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Roles of CD4+CD25^{high} FOXP3+ Tregs in lymphomas and tumors are complex

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TABLE OF CONTENTS

1. ABSTRACT

CD4+CD25^{high}FOXP3+ regulatory T cells (Tregs) play an important role in the maintenance of immunological self-tolerance by suppressing autoimmune responses and anti-tumor immune responses. The current model suggests that epithelial tumor cells recruit Tregs to inhibit anti-tumor immunity in the tumor microenvironment, which thus limits the efficiency of anti-tumor immune responses and immunotherapy. However, recent findings on Tregs in lymphomas have complicated this working model. The biopsy specimens of some lymphomas have significantly higher percentages of Tregs than that in tumor-free lymph nodes and normal peripheral mononuclear cells. Higher Tregs numbers in these lymphomas predict improved survival and prognosis of patients. In this brief review, we summarize the progress in following topics: (1) Tregs; (2) Tregs and T cell co-stimulation; (3) Tregs in lymphomas; and (4) Tregs in other Tumors. Further characterization of Tregs in lymphomas and other tumors will provide insight on the differential regulation of Tregs' function and survival, and define the potentials of Tregs-based immunotherapeutics.

2. TREGS

Significant progresses have led to the renaissance of tumor immunology and studies on anti-tumor immunotherapy (1, 2). Despite this (3-17), current anti-tumor immunotherapies are far less than satisfactory, which reflects the urgent need to improve our understanding of immune tolerance on the responses elicited by self-tumor antigens and the suppression of anti-tumor immune responses. In order to develop effective antigen specific anti-tumor immunotherapies and to monitor the responses to these immunotherapies in patients with tumors, several important questions need to be addressed including: (1) What roles do CD4+CD25^{high}FOXP3+ regulatory T cells (Tregs) play in anti-tumor immune responses? (2) What roles do T cell co-stimulation molecules play in the generation and homeostasis of Tregs? (3) What is the significance of Tregs in the treatment of lymphomas and other malignancies? This brief review will overview the progress in these regards.

2.1. Tregs phenotypes

Regulatory T cells are a specialized subpopulation of T cells. As an active mechanism of immune suppression, distinct subsets of regulatory T cells

suppress the activation of autoreactive T cells that have escaped the immune tolerance mechanisms of thymic clonal deletion and T cell anergy (18) and other immune cells, and thus, maintain homeostasis of the immune system and self-tolerance. Several regulatory T cell subsets including CD4+CD25^{high}FOXP3+ T cells, CD4+CD25-PD-1+ T cells (19), CD8+ cells (20), CD8+CD25+ T cells (21, 22), CD8+CD28- T cells (31), TCRγδ+ T cells (20), NKT cells (20), and CD3+CD4-CD8-αβTCR+ T cells (23) have been reported, among which CD4+CD25^{high}FOXP3+ regulatory T cells (Tregs) are best characterized (24-28). Comprising 5-10% of peripheral CD4+ T cells (56-59), Tregs exhibit potent immunosuppressive functions (29) and play an important role in the regulation of anti-tumor immunity, autoimmunity, pathogenesis of atherosclerosis, allergy and asthma (30), transplantation immunity (31, 32), and anti-microbial immunity. There are two subsets of Tregs, (*a)* peripherally adaptive Tregs [aTreg cells, transforming growth factor-β (TGF-β) secreting, and IL-10 secreting] (33, 34), and (*b)* naturally occurring Tregs (thymic generated, nTregs). After encountering foreign antigens (35), aTregs can be generated (36) with a standardized protocol (37), which is defined by their cytokine profile, including two subsets: Treg type 1 (T_R 1) cells that secrete high levels of IL-10, and Th3 cells that secrete high levels of TGF-β (38). aTregs are concerned with ablating an ongoing immune response (39) whereas, nTregs, generated in the thymus, are primarily engaged in the maintenance of self-tolerance and down-regulation of various immune responses via a cell-cell contact manner. nTregs can be defined by the cell surface and intracellular marker profile (40)[CD4+, CD25^{high}, intracellular FOXP3+ (41), GITR+ (42), CD62L$^{+/high}$ (82), CD5+, CD27+ (43), CD38+, CD39+ (44), CD45RB^{low}, CD45RA^{low}, CD45RO^{high}, CD73+ (44), CD103 (integrin αEβ7)+ (45), intracellular CTLA-4^{high}, surface CTLA-4^{low} (86, 87), HLA-DR^{high}, CD122^{high}, CD127$^{low/-}$ (46), CD130- (47), CD134 (OX40)^{high}, LFA-3^{medium}, CCR4+ (48), CCR7+ (49), CCR8+ (50), TNFR2+ (51)] [also see excellent reviews (52, 53)].

2.2. Targets and suppression mechanisms of Tregs

nTregs can be activated by self-antigens and non-self-antigens (54). Once activated, nTregs can suppress T cells in antigen-specific and antigen-nonspecific manners. Interestingly, the suppressive effects of these cells are not restricted to the adaptive immune system which include CD4+ T cells, CD8+ T cells (55), NK cells (56, 57), and B cells (58), but can also affect the activation and function of innate immune cells including monocytes, macrophages, dendritic cells (33), and neutrophils (59). nTreg suppression *in vitro* is dependent on cell contact with target cells as shown by experiments utilizing transwells (39). One study shows that Tregs directly inhibit NKG2D-mediated NK cell cytotoxicity, largely by a TGF-β-dependent mechanism, and independently of IL-10, Tregs can effectively suppress NK cell-mediated tumor rejection (57). Blocking antibodies to IL-10 and TGF-β does not affect the nTreg suppression, and nTregs isolated from IL-10- or TGF-β-deficient mice are also functional *in vitro* (39). nTregs suppress target cells by at least four mechanisms: nTregs may kill target cells via a granzyme B-dependent pathway (60), perforin pathway (61), programmed death-1/programmed death-1 ligand pathway (62), or Fas-Fas ligand interaction in an epitope-specific manner (58).

