Review

Immunotherapy Approaches Targeting Regulatory T-Cells

HIDEYA ONISHI[1], TAKASHI MORISAKI[2] and MITSUO KATANO[1]

[1]*Department of Cancer Therapy and Research, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan;*
[2]*Fukuoka Cancer General Clinic, Fukuoka, Japan*

Abstract. *The immune system prevents establishment and progression of cancer through innate and adaptive surveillance. However, some cancerous cells successfully evade the immune system. Regulatory T-cells (Tregs) facilitate such evasion. Tregs may be the factor responsible for the limited success of human tumor immunotherapy to date. To improve immunotherapy, it is thought that the number of Tregs and their functions should be inhibited in patients with advanced cancer. In this review, we focus on recent immunotherapy efforts targeting Tregs.*

Certain T-cells react to self-antigen. Consequently, the immune system consists of various regulatory systems that suppress induction of autoreactive cytotoxic T-lymphocytes (CTLs). One such regulatory system is composed of regulatory T-cells (Tregs). Tregs play an essential role in maintaining immunological unresponsiveness to self-antigens. Since many cancer antigens are self-antigens, Tregs are thought to suppress antitumor immunity (*e.g.* CTLs) and promote tumor progression. In this review we provide a brief summary of the importance of Tregs in cancer and focus on therapy targeting Tregs.

Fundamental Role for Tregs

Tregs represent a unique $CD4^+$ T-cell subpopulation that suppresses the activation and proliferation of autoreactive lymphocytes and induces self-tolerance. There are two kinds of self-tolerance, central tolerance and peripheral tolerance. The former contributes to lymphocyte differentiation through the elimination and inactivation of autoreactive lymphocytes in primary lymph organs. The latter contributes to immune tolerance to self-antigen in mature lymphocytes circulating in peripheral blood. Tregs are believed to contribute to the induction and maintenance of peripheral tolerance. Generally, Tregs are recognized as naturally occurring Tregs (nTregs), having differentiated from $CD4^+CD8^+$ T-cells in the thymus. nTregs, like all T-cells, arise from progenitor cells in the bone marrow and undergo lineage commitment and maturation in the thymus (1). nTregs comprise a small population, just 5-10% of peripheral $CD4^+$ T-cells (2), but play a critical role. nTregs migrate from the thymus into the periphery when just 3 days old, and thymectomy of mice at day 3 results in lethal autoimmunity due to the lack of peripheral Tregs (3). Recently, inducible Tregs (iTregs) have attracted attention regarding tumor immunity. iTregs are derived from naïve $CD4^+$ T-cells (Tn cells) that have migrated from the thymus to the periphery, where they differentiate and induce cytokines such as transforming growth factor-β (TGF-β). The function of nTregs and iTregs are quite similar and it is difficult to distinguish nTregs from iTregs. $CD4^+CD25^+$ forkhead box P3 $(FOXP3)^+$ Tregs include nTregs and iTregs and are thought to be the major Tregs population. nTregs differentiate in the thymus and decrease in number with age since the thymus undergoes rapid atrophy after adolescence. On the other hand, the decrease of iTregs that is induced in the periphery with age is slow compared to the one of nTregs. Therefore, conversion of memory T-cells to iTregs is thought to be required for Tregs maintenance (4-5). $FOXP3^-$ T-cells with suppressive activity have been described based upon their cytokine induction profile. Type 1 regulatory T cells (Tr1) are induced by interleukin-10 (IL-10) (6), and T-helper 3 (Th3) cells, are induced by TGF-β (7). Natural killer (NK) T-cells have also been studied for their regulatory properties (8). Although the majority of NK T-cells express CD4, most of the remaining cells express neither CD4 nor CD8, although in humans there is a small subset of $CD8^+$ NK T-cells (9-10).

Correspondence to: Hideya Onishi, Department of Cancer Therapy and Research, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka, 812-8582, Japan. Tel: +81 926426220, Fax: +81 926426221, e-mail: ohnishi@surg1.med.kyushu-u.ac.jp

Key Words: Dendritic cells, CD4+ T-cell, Treg, immunotherapy, regulatory T-cells, review.

There are other Tregs that are not of the $CD4^+$ lineage. An *in vitro* study identified a subset of human $CD8^+$ T-cells ($CD8^+CD28^-$) that was able to confer tolerance by preventing up-regulation of the co-stimulatory markers CD80 and CD86 on antigen presenting cells (APCs), by $CD4^+$ T-cells (11). Unlike $CD4^+$ iTregs, $CD8^+$ Tregs are dependent on interferon-γ (IFN-γ) to secrete TGF-β (12). How $CD8^+$ Tregs are generated is still unknown, however, and multiple subsets of $CD8^+$ Tregs, both thymus-derived and peripherally induced, have been described in human and mouse (13). γδ-T-cells which can be developed extrathymically also seem to be important Treg elements of the immune system (14-15). It has been shown that γδ-T cells are capable of enhancing inflammatory responses in autoimmune disease, such as systemic lupus erythematosus, rheumatoid arthritis (15, 16), graft-*vs*-host disease (17), and delayed-type hypersensitivity (18). Figure 1 summarizes the differentiation pathways of Tregs described in this review (19).

