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COMMENTARY

Anti-regulatory T cell vaccines in immunotherapy: focusing on FoxP3 as target

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ABSTRACT

Anti- tumor vaccination elicits imperfect immune responses against tumor cells; that is related to the presence of suppressive obstacles in the tumor microenvironment. The main members of suppressive milieu of tumor are heteroogenous groups of immune cells in which regulatory T cell is a substantial component. Tregs express different immunomodulatory molecules such as FoxP3. Transcription factor, FoxP3, is a specific intracellular marker of Treg and crucial for Treg development. Therefore it is an attractive target for cancer treatment. This article reviews some recent anti-Treg vaccine focusing on FoxP3 to ameliorate anti-tumor immune responses. Among them, fusion vaccine of FoxP3-Fc(IgG) recombinant DNA vaccine and its accordant protein vaccine represents effective results.

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Background

To date, various means of active immunotherapy against tumors have been employed to empower immune system including anti-tumor vaccination combined with other means (for example by depletion of suppressory factors of immune system) to improve immunological situation for more effective immune responses[1] Depletion of suppressory factors in tumor microenvironment had been assessed in different studies in which targeting regulatory T cells (Treg) is crucial as the main obstacle tempering successful immunotherapy and active vaccination[1]

The major components of the suppressive compartment in tumor microenvironment are a group of heterogeneous immune cells and some secretory mediators[2] Cancer associated fibroblasts (CAFs) are a specialized subpopulation of fibroblasts which play their role actively in tumor growth and metastasis. Because of production of cytokines, chemokines and release of proinflammatory and proangiogenic factors, CAFs provide a proper condition for tumor[3] Mesenchymal stem cells (MSCs) are multipotent, non-hematopoietic cells which are able to migrate to tumor microenvironment following induction by chemokines or inflammatory factors. MSCs recruited to the tumor microenvironment play different tumor promoting roles such as increasing stemness of tumor cells, inducing migration, mediating angiogenesis, suppressing immune system and inducing drug resistance[4] Besides, myeloid- derived suppressor cells (MDSCs) are a heterogeneous cell population composed mainly of myeloid progenitor cells that do not completely differentiate. This subset of myeloid cells can be scaled up to 10 fold in various cancers; which is accompanied with nearly blocked differentiation and acquisition of suppressive activities.[5] Therefore, MDSCs are important regulators of anti-tumor immunity due to their inhibition of both tumor specific and non-specific T cell responses[6-9] Meanwhile, tumor associated macrophage (TAMs) which are M2-polarized could compose of a malignant tumor mass. TAMs with M2 phenotype are characterized by production of low amount of inflammatory cytokines and high levels of Tumor growth factor-β (TGF-β) and also are capable of promoting tumor growth, neoangiogenesis, invasion and metastasis.[10,11]

Treg

Since their first observation in 1970s, Tregs were defined as antigen specific and once activating cells which could target CD4 + T helper to block activation and progression of both humoral and cellular immunity[12] Tregs are universally characterized by concurrent expression of CD4, CD25 (IL2 receptor component) and intracellular expression of the transcription factor FoxP3, have crucial role in immune homeostasis.[13]

To date, there are about five defined populations of Tregs: 1) Naturally occurring Tregs (nTregs) which are thymic- derived cells entering peripheral blood after propagation and can be activated by antigen-MHCII complex[1] 2) Inducible Tregs (iTregs) such as T CD4+ CD25- FoxP3- T cells.

nTregs acquire their immunosuppressive characteristics in function and peripherally under the influence of cytokine microenvironment[12] Huge mass of self-antigens in the tumor microenvironment and regional draining lymph nodes convert existing dendritic cells (DCs) into tolergenic DCs which express inhibitory coreceptors and induce conversion of naïve T cells into iTregs.[1,14,15] iTregs need antigen in the presence of MHCII in addition to stimulation of costimulatory molecules to be activated[12]

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3) Adoptive Tregs(Tr1) differ with nTreg due to their high amount release of TGF-β and IL10 which enables them to suppress function of both memory and naïve T CD4 +[12] 4) T helper3(Th3) is another subset of Tregs which are crucial in maintenance of oral tolerance[12] 5) CD8+ Tregs which are induced by plasmacytoid dendritic cells in tumor microenvironment and inhibit the function of tumor antigen specific effector T cells by producing IL10.[12]