2.3. Critical signals in Tregs

Signaling through the high-affinity interleukin-2 (IL-2) receptor has been shown to be critical for Tregs differentiation and survival *in vivo*. Mice deficient in IL-2 or its associated receptor α-chain (CD25), β-chain (CD122), or in downstream signaling molecules, including JAK-3 and STAT-5, do not develop a stable population of Tregs and subsequently, acquire lymphoproliferative disease and autoimmunity (63). Moreover, *in vitro*, IL-2 is required to expand Tregs and to induce their suppressive characteristics (63-65). IL-10 and TGF-β have been implicated in the induction of antigen-specific Tr1 and Th3 cells *in vitro* and *in vivo* (66, 67). IL-2, TGF-β, and IL-10 also influence the ability of naturally occurring and antigen-induced CD4+CD25+ aTregs to convert CD4+CD25− cells to suppressive Tregs. TGF-β plays an important role in the induction and maintenance of FOXP3 expression, suppressive function, and homeostasis in peripheral CD4+CD25+ Tregs (68, 69). FOXP3 acts as a master transcription factor for inducing a Treg phenotype and lineage. Tregs are either abnormally regulated or nonexistent in mice with genotype of FOXP3−/−, which developed some form of fatal lymphoproliferative disease. This suggests a critical role for FOXP3 in the development of self-tolerance mediated through Tregs (70, 71). In some tumors, the presence of Tregs in the tumor microenvironment diminishes anti-tumor immune responses and might explain why current clinical trials using cancer peptides or dendritic cells (DCs) pulsed with antigenic peptides induce only transient immune responses and fail to have a therapeutic benefit. The enthusiasm in exploring the therapeutic potentials of Tregs has tremendously increased, driven by the need to develop strategies that prevent autoimmune diseases, transplant rejection, and inhibit progression of tumors and chronic viral diseases.

3. TREGS AND T CELL CO-STIMULATION

3.1. Co-stimulatory and co-inhibitory pathways

Immature DCs tend to induce immune tolerance rather than immunity by either deleting reactive T cells or inducing regulatory T cells (72). Some studies suggest that in addition to eliciting potent effector T cell responses, DCs have the ability to induce and expand CD4+ Tregs (73). Numerous factors have been found affecting the generation, homeostasis, and suppressive function of Tregs, including T cell co-stimulatory molecules [also see a recent review by Yang, XF (40)]. Among the factors well-characterized or under characterization, T cell co-stimulatory molecules expressed on the cell surface of DCs play a major role. Two major superfamilies of co-stimulatory/co-inhibitory molecules have been characterized; tumor necrosis factor receptor (TNFR) superfamily and immunoglobulin superfamily. The TNFR family includes several interaction pairs of molecules, such as CD30L/CD30, LIGHT/HVEM, OX40L/OX40, 4-1BBL/4-1BB, CD70/CD27, and GITR-

L/GITR. The immunoglobulin co-stimulatory family includes pairs of molecules, such as B7-1/2/CD28, ICOSL/ICOS, B7-1/2/CTLA-4, PD-L1/2/PD-1, and HVEM/BTLA. The interaction of B7-1/2/CD28 and ICOSL/ICOS provides co-stimulatory signals whereas the interaction of B7-1/2/CTLA-4, PD-L1/2/PD-1, and HVEM/BTLA provide co-inhibitory signals (74). As magic bullets to target tumors, the exquisite specificity and unlimited supply of monoclonal antibodies (mAbs) are being discovered with 18 mAbs now having FDA (Federal Drug Administration) approval for therapeutic use (74). Recent reports showed that some of the mAbs to co-stimulatory molecules have significant potential for clinical use in regulating the homeostasis and suppressive function of Tregs (75).

3.2. B7-CD28

CD28 is one of the molecules expressed on T cells that provide co-stimulatory signals, which are required for T cell activation. CD28 is the receptor for B7.1 (CD80) and B7.2 (CD86). As low as 10% in B7 deficient mice and 25% in CD28 deficient mice have decreased nTregs in the spleen in comparison to that in wild-type mice (76). CD28-B7 co-stimulatory interactions may also play an important role in Treg development, as illustrated by the reduced Treg populations in both CD28−/− and B7-1/B7-2 double knockout mice. In addition, the use of super-agonist anti-CD28 antibodies *in vivo* results in the rapid expansion of Tregs (77), and rodent experimental data suggest that they may be useful in the treatment of autoimmune diseases (78) and graft-versus-host disease (75, 79). Thus, B7/CD28 T cell co-stimulation plays an essential role in the generation and survival of nTregs (76, 80-86). It is important to note that CD28 co-stimulation provides more IL-2 compared to developing Tregs, as CD28 co-stimulation of TCR-signaled double-positive thymocytes induces expression of FOXP3 as well as GITR and CTLA-4, two proteins highly expressed on Tregs (87). Some investigators showed that the CD28-mediated homeostasis of nTregs is independent of IL-2, OX40, CD40L, and survival factor Bcl-xL (88). However, others have found that IL-2 plays a critical role in the maintenance of nTregs *in vivo* by regulating FOXP3 expression through a STAT-dependent mechanism and inducing the expansion of these cells *in vivo (89)*.

CD7 and CD28 are T cell Ig superfamily molecules that share common signaling mechanisms. CD28-deficient mice have decreased nTregs in the spleen compared with wild-type mice, and CD7/CD28-double-deficient mice have further decreased numbers of nTreg cells in both the thymus and spleen compared with both wild-type and CD28-deficient mice (83). Tregs from CD28-deficient mice and CD7/CD28-double-deficient mice may mediate suppression of CD3 mAb activation of CD4+CD25- wild-type T cells, but are less potent than wild-type Tregs (83). These results suggest that there is a synergy of CD7 and CD28 T cell co-stimulation in regulating the generation and suppressive function of Tregs (83).

It was recently reported that the B7 molecule is required for the conversion of naïve T cells to Tregs *in vivo* (90). Therefore, tumor-infiltrating APCs and co-stimulatory molecules can contribute to the conversion of naïve T cells to Tregs.