Tregs in Cancer

Tregs comprise 5-10% of $CD4^+$ T-cells in peripheral blood and are normally found in lymph organs (20-23). The reason why Tregs are enriched in lymph nodes may be due to the fact that they express homing receptors, including CC chemokine receptor1 (CCR1), CCR2, CCR4, CCR5, CCR6, CCR8, CCR9, CXC chemokine receptor3 (CXCR3), CXCR4, CXCR5,CXCR6, α4β1 integrin, αEβ7 integrin, α4β7 integrin, and the P- and E- selectin ligands (24). The study also showed that dendritic cell (DC) preferentially attract Tregs by secreting chemokines CCL17 and CCL22, which are ligands for the CCR4 receptor (24). Tregs numbers in peripheral blood mononuclear cell (PBMC) from patients with non-small cell lung cancer or ovarian cancer are higher than the ones observed in healthy volunteers (25). Similar results have been noted in breast cancer, colon cancer, esophageal cancer, gastric cancer, hepatocellular carcinoma, leukemia, lymphoma, malignant melanoma and pancreatic cancer (26-28). Moreover, Tregs may increase in malignant ascites (25, 29). There are three possible reasons why Tregs accumulate in cancer. Firstly, iTregs are induced or proliferate locally due to cytokines such as TGF-β, IL-10, and vascular endothelial growth factor (VEGF), which are reportedly important factors for induction and promotion of iTregs differentiation. Secondly, immature DCs exposed to TGF-β, IL-10, and VEGF induce Tregs. Much current vaccine-based immunotherapy for cancer is dependent on DC function, hence the need for modulating Tregs to maximize the effect of such vaccines (30). Thirdly, Tregs proliferate through signaling of T-cell receptor (TCR), CD28, and IL-2. Thus, Tregs may promote self-tolerance to impede immune surveillance against cancer in healthy individuals and suppress potential responsiveness to autologous tumors in cancer patients (31).

Many investigators define T-cells that express $FOXP3^+$ as Tregs, and suggest a negative correlation between prognosis and the number of tumor-infiltrated Tregs (32-36). On the other hand, several recent studies present conflicting prognostic data for some hematological malignancies, especially B-cell lymphoma in which an elevated number of $FOXP3^+$ cells were shown to correlate with improved survival (37). The assertion that there is a correlation between Tregs and prognosis is still controversial. Importantly, a reduced ratio of $CD8^+$ T-cells to $CD4^+CD25^+FOXP3^+$ in Tregs, as well as Tregs numbers in tumors, correlates with poor prognosis in patients with breast (35), gastric (38), ovarian (32, 35, 38-39), and colon cancer (40).

Immunotherapy Targeting Tregs

This review focuses on therapy directed at Tregs aiming to reduce and eliminate them. Figure 2 summarizes potential approaches for the elimination and inhibition of Tregs, as described below.

Anti-CD25 therapy. Basiliximab which is an anti-CD25 monoclonal antibody is the arsenal of current immunotherapies being used in kidney transplant patients (41). Bluestone *et al* showed that basiliximab caused a transient loss of $FOXP3^+$ and $FOXP3^-CD25^+$ T-cells in the circulation (41). Denileukin diftitox (Ontak) is a recombinant fusion protein product of diphtheria toxin and IL-2 that selectively binds to the IL-2 receptor of cells and, following internalization, inhibits protein synthesis (42). Rasku *et al* showed that Ontak caused a transient depletion of Tregs (43). Telang et al showed the promising result of Ontak in patients with unresectable stage IV melanoma in phase II trial (44).

Immunotoxin LMB-2 is another agent used for relative selective destruction of Tregs. LMB-2 consists of a single-chain Fv fragment of anti-CD25 monoclonal antibody fused to a truncated form of the bacterial *Pseudomonas* exotoxin A, from which two amino acids have been deleted (45). Major trials testing this immunotoxin in patients with $CD25^+$ hematological malignancies (46) and refractory hairy cell leukemia (47) have shown promising results.

However, one of the problems is that CD25 can be expresed in activated effector T-cells, thus anti-CD25 therapy may also affect activated T-cells.

Monoclonal antibodies against Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4). CTLA-4 is expressed on Tregs and is thought to play a pivotal role in their suppressive function. Ipilimumab, a monoclonal antibody against CTLA-4, was evaluated extensively as a possible therapeutic agent for the treatment of several kinds of melanoma (48). A phase III study of ipilimumab was published, and significant improvement in overall survival and drug tolerance among patients with