Different markers have been proposed to further define the phenotype of Tregs, including CD25, cytotoxic T lymphocyte antigen-4(CTLA-4), glucocorticoid induced TNFR- related protein(GITR), lymphocyte activation gene 3(LAG3), CD127 and FoxP3.[16–20] Among them FoxP3 is the most specific identifier of this population; since most activated CD4+ and CD8 + T cells can transiently express other mentioned markers[21]

Engagement of Tregs in progression of cancer was first identified in 1970. As several animal studies have demonstrated that highly immunogenic tumors will progress and no tumor depletion occurs despite induced antitumor responses.[1,22] Increased number of Tregs have been reported in the peripheral ascites, tumor tissue and draining lymph nodes of tumors in vast types of solid tumors in lungs, head and neck, digestive system and ovary. A direct correlation has been shown between the accumulation of Tregs in solid tumors and bad prognosis of the disease[23] Treg cells express CC-chemokine receptor 4(CCR4) and CC-chemokine receptor 8(CCR8). On the other side abundant expression of CC-chemokine ligand 2 and 22(CCL2 and CCL22 the ligands for CCR4) by tumor cells stimulate Tregs tumor infiltration. As well as tumor cells, dendritic cells and tumor infiltrating macrophages could produce CCL22 to recruit CCR4 expressing Tregs to the tumor site.[23–25]

Possible suppressive mechanisms of regulatory T cells which have been addressed in different mouse model studies consist of: (a) induction of B7-H4 inhibitory molecules expression by APCs which can negatively regulate T cell responses. (b) secretion of perforin and granzyme B by activated Tregs which induce apoptosis in effector T cells and APCs. (c) induction of indoleamine 2,3 dioxygenase (IDO) expression by APCs which in turn suppress effector T cell activation by reducing essential amino acid, tryptophan. (d) Release of IL10 and TGF-β to inhibit T cell activation and suppress APC function.[1,26,27]

Means of Tregs depletion

A potential application of anti-Treg strategies is to augment the immune response in the field of immunotherapy. Tumor growth is proposed to be consequence of the lack of proper immune response to tumor antigens, and increased Treg amount may lead to poor immune response to cancer. Diminishing the count of Treg in the body may improve the immune response to weak tumor antigens[28,29] Tumor vaccines for treatment or prevention of recurrence of cancer have been of great interest, but there is a challenge to their efficacy about an immune suppressive effect of the tumor microenvironment on T cell expansion by suppressory cells such as Tregs. Several studies demonstrated the role of Tregs in the impaired host immune response against tumor. Augmented Treg levels in the peripheral blood, regional draining lymph nodes and the tumor microenvironment are accompanied with reduced survival. Depletion of Tregs in experimental models led to elevated anti-tumor responses. Human studies have also implicated the contribution of Treg depletion before immunization in enhancement of tumor antigen-specific T cell responses[30-33]

To date, multiple strategies have been proposed for depleting regulatory T cells.

One of the first considered means was using low doses of cyclophosphamide as a chemotherapy agent which strikingly induced inhibition of Treg function and expansion as well as decreased tumor growth. Although the effects of cyclophosphamide on Tregs was not specified and could also deplete tumor antigen-specific T CD8+ cells. Collectively increased evidence implicated that in the absence of Tregs by using cyclophosphamide, the process of immune priming would be also influenced and devastated the efficacy of this treatment.[15,23] Some other chemotherapy drugs as standard treatments in controlling Tregs are gemcitabine, mitoxantron, fludarabin and COX inhibitors. However, suppression of Tregs is not the main mechanism of these drugs at all; but as a second effect they could spoil Tregs.[12]