3.3. Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4)

CTLA-4 is a CD28-family receptor expressed mainly on CD4+ T cells. It binds the same ligands as CD28 (CD80 and CD86 on B cells and dendritic cells), but with higher affinity. However, in contrast to CD28, which enhances cell function when bound at the same time as the T cell receptor, CTLA-4 inhibits the T cell and prevents it from functioning. CTLA-4 expression is not required for nTreg development or function since in CTLA-4-deficient mice Treg development and homeostasis is normal (91). However, non-activating anti-CTLA-4 antibodies block the suppressor activity of Tregs *in vitro*. Of note, clinical application of co-stimulatory blockade using agents such as CTLA-4Ig in the treatment of autoimmune disease results in complicated outcomes (88). Based on the results of anti-CTLA-4 therapy in mouse tumor models, two human anti-CTLA-4 antibodies, MDX-010 (Ipilimumab) and CP-675, 206 (Ticilimumab) were developed and have entered into clinical trials at multiple centers. Anti-CTLA-4 monotherapy is capable of inducing objective tumor responses, partial responses, or even complete responses in patients with melanoma, renal cell, prostate carcinoma, and non-Hodgkin's lymphoma (NHL) (92, 93).

3.4. PD-1/PD-L1

Adult T-cell leukemia/lymphoma (ATLL) is a CD4+CD25+ T-cell malignancy infected with human T-cell leukemia virus type-I (HTLV-I) (94, 95). HTLV-I infection causes T-cell dysfunction, which contributes to the immuno-deficient state of the patients. Programmed death-1 (PD-1) can downregulate T-cell response and allo-immune responses (96) when its ligand, PD-L1 or PD-L2 which is mainly expressed on antigen presenting cells, binds to this B7 family receptor. The levels of PD-1 expression in both CD4+CD25+ and CD4+CD25- T-cell populations are increased in ATLL patients compared to normal healthy volunteers, while PD-1 levels on CD8+ T-cells are comparable between the patients and normal subjects. With the stimulation of an anti-CD3 antibody, the proliferation of PD-1-expressing T-cells from ATLL patients is weak compared to that of PD-1-nonexpressing normal T-cells. In addition to PD-1, PD-L1 is co-expressed on ATLL cells in some patients, and PD-L1 expression is enhanced by the stimulation with an anti-CD3 antibody. These findings suggest that CD4+ T-cells are the main PD-1-expressing cells rather than CD8+ T-cells in ATLL patients, and both neoplastic and normal CD4+ cells are exhausted as a result of PD-1 expression. B7-H1 (PD-1L)/PD-1 interaction is involved in Tregs-mediated suppression of infiltrating CD4+CD25-T cells in B-cell NHL (97). Blocking the interaction between B7-H1 and PD-1 partly attenuates Tregs-mediated inhibition of CD4+CD25-T cells (98), suggesting that there is a therapeutic potential of blocking PD-1L-PD-1 interaction. In addition, PD-L1, but not PD-1 or PD-L2, blockade accelerates the rejection of MHC class II-mismatched mouse skin graft [bm12 (I-Abm12) into B6 (I-Ab)] in a similar manner as CTLA-4 blockade (96), suggesting that PD-L1 blockage may inhibit Treg suppression function.

3.5. GITR and other TNF superfamily members

Glucocorticoid-induced tumor necrosis factor (TNF), receptor-related protein (TNF superfamily member 18, TNFSF18, GITR), and its ligand (GITR-L) play an important role in the control of nTreg activity. GITR is a constitutively expressed marker for nTregs, but is also upregulated on activated CD4+ T cells (42). Blocking GITR with an anti-mouse GITR mAb inhibits nTreg suppression function *in vitro* and leads to the induction of autoimmunity (99). Furthermore, GITR-L blocks *in vitro* suppression mediated by nTregs (100, 101). These results suggest that the interaction of GITR-GITR-L is important for Treg suppression.

3.6. OX40 (CD134)

OX40 (CD134) is a member of the TNF receptor family that is transiently expressed on T cells after TCR ligation. Both naive and activated Tregs express OX40. Triggering of OX40 on CD4+CD25+ Treg cells with agonist antibodies blocks their inhibitory activity (102), suggesting that OX40 co-stimulation turns off FOXP3+ Tregs suppression (103).

3.7. 4-1BB (CD137)-4-1BB ligand

4-1BB (CDw 137), a member of the TNFR superfamily, is a costimulatory receptor primarily expressed on activated T cells. The administration of agonistic anti-4-1BB mAb enhances tumor immunity and allogenic immune responses. nTregs from 4-1BB-deficient mice are able to prevent naive CD4+ T cell-induced colitis. Paradoxically, others reported that administration of agonistic anti-4-1BB monoclonal antibody leads to the increased number of splenic Tregs and amelioration of TNBS-induced inflammatory bowel disease (colitis). Taken together, both immunoglobulin superfamily and TNF superfamily co-stimulatory /co-inhibitory members play critical roles in regulating Tregs survival as well as suppressing the function of Tregs. This suggests again that new mAbs-based therapeutics which either stimulate or inhibit co-stimulatory molecules may have significant potential for clinical use in regulating the homeostasis and suppressive function of Tregs (75).

