. Hu, Depletion of regulatory T cells by anti-ICOS antibody enhances anti-tumor immunity of tumor cell vaccine in prostate cancer, Vaccine 35 (43) (2017) 5932–5938, http://dx.doi.org/10.1016/j.vaccine.2017.08.093.
[170] L. Berod, F. Puttur, J. Huehn, Sparwasser T.Tregs in infection and vaccinology: heroes or traitors? Microbiol. Biotechnol. 5 (2) (2012) 260–269, http://dx.doi.org/10.1111/j.1751-7915.2011.00299.x.
[171] C. Ustun, J.S. Miller, D.H. Munn, D.J. Weisdorf, B.R. Blazar, Regulatory T cells in acute myelogenous leukemia: is it time for immunomodulation? Blood 118 (19) (2011) 5084–5095, http://dx.doi.org/10.1182/blood-2011-07-365817.
[172] A. Batista-Duharte, D. Portuondo, O. Pérez, I.Z. Carlos, Systemic immunotoxicity reactions induced by adjuvanted vaccines, Int. Immunopharmacol. 20 (1) (2014) 170–180, http://dx.doi.org/10.1016/j.intimp.2014.02.033.
[173] R.F. Wang, H.Y. Wang, Immune targets and neoantigens for cancer immunotherapy and precision medicine, Cell Res. 27 (1) (2017) 11–37, http://dx.doi.org/10.1038/cr.2016.155.
[174] J.K. Simon, R. Edelman, Clinical evaluation of adjuvants, in: V.E.J.C. Schijns, D.T. O'Hagan (Eds.), Immunopotentiators in Modern Vaccines, Academic Press, Burlington, MA, 2006, pp. 319–342.
[175] A. Batista-Duharte, Vaccines and autoimmunity: a strange association under debate, Rev. Peru Med. Exp. Salud Publica 29 (2) (2012) 265–271 (PMID:22858777).