Early animal studies have showed that in vivo administration of CD25-specific antibody prophylactically and just before tumor induction could induce effective anti-tumor responses even to liquidation of tumor. Although administration of anti-CD25 after tumor induction was damnably less effective and the reason was due to expression of CD25 on activated CD4+ and CD8 + T cells whose expansion was hindered because of anti-CD 25.[15,23] Several lines of evidences have designed some toxic recombinant proteins to target high expressing CD25 Tregs. LMB2 is a fusion protein of the anti-CD25 monoclonal antibody as single chain variable fragment(SCFV) attached to a fragment of exotoxin A of pseudomonas. This recombinant protein has shown positive effects in CD4+ CD25+ Treg depletion. In addition, denileukin diftitox (DD) or ONTAK is another fusion protein composed of the active domain of diphtheria toxin and IL2. Denileukin diftitox binds to cells expressing high levels of CD25, whereupon it will be internalized leading to direct anti-tumor activity against malignancies[34,35] GITR is a costimulatory molecule which is expressed on the surface of Treg cells but is also expressed to various degrees on CD4+ and CD8 + T cells. Evidences have reported that administration of GITR-specific antibody or GITR ligation directly could inhibit suppressory activity of CD4+ CD24+ Tregs[15,23] CTLA-4 which is an inhibitory coreceptor is expressed by activated T cell to sustain hemostasis of immune responses. CD4+ CD25+ Treg cell constitutively express CTLA-4 and increase its expression after TCR stimulation and inhibition of CTLA-4 on Tregs led to anti-tumor responses.[15,23] In addition to molecules mentioned above, OX40 is also a costimulatory molecule belonged to TNF-receptor superfamily which is expressed temporarily on activated T cells and constitutively on Tregs. Several reports have demonstrated that using agonistic anti-OX40 monoclonal antibody not only stimulated effector T cells but also blocked inhibitory activity of Tregs.[36] FoxP3+ Tregs in rodent express high level of folate receptor 4(FR4) compared to FoxP3- naïve T cells

after TCR stimulation. Accordingly activated Tregs and effector T cells can be distinguished, then administration of anti-FR4 monoclonal antibody could considerably deplete activated Tregs meanwhile maintaining activated effector T cell which leads to impressive stimulation of anti-tumor immunity[23] Studies have reported toll like receptor (TLR) signaling in DCs caused resistance in T cells against Treg induced suppression. TLR ligands could also directly conquer suppressory function of Tregs[12]

There are some other surveys on means which could be applied in suppression of Tregs. Imatinib is an inhibitor of tyrosin-kinase which could significantly decrease expression of CD69, GITR, CTLA-4, FoxP3 and secretion of IL10 and TGF-β by Treg in a dose-dependent manner. Therefore, Imatinib is an effective drug in suppression of Tregs and intensifying effects of anti-tumor immunotherapy.[12] Bevicuzimab is an antibody effective in rejection of tumor angiogenesis which operates by inhibition of tyrosin-kinases. In cancer patients treated by Bevicuzimab a slump of Tregs count was reported among clinical responders, although the real mechanism is still undetermined. Recently an in vitro study has shown that Lenalidomide and CC-4047 could inhibit expression and function of Treg which could be concluded from induction of diminution in FoxP3 expression[12]

Anti-Treg vaccine focusing FoxP3

One of the most important advances in the field of Treg investigations achieved due to detection of the transcription factor called FoxP3. This factor considerably helped the phenotypic distinction of suppressory CD4+ CD25 + T cells from effector cells.[14] FoxP3 transcription factor has been presented as a specific intracellular marker of Tregs, which is expressed not only in CD4+ CD25+ and CD4+ CD25low/- cells but also in CD8+ cells with inhibitory performance. Thereafter, the notion of targeting FoxP3 instead of CD25 was revived and thereupon tumor immunotherapy was evolved[37] Indeed, FoxP3 is a nuclear product which is not expressed on the cell surface unlike CD25. Hence, usage of monoclonal antibodies is not effective for destroying of FoxP3 expressing cells[37] conventional immunotherapy reinforced the immune system and also applied required immune components, such as tumor specific antigens, antigen presenting cells(APCs), effector T cells, cytokines and chemokines to intensifying tumor specific antigens immunity. Some clinical trials with conventional immunotherapy have been hopeful, as regards it still needs developments in clinical effectiveness[1] Recent strategies in immunotherapy have aimed immunosuppressive elements of tumors such as Tregs, inhibitory molecules and dysfunctional APCs to recover tumor specific immunity. Combinatorial therapy targeting Tregs and suppressory molecules which contains traditional therapy, conventional and novel immunotherapy is essential to attain efficient, comprehensive and reliable clinical treatments.[1] Vaccination focusing on FoxP3 may provide a simple and specific protocol for the extended control of Tregs leading to diminished probability of autoimmunity. This achievement offers a strategy for specific elimination of cells not exclusive for targeting cell surface products of the cells; since FoxP3 is not expressed on the cell surface and unlike CD25 cannot be targeted by antibodies.[37] In a study by Smita Nair et. al published in 2007, depletion of FoxP3+ Tregs using DCs pulsed with FoxP3 mRNA led to strong CTL response against FoxP3+ Treg and its accompaniment with DC vaccine would intensify induced anti-tumor response by DC vaccine[37] Furthermore in some other researches, FoxP3 has been targeted to deplete Tregs. For instance, transgenic mice expressing diphtheria toxin receptor gene under control of FoxP3 gene promoter have been designed; in which injection of diphtheria toxin might cause depletion of FoxP3 expressing cells. Results at above study suggested positive effects for depleting FoxP3 expressing cells on promotion of anti-tumor vaccine[38,39]