4. TREGS IN LYMPHOMAS

4.1. Malignant lymphomas

Primary neoplasms of lymphoid tissue derived from lymphocytes occur as solid tumors, usually within lymph nodes and less often in extranodal lymphoid tissues such as the tonsil and gastrointestinal tract. Lymphomas are a group of serious and frequently fatal diseases. Malignant lymphomas are classified as NHL, which are derived from lymphocytes, and Hodgkin's lymphomas (HL). Hodgkin's lymphomas have retained their eponymous designation because the cell of origin remains uncertain. According to the World Health Organization Classification of Tumors (104), lymphomas are variable in clinical characteristics, and the molecular and cellular mechanisms responsible for the clinical heterogeneity of lymphoma are largely unknown. A recent report from Ke XY' s team showed that detection rates of t (11;18) translocation in Chinese patients with MALT lymphoma is different from that found in other countries, also suggesting the heterogeneity of lymphomas (105). Bearing the heterogeneity of lymphomas in mind, ones should easily expect a complicated scenario for Tregs function in lymphomas. Based on Tregs roles characterized in lymphomas, we can categorize various roles of Tregs into three groups (106): (1) Malignant Tregs: FOXP3 is a selective marker for a subset of adult T cell leukemia/lymphoma, suggesting that Tregs can be malignant (107); (2) Direct tumor-killing Tregs: Some B cell lymphoma cells can be target cells for Tregs suppressive cytotoxicity, suggesting that Tregs are tumor cell-killers (106); and (3) Suppressor Tregs: Tregs suppression of anti-tumor CD8+ T cell-mediated immune responses is also observed in various lymphomas, which is similar to that reported for other solid tumors (108). The classification into three groups is significant in designing Tregs-based immunotherapies for treating lymphomas in the future. For example, in the patients with lymphomas where Tregs serve as either malignant Tregs or suppressor Tregs, decreased numbers of Tregs are associated with a good prognosis. In contrast, patients with lymphomas where Tregs serve as tumor-killing Tregs, increased numbers of Tregs are associated with a good prognosis.

4.2. Malignant Tregs

Although ATLL cells (94, 95) display an activated helper/inducer T-cell phenotype, CD4+ and CD25+, they are known to exhibit strong immunosuppressive activity. In primary ATLL cells, the expression levels of FOXP3 and GITR are significantly higher than those from healthy adults (107). Furthermore, ATLL cells are unresponsive *in vitro* to concanavalin A stimulation, and suppress the proliferation of normal T cells. Taken together, ATLL cells may originate from HTLV-I-infected Tregs. GITR seems to be involved in the progression to ATLL (109). Analysis of the expression of FOXP3 on ATLL cells from 17 patients showed that FOXP3 expression in 10 ATLL cases, but was relatively down-regulated compared with Tregs from normal subjects (95).

In addition, in a subset of patients with CCR4+ (a chemokine receptor) T-cell leukemia/lymphoma, the tumor cells themselves function as Tregs, contributing to tumor survival in the face of host anti-tumor immune responses. In other types of cancers, the chemokines TARC/CCL17 and MDC/CCL22, specific ligands for CCR4 that are produced by tumor cells and the tumor microenvironment, attract CCR4+ Tregs to the tumor, where they create a favorable environment for tumor escape from host immune responses. Ishida T *et al* are now conducting a phase I clinical trial of an anti-CCR4 mAb (KM2760) (110) in patients with CCR4+ T-cell leukemia/lymphoma in Japan (clinical trials gov. identifier: NCT00355472). Anti-CCR4 mAb may be an ideal treatment modality for many different cancers, not only to directly kill the CCR4+ tumor cells, but also to overcome the suppressive effect of CCR4+ Tregs on the host's immune response to tumor cells (111, 112). These reports show that ATLL cells from a subset of patients function as Tregs in an autologous setting (113, 114).

Mycosis fungoides (MF) is a low-grade lymphoma cluster of differentiation CD4+, CD45RO+, cutaneous leukocyte antigen (CLA)+ T cells that homes to the skin. In the analysis of PBMCs in MF, data shows that CTLA-4 is stimulated by phorbol myristate acetate/A23187 to greater levels compared to the normal. This defect was seen in the dominant clones of T cells. The increased CTLA-4 expression was significant between normal patients and those with MF, with a correlation between higher expression of CTLA-4 and a higher grade of MF. In a patient whose disease progressed, the CTLA-4 level increased. The abnormal level of CTLA-4 was confirmed at both the transcription and translation levels. Although MF is associated with a Th2 bias, Th1 cytokines IL-2 and IFN- γ enhanced CTLA-4 expression while IL-4 did not. These findings reveal an abnormal regulation of CTLA-4 expression in MF and show that PBMCs from patients with MF have properties that are divergent from those of normal T cells (115). If indeed MF/SS is a tumor of Tregs, this may be an additional compelling explanation behind the immunosuppression seen in advanced disease, which is further supported by a recent report that Tregs phenotype may be associated with large cell transformation of MF (116). Moreover, cutaneous T-cell lymphoma (CTCL) cells also adopt a T-regulatory (Treg) phenotype expressing CD25/CTLA-4 and FoxP3 and secreting IL-10 and TGF-β (117). In ALCL, the Nucleophosmin/anaplastic lymphoma kinase **(**NPM/ALK)-carrying T cell lymphoma (ALK+TCL) cells secrete IL-10 and TGF-β and express FOXP3, indicating their Tregs phenotype. The secreted IL-10 suppresses proliferation of normal immune CD3/CD28-stimulated peripheral blood mononuclear cells and enhances viability of the ALK+TCL cells. The Treg phenotype of the affected cells is strictly dependent on NPM/ALK expression and function as demonstrated by transfection of the kinase into BaF3 cells and inhibition of its enzymatic activity and expression in ALK+TCL cells. NPM/ALK, in turn, induces the phenotype through activation of its key signal transmitter, signal transducer and activator of transcription 3 (STAT3). These findings identify a mechanism of NPM/ALK-mediated oncogenesis based on the induction of the Treg phenotype of transformed CD4+ T cells (118).

4.3. Direct tumor-killing Tregs

In addition to the malignancy of Tregs (107), Tregs can directly suppress B cell lymphoma cells (106), presumably due to the Tregs' capability of suppressing B cells (119, 120) via the suppression mechanisms discussed in section 2.2. In solid tumors, elevated numbers of Tregs are associated with a poor prognosis. In contrast, Gjerdrum *et al* reported that in the 85 cases with mycosis fungoides (MF) and cutaneous T-cell lymphoma (CTCL) unspecified, the atypical neoplastic infiltrate was either FOXP3 negative (n=80) or contained only very occasional weakly positive cells (n=5). MF patients with early or infiltrated plaques had significantly higher numbers of FOXP3+ Tregs than CTCL unspecified or advanced MF patients with tumors or transformations to large cell lymphomas. An analysis of all patients demonstrated that increasing numbers of FOXP3+ Tregs are associated with improved survival in patients with both MF and CTCL unspecified. This report indicates that the presence of FOXP3+ Tregs in CTCL is associated with disease stage and patient survival (121). Under certain *in vitro* conditions, malignant CD4+ T cells derived from patients with CTCL can be induced to demonstrate a CD25+ Treg phenotype (117). CTCL cells adopt a Treg phenotype expressing CD25/CTLA-4, FOXP3 and secreting IL-10 and TGF-β. Tregs CTCL cells suppress normal T-cell antigen-driven secretion of IL-2 and IFN-γ. Blocking DC MHC class II expression or transport inhibited the CTCL cell adoption of a Tregs phenotype.

Similarly, Tiemessen MM *et al* investigated the percentage, phenotype, and suppressive function of Tregs from peripheral blood of CTCL patients. Although the percentage of Tregs did not differ significantly between patients and controls, functional assays demonstrated a dichotomy in Tregs function: in four out of 10 patients, CD4+CD25+ T cells were incapable of suppressing autologous CD4+CD25- T-cell proliferation, whereas suppressive function was intact in the other six patients. Suppressive activity of Tregs inversely correlated with the peripheral blood tumor burden. In the dysfunction patients, expression of FOXP3 in the CD4+CD25+ Tregs was reduced compared with the normal function CTCL patients and controls. These findings indicate a dysfunction of peripheral Tregs in certain CTCL patients, which correlates with tumor burden (115). In other words, the presence of increased numbers of activated intra-tumoral CD4+T cells predicts a better overall survival in patients with large B-cell NHL(122). In addition, Carreras J *et al* examined samples from patients with follicular lymphoma (FL) and diffuse large B cell lymphoma (DLBCL). Their results showed that patients with very low numbers of Tregs (< 5%) presented more frequently with refractory disease. The prognostic significance of Tregs numbers was independent of the follicular lymphoma international prognostic index (FLIPI). DLBCLs had lower Tregs percentages than the grades 1, 2 or 3 of FL, which indicates that in FL, higher Tregs numbers predict better survival of FL patients. Piris' lab also found that low infiltration of FOXP3+ cells in conjunction with high infiltration of TIA-1+ cells in cHL may represent biological markers predicting an unfavorable outcome (98). Moreover, in addition to Tregs numbers, Lee *et al* showed that CD4 and FOXP3 expression were significantly different between the long-survival group (live longer than 15 years) and short survival group (live less than 5 years). Samples from the long-survival group were more likely to have CD4+ staining cells and FOXP3-positive cells in a perifollicular location (123). Andersson's lab showed that the number of tumor-infiltrating TIA-1+ cytotoxic T cells, but not FOXP3+ regulatory T cells, predicts outcome in DLBCL, suggesting that more work needs to be done in order to consolidate the finding regarding direct tumor killing Tregs and survival (124).

4.4. Suppressor Tregs

Similar to the reports for other solid tumors (108), Tregs suppression of anti-tumor immune responses is also observed in various lymphomas. HL is characterized by the presence of a small number of tumor cells in a rich background of inflammatory cells, but the contribution of the abundant non-tumor cells to HL pathogenesis is poorly understood. Migratory CD4+ cells

induced by HL cells were hypo-responsive to T cell receptor stimulation and suppressed the activation/proliferation of the effector CD4+ T cells in an autologous setting. HL cells in the affected lymph nodes were surrounded by a large number of lymphocytes expressing both CC chemokine receptor 4 (CCR4) and FOXP3 (125). These findings indicate that the migratory cells induced by HL cells function as Tregs so that these cells may create a favorable environment for the tumor cells to escape from the host's immune system. A chimeric anti-CCR4 monoclonal antibody (mAb) could deplete CCR4+ T cells and inhibit the migration of CD4+CD25+ T cells *in vitro*. Recognition of the importance of CCR4+ Tregs in the pathogenesis of HL will allow the rational design of more effective treatments, such as an anti-CCR4 mAb to overcome the suppressive effects of CCR4+ Tregs on the host's immune response to tumor cells. In B-cell NHL, the frequency of Tregs is significantly higher in biopsy specimens from B-cell NHL compared with those from peripheral blood mononuclear cells (PBMCs) in healthy individuals or tumor cell-free lymph nodes. Intra-tumoral Tregs inhibit the secretion of IFN-γ and IL-4 by infiltrating CD4+CD25- T cells in B-cell NHL (126). The inhibition of cytokine production by infiltrating CD4+CD25- T cells may be one of the mechanisms by which Tregs mediates suppression of cell division in addition to the Tregs suppression via several cytotoxic mechanisms described in the section 2.2.

The following reports suggest various mechanisms in inducing high numbers of Tregs in lymphomas. A flow cytometry analysis demonstrated that HLILs (HL-infiltrating lymphocytes) contained large populations of CD4+CD25+ Tregs, and LMP1 epitopes can induce HLIL Tregs (127). In addition, a recent report from Shipp's team showed that the AP-1 dependent secretion of galectin-1 by Reed Sternberg cells fosters immune privilege in classical HL by developing and maintaining an immunosuppressive Th2/Tregs-skewed microenvironment (128). A report using Hodgkin's Reed-Sternberg cell line (KM-H2) also showed Reed-Sternberg cells promotes differentiation of Tregs (129). Ansell's lab reported that in B-cell NHL, CD70+ lymphoma B cells significantly contributed to the activation-induced FOXP3 expression in intratumoral CD4+CD25- T cells. The blockade of CD27-CD70 interaction by anti-CD70 antibody abrogated lymphoma B-cell-mediated induction of Foxp3 expression in intratumoral CD4+CD25- T cells. These studies reveal a novel role for NHL B cells in the development of intratumoral regulatory T cells (130). In addition to the mechanisms of Tregs induction, several labs reported that intratumoral CD4+CD25+ regulatory T cells from follicular lymphoma (131) and B-cell NHL (132) suppress autologous and allogeneic CD8+CD25- and CD4+CD25- T cells.