Recently in a study by Franco-Molina M. A. et. al inoculation of FoxP3-silenced B16F10 melanoma cell line revealed delayed tumor appearance, decreased weight of tumor and production of IL10, IL2 and TGF-β and increased time of survival compared with mice injected with wild type cell line[40] Their results highlights the crucial role of FoxP3 expression not only in Tregs but also in tumor cells in inducing tumor growth. Since FoxP3 partly induce tumor growth by modifying the immune system and tumor environment toward an immunosuppressive profile.[40] Likewise, tumor cell vaccine combined with FoxP3 gene silencing can empower the efficacy of therapeutic antitumor vaccination[41]

Considering the above evidences makes FoxP3 an attractive target for cancer treatment.

Improved Anti-FoxP3+Treg vaccine

As we know, CD8+ CTLs can distinguish each cellular product in association with MHC I molecules on the cell surface.[42] Accordingly, in our recent study, we could achieve FoxP3-Fc (IgG) expressing DNA vaccine and its correspondent recombinant protein through cloning truncated FoxP3 gene (lacking effector function) fused to fragment C (Fc) IgG in proper vectors.[43] Subsequently, vaccination of mice with Fox-Fc DNA vaccine/recombinant FoxP3-Fc fusion protein was performed to induce CTL response against FoxP+ Treg[44] and finally to access the effect of this protocol of vaccination in improvement of anti-tumor DC vaccination. (Unpublished data)

The present strategy has been employed in multiple projects aiming to increment of vaccination efficiency. Whole obtained outcomes demonstrated wide increase of antigen-specific responses about CD4 + T helper cells, CD8+ cytotoxic T cells and B cells.[45–48] Due to the results, the strategy of inserting Fc in vaccine design and immunization protocol of DNA/protein could elicit CTL responses against FoxP3 in mice model.

Flocytometric analysis stated the impact of anti-FoxP3 CTL responses in reducing significantly FoxP3 expressing Tregs in spleen of mice[44] In this protocol of vaccination against FoxP3 expressing cells, tolerance for FoxP3 was broken up not only in CTL responses but also in IFN-γ producing T helper cells (showed by ELISA evaluation) and in T helper1-dependent humoral responses (IgG2b).[44,49]

What could be the reasons for the absence of tolerance to FoxP3 in T helper1 compartment? One possibility is that the DNA vaccines could properly trigger both pathways of antigen presentation. Owing to presentation of included gene product associated with MHC I to cytotoxic T cells and also

along with MHC II to helper T cells, DNA vaccine is capable of stimulation of both cell compartments[50] A second reason could be about upgraded immunization process related to vaccine design in the form of fusion vaccine containing Fc(IgG); which can potently capture and present antigens. This might provide immune system with improved and comprehensive stimulation against antigens.[45]

Conclusions

It seems, drastic immunotherapy inevitably will need supplementary arrangements to successfully deplete immunosuppressory agents in tumor condition. At the moment, vast infiltration of Tregs in to tumor and regional lymph nodes is a crucial reason for incapacitation of anti-tumor responses. Future studies will need to explore the mechanism underlying suppression of anti-tumor responses by Treg. Though some studies suggested decrease in count and function of cytotoxic CD8 + T cell as a main cause, whereas there is a reverse relation between presence of Tregs and the power of anti-tumor cytotoxic responses[51] As a major obstacle for prospering immunotherapy Treg is a highly suitable target in novel means of immunotherapy[39]

To date, several strategies have been described to target Tregs, among them depleting Treg by different means was the most important[37-39,44,52] Elimination of Tregs has been studied abundantly from which, some have reached to clinical trial.[51]

In summary, application of Fc(IgG) fusion in vaccine design and performing vaccination protocol of DNA vaccine for priming and its recombinant protein counterpart for boosting could set up favored immunization procedure against FoxP3.[44,49]

Disclosure of potential conflicts of interest

No potential conflict of interest was reported by the authors.

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