5. TREGS IN OTHER TUMORS

5.1. Several new models in tumor immunology

The current paradigm in tumor immunity suggests that tumors express tumor-associated antigens that can be captured by host professional antigen presenting cells (APCs). The most notable of these APCs, DCs, may prime naive T cells (through the expression of cosignaling molecules, the production of soluble factors, and other mechanisms) to become antigen-specific CD8+ cytotoxic T lymphocytes (CTLs). These CTLs, when in sufficient abundance and directed against the appropriate tumor antigens, then eradicate the tumor. Despite the presence of such tumor-reactive T cells, it is rare to observe spontaneous regression of cancers (108). According to this paradigm, in order to engender curative anti-tumor immunity, one must supply more antigen or the appropriate antigen, augment the effects of the co-signaling molecules and/or soluble factors that induce the generation of antigen-specific CD8+ CTLs, or boost sufficient numbers of APCs and/or antigen-specific T cells. On the basis of this paradigm, numerous anticancer immunotherapeutic strategies have been developed. These include (i) infusing additional tumor antigen or antigen-pulsed APCs, (ii) supplying T cells generated from tumor-infiltrating lymphocytes, (iii) T cells together with a soluble growth factor, (iv) T cells activated *ex vivo* with cytokines, or (v) T cells engineered to express receptors for specific tumor-associated antigens; and (vi) boosting the effect of co-signaling molecules or activating cytokines (72). Current anti-tumor immunotherapies are far less effective than expected, which may be partially explained by several new models. *First,* Yang XF's lab found that tumor antigen CML66 has two alternatively spliced isoforms, CML66L and CML66S. CML66L, but not CML66s is the immunogenic isoform, which suggests that alternative splicing of tumor antigen regulates the immunogenicity of tumor antigens (12). Based on data from Yang XF's team and others, Yang XF recently proposed a new model of stimulation-responsive splicing for the selection of autoantigens and self-tumor antigens (5). This new model theorizes that the significantly higher rates of alternative splicing of autoantigen and self-tumor antigen transcripts that occur in response to stimuli may induce extra-thymic expression of untolerized antigen epitopes for the elicitation of autoimmune and anti-tumor responses. This model suggests that inefficiency of current antigen-immunotherapies may be due to the failure of choosing highly immunogenic isoforms of tumor antigens as immunogens (5). To extend this new model of stimulation-responsive splicing, Yang XF's team has recently reported that the expression of unconventional tumor antigens encoded by either introns of genes or secondary reading frames of mRNAs also significantly contributes to the stimulation-responsive expansion of self-antigen repertoire (14, 15). *Second,* the observation of similarities between the self-renewal mechanisms of stem cells and cancer cells has led to the new concept of the cancer stem cell. In 1994, the presence of cancerous stem cells in acute lymphocytic leukemia was documented by cloning such cells and documenting their self-renewing capacity (133). A self-renewing cancer stem cell population has been identified in solid tumors such as breast (134) and brain (135). These cancer stem cells represent approximately 1% of the tumor and are the only cells in the tumor generating tumors in nude mice (136). In cases of multiple myeloma, cells with a high self renewal potential have also been identified (137). Many researchers now suspect that all cancers are

composed of a mixture of stem cells and proliferative cells with a limited lifespan (136). The implications of this research are far reaching. The relapse of many cancers following therapy could be the result of the survival of the cancer stem cells. Therefore, it is critical to fully characterize the immunological features of these cells and to develop immunotherapeutic approaches to eliminate these cancer stem cells without excessive toxicity to normal stem cells. Of note, since cancer stem cells represent approximately 1% of the tumor cells (136), tumor antigens highly expressed in cancer stem cells may not be the tumor antigens highly expressed in tumors. Current anti-tumor antigen-specific immune therapies focused on tumor antigens highly expressed in tumor cells are not capable in eliciting effective anti-cancer stem cell immune responses and inhibiting cancer stem cell growth and cancer relapse after initial treatment. Therefore, future immunotherapy could be in a combinational format, including cancer cell antigen-specific immunotherapy and cancer stem cell antigen-specific immunotherapy (6). *Third,* anti-tumor immune responses elicited by immunotherapies may be suppressed by Tregs. In general, Tregs identified from most human cancers are Tregs population and Tregs possess potent ability to suppress immune responses *in vitro*. However, Munshi's lab found that even when Tregs are added in higher proportions, Tregs in patients with multiple myeloma and monoclonal gammopathy of undetermined significance are unable to suppress anti-CD3-mediated T-cell proliferation (138).

5.2. Association of high percentage of Tregs in cancers with the poor prognosis

Tregs within the tumor microenvironment may play a significant role in the suppression of anti-tumor immune responses against cancer cells. In recent studies, many groups reported elevated percentages of $CD4+CD25^{high}FOXP3+$ Tregs in the total T cell population isolated from tumor tissues or peripheral blood in a variety of cancers, including lung cancer (73), breast cancer (139), ovarian (140), melanoma (141), liver cancer (142), gastric cancer (143), and lymphoma (126). Curiel TJ *et al* reported that the high percentage of Tregs in ovarian cancer is associated with the poor prognosis of cancer patients (140), and a recent study demonstrates that the ratio of CD8+ T cells to Tregs is a better predictor of patient survival than looking at Tregs alone (144). Therefore, direct correlation or association of high percentage of Tregs and clinical prediction of patient survival requires further investigation.

5.3. Mechanisms of elevated Tregs in tumors

Although current anti-tumor immunotherapeutic approaches are generally sound and well executed, they ignore the important reality that many patients harbor abundant tumor antigens, DCs, and antigen-specific effector cells, but do not eradicate tumors. Therefore, it is becoming clear that current immune-based approaches to cancer therapy require substantial rethinking. Recent discoveries show that tumors actively fight back by producing numerous immunosuppressive factors such as IL-10, TGF-β, VEGFs, colony-stimulating factor (CSF)1, and IL-10, etc (145). These factors and other immunosuppressive mechanisms induced by the tumor are likely to arise as efforts by the immune system to purge abnormal tumor tissue simultaneously elicit intrinsic mechanisms that protect against the autoimmunity engendered by most antitumor immune responses (72). *First,* the generation and maintenance of Tregs have long been speculated to require the presence of target antigen, tissues (66) or cancer vaccination (146). Some tumor antigens have been identified as self antigens to stimulate Tregs at tumor sites. They have the potent ability to suppress naïve T cell proliferation through a cell contact dependent mechanism. As tumor cells express an array of tumor-specific antigens as well as self antigens, it is likely that Tregs specific for tumor-specific antigens as well as Tregs specific for nonmutated self antigens co-exist in the tumor microenvironment. Dna J-like 2, an autoantigen identified by recombinant expression cloning (SEREX), tends to elicit CD4+CD25+ Tregs for immune suppression in animals (67). CD4+CD25- T cells can also be converted to Tregs after TCR-mediated activation (i.e. antigen or anti-CD3 stimulation) (69), using a low antigen dose in the absence of co-stimulatory molecules favors the conversion of CD4+CD25- T cells into Tregs (147, 148), or by tumor cells in the absence of thymus and proliferation (149). All of these studies provide compelling evidence that antigen-specific Tregs are present at tumor sites and mediate antigen-specific local immune suppression. *Second,* chemokine-mediated attraction of Tregs to tumor sites (67), ligands, and/or antigens expressed by cancer cells, might play a crucial role in the recruitment, maintenance, and expansion of Tregs, leading to elevated percentages of Tregs at tumor sites. Ansell's lab showed that CCL22 secreted by lymphoma B cells is involved in the chemotaxis and migration of intratumoral Tregs that express CCR4, but not CCR8 (126). *Third,* IL-2 signaling is thought to be crucial for the development of Tregs as there are reduced numbers of CD4+ Tregs in IL-2-deficient mice. Recent studies revealed that IL-2 is essential for the peripheral maintenance of Tregs but not for their generation and development in the thymus (150, 151). *Fourth,* IL-10 and TGF-β have been implicated in the induction or conversion of Tregs. IL-10 is required for the induction of antigen-specific Tr1 cells *in vitro* and *in vivo*; TGF-β is a crucial cytokine required for the conversion of CD4+ naïve T cells into Tregs (152). Tumor cells not only secrete TGF-β and/or IL-10, but also induce immature myeloid DCs or immature myeloid suppressor cells to secrete TGF-β and/or IL-10, thus promoting the generation of Tregs from CD4+CD25- naïve T cells in the tumor microenvironment (153, 154). These studies indicate that tumor cells or tumor-infiltrating immune cells can secrete TGF-β and/or IL-10 and create a tumor microenvironment that facilitates the conversion of CD4+CD25- T cells into Tregs. Therefore, the success of peptide-based immunotherapy against cancer and other diseases will depend upon the nature of antigens that preferentially activate Treg or Th cells, thus providing a new opportunity to modulate the balance between Treg and effector T cells. *Fifth,* Indoleamine 2,3-dioxygenase (IDO) is a novel immunosuppressive enzyme expressed in some subsets of normal and neoplastic cells (155), which catalyzes the rate-limiting step of tryptophan degradation along the kynurenine pathway (156). IDO catalyzes the initial and

rate-limiting step of tryptophan degradation, resulting in tryptophan deficiency. Because tryptophan is an essential proliferative stimulus for effector T cells, these cells undergo apoptosis in a tryptophan-deprived environment. In addition, CD4+CD25+ T cells induce activation of indolamine 2,3-dioxygenase in DCs. Interestingly, IDO correlates with increased circulating Tregs in patients with acute myeloid leukemia (AML) (157). These findings provide a new insight of IDO-based immunoregulation that combines the regulatory function of Tregs with DCs acting as the final mediators of tolerogenic responses. *Sixth,* in addition to the conversion of CD4+CD25- T cells into Tregs, Schultze's lab showed *in vivo* peripheral expansion of naive Tregs in patients with multiple myeloma, suggesting another mechanism of Tregs expansion in patients with tumor (158). *Seventh,* at least in cutaneous T-Cell lymphoma, induction of a CD4+CD25+FOXP3+ T-cell phenotype is associated with HTLV-1 infection (94).

5.4. Tregs-based experimental therapeutics

A key question in cancer immunotherapy is how to eliminate or to reverse the suppressive function of Tregs. Using the A20 B cell lymphoma as a transplantable tumor model, Shirwan's team showed that Tregs serve as an important therapeutic target for patients with early stages of cancer (159). One approach is to eliminate CD25+ Tregs by Denileukin diftitox or Ontak (a IL-2–toxin fusion protein). Denileukin diftitox is a recombinant fusion protein consisting of IL-2 and diphtheria toxin that has been approved by the FDA to treat cutaneous T cell leukemia/lymphoma, which is characterized by large numbers of malignant CD4+CD25+ T cells (160). CD25 is the IL-2 receptor α chain. Denileukin diftitox binds the IL-2 receptor and inhibits protein translation following internalization, leading to apoptosis. Administration of the anti-CTLA-4 antibody together with an anti-CD25 antibody, which substantially reduced the number of CD4+CD25+ T cells, resulted in enhanced tumor immunity. This may partly be due to the blockade of CTLA-4 molecules expressed on the residual CD4+CD25+ regulatory T cells and consequent interference with CD4+CD25+ T-cell-mediated immunoregulation (161). However, because the CD25 marker is not specific for Tregs and many T cells are positive for the CD25 marker, this approach with either a toxin or an anti-CD25 antibody (162) might not efficiently eliminate Tregs, and may deplete both Tregs and activated effector cells (106, 163). It also might accelerate the return of Tregs through the conversion of Teff (effector T cells) to Tregs (106, 163). Thus, more specific and effective reagents are needed to deplete Tregs in cancer patients.

Secondary lymphoid tissue chemokine and Fas ligand together can attract an array of immune cells and induce apoptosis in Tregs, whereas 4-1BBL and TRANCE together can stimulate T cells and DCs. Liu S *et al* showed that the transfer of all four molecules increases tumor-infiltrating neutrophils, DCs, and CD4+ and CD8+ T cells and decreases intra-tumoral Tregs. Compared with transfer of the chemotactic molecules alone or co-stimulatory molecules alone, treatment of all four molecules *in vitro* demonstrates stronger anti-tumor responses against established tumors. Treatment also favors the generation of a Th1 cytokine milieu at the tumor site, which is attributed not only to an increase in IL-12-producting DCs and IFN-γ-producing CD8+ T cells, but also to a decrease in IL-10-producing Tregs (164). Of note, Yang XF's lab recently reported that Tregs express higher levels of pro-apoptotic protein Bax compared to CD4+CD25- T cells, and removal of Tregs via a Bax-dependent apoptotic pathway significantly enhances anti-self antigen immune responses. This finding demonstrated for the first time the proof of principle that the apoptosis pathway of Tregs is a new therapeutic target (11, 40). In addition, it was also reported that the use of FasL to pre-deplete intratumoral Tregs may also provide a useful means for optimizing adoptive therapy (165).

Strong evidence for Tregs anti-tumoral immunity was found when depletion of CD25+ T cells and/or blocking CTLA-4 or GITR *in vivo* with antibodies could increase reactivity to known self-antigens and enhance tumor rejection (166-168). Tumor effector cells can be generated *in vitro* by simply eliminating CD4+CD25+ T cells from splenic cell suspensions prepared from tumor-non-sensitized mice (169). In this *in vitro* induction of tumor immunity, CD4+CD25- T cells responding to self-peptides/class II MHC molecules expressed on syngeneic APCs spontaneously proliferated upon the removal of Tregs. A large amount of IL-2 produced by such CD4+ self-reactive T cells, generated natural killer-like tumor effector cells as lymphokine-activated killer cells, capable of promiscuously killing various tumor cells. Thus, removal of Tregs can abrogate immunological unresponsiveness to syngeneic tumors *in vivo* and *in vitro*, which leads to the spontaneous development of tumor-specific effector cells as well as tumor-non-specific ones. Data suggests that Tregs may be important therapeutic targets for patients with early stages of cancer. More vigorous combinatorial approaches simultaneously targeting multiple immune evasion, as well as immunosurveillance mechanisms for the generation of a productive immune response against tumor, may be required for effective immunotherapy in patients with advanced disease (159).

RNA interference (RNAi) describes the sequence specific degradation of mRNA in animals and plants initiated by double-strand RNA molecules (dsRNA), which consist of two 21 to 22 nucleotide long short-interfering RNA strands (siRNA) (170). To overcome the instability and relatively poor delivery of unmodified siRNA into mammalian cells *in vivo*, noncovalent complexation of synthetic siRNAs with low molecular weight polyethylenimine (PEI) efficiently stabilizes siRNA. The systemic (intraperitoneal, i.p.) administration of PEI-complexed siRNA targeting the c-erbB2/neu (HER-2) receptor results in marked reduction of tumor growth through siRNA-mediated downregulation of HER-2 (171). It has been proposed that application of siRNA targeting the critical transcription factor FOXP3 might abrogate the Tregs *in vivo*, thereby facilitating the generation of an efficient anti-tumor immune response (172).

In the following section, we analyze reports on the effects of some commonly used chemotherapy drugs on Tregs homeostasis and survival. Fluticasone propionate increases nTreg suppression of allergen-stimulated T effector cells by means of IL-10-dependent mechanism (173). In addition, inhaled or systemic glucocorticoids have been found to induce FOXP3 and IL-10 expression and generation of aTregs (236). The number of Tregs in the blood is significantly lower in untreated myasthenia gravis patients than in age-matched healthy subjects, whereas it is normal or elevated in patients on immunosuppressive therapy (prednisone frequently associated with azathioprine) (174). The following mouse studies have demonstrated that murine Tregs show differential response to dexamethasone-induced cell death when compared to CD4+CD25- T cells (175). Administration of dexamethasone into BALB/c mice enhances the proportion of Tregs and the ratio of Tregs to CD4+CD25- cells in lymphoid organs, particularly the thymus. This correlates with the *in vitro* observation that Tregs express higher levels of glucocorticoid receptor (175), and are therefore more resistant to dexamethasone-mediated cell death than CD4+CD25- T cells. Rapamycin is an immunosuppressive compound that is currently used to prevent acute graft rejection in humans. Rapamycin selectively expands Tregs *in vitro*. These expanded nTregs suppress proliferation of syngeneic T cells *in vitro* and prevent allograft rejection *in vivo*. Cyclosporine A, but not rapamycin and mycophenolate mofetil, inhibits Treg function by reducing IL-2 production (176). Immunosuppression with tacrolimus is superior to cyclosporine A after pulmonary allotransplantation, which is associated with the induction of Tregs (177). Cancer chemotherapy and the immunosuppressive drug Cyclophosphamide could have the following effects on Tregs: *1)* enhance apoptosis and decrease homeostatic proliferation of Tregs; *2)* abolish the suppressive function of Tregs (178); *3)* markedly enhance the magnitude of secondary but not primary CTL responses induced by dendritic cell-derived exosomes vaccines; *4)* synergize with dendritic cell-derived exosomes in therapy but not prophylaxis tumor models (172). Fludarabine is a cytotoxic analog of deoxyadenosine monophosphate and has high efficacy in the treatment of chronic lymphocytic leukemia (CLL). In CLL patients receiving fludarabine therapy, the inhibitory function of Tregs is decreased or even abrogated. The immunosuppressive drug FK778, an analogue of the active metabolite A77 1726 in leflunomide (179), induces regulatory activity in stimulated human CD4+CD25- T cells (180). This anergic state is reversible by exogenous IL- 2 and is induced independent of nTregs. Taken together, the promotion of Tregs survival and Tregs function are underlying the pharmacological mechanisms of these immunosuppressive drugs.

6. CONCLUSION

The immunosuppressive T cells – Tregs – play an important role in the treatment of lymphomas and tumors, but the mechanisms remain poorly defined. Compared to carcinomas, Tregs in lymphomas are more complex, especially in some types of T cell lymphomas, which can be classified into three groups. Therefore, the utility of Tregs as prognostic factors and therapy strategies in different cancers is under intense investigation, but much remains to be understood.

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