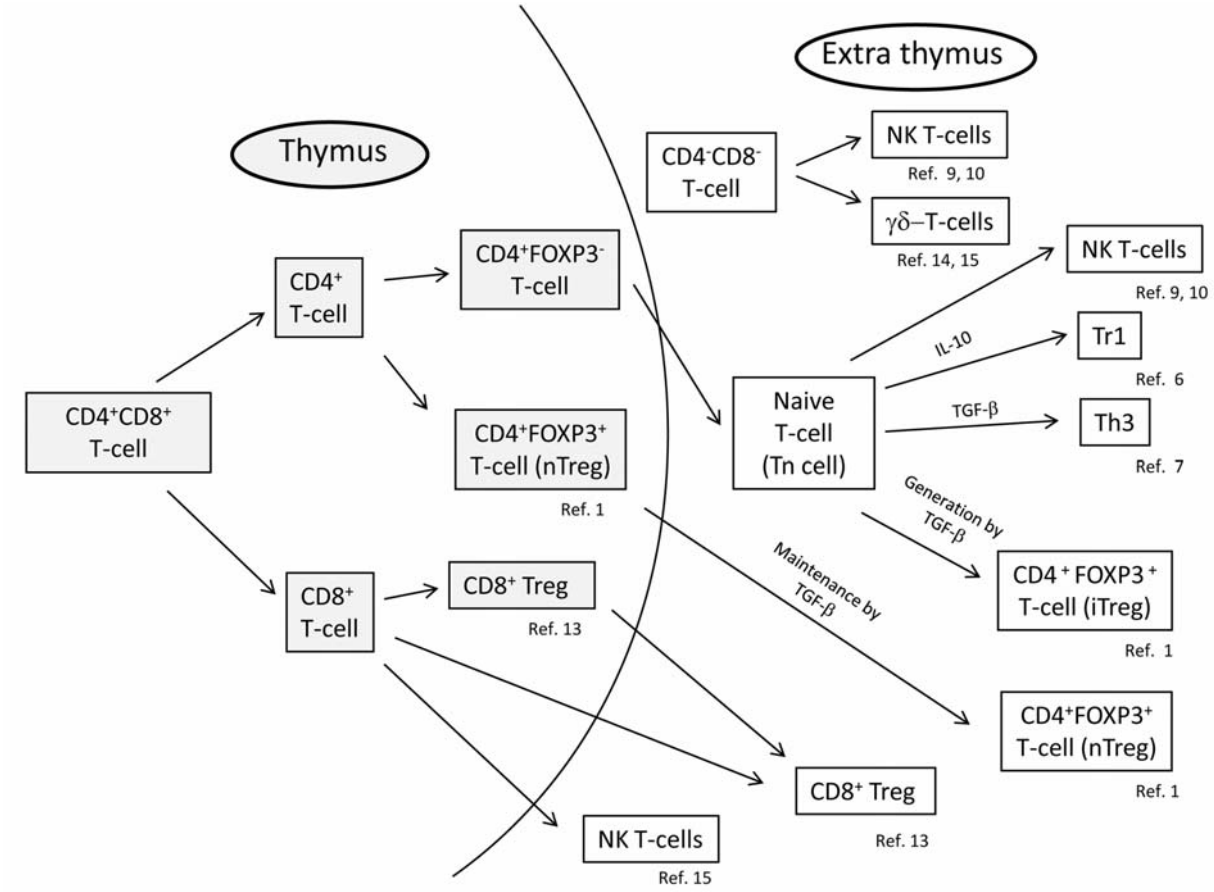

Figure 1. *A schema illustrating how regulatory T-cells (Tregs) differentiate in both the thymus and periphery. Natural killer T-cells; NK T-cells, forkhead box P3; FOXP3.*

metastatic melanoma was observed (49). The US Food and Drug Administration approved ipilimumab injection for unresectable or metastatic melanoma on March 25, 2011. Another monoclonal antibody against CTLA-4, tremelimumab, a fully human IgG_2 antibody developed by Pfizer Pharmaceuticals, is undergoing clinical investigation (48).

Antibody against glucocorticoid-induced tumor necrosis factor (TNF)-receptor. Glucocorticoid-induced TNF-receptor (GITR) contributes to the immunosuppressive function of Tregs. Interestingly, anti-GITR antibody may ameliorate the suppressive function of Tregs *in vivo* and *in vitro* (50). Preclinical evidence has demonstrated that signaling through GITR such as anti-GITR antibody, soluble GITR ligand could modulate the activity of Tregs with loss of FOXP3 expression (51).

FOXP3 vaccination. FOXP3 is a Tregs-specific marker and may offer the most rational approach to target Tregs. The concept of Tregs depletion *via* vaccination is to enhance the efficiency of previous antitumor vaccinations that have led to patented products (48). Patent WO 2008/081581 describes the invention of a vaccine using nonapeptides and decapeptides derived from FOXP3 that bind HLA molecules (48).

Toll-like receptor 8 (TLR8). The TLR8-myeloid differentiation factor 88 (MYD88)- Interleukin-1 receptor associated kinase 4 (IRAK4) signaling pathway can reverse the suppressive function of different Tregs populations (52). It is not entirely clear why only TLR8 ligands can reverse the suppressive function of Tregs. One reason may be that Tregs express a relatively high level of TLR8 (52). Poly-G oligonucleotides or similar ligands might be useful in clinical settings to enhance the efficacy of immunotherapy directed toward cancer.

Drug-induced Tregs inhibition. Recent effects of drug-mediated Tregs inhibiton have been reported. Cyclophosphamide increases antitumor effects by reducing Treg numbers and function (53). Cyclosporine A and tacrolimus also reduce the Tregs numbers as a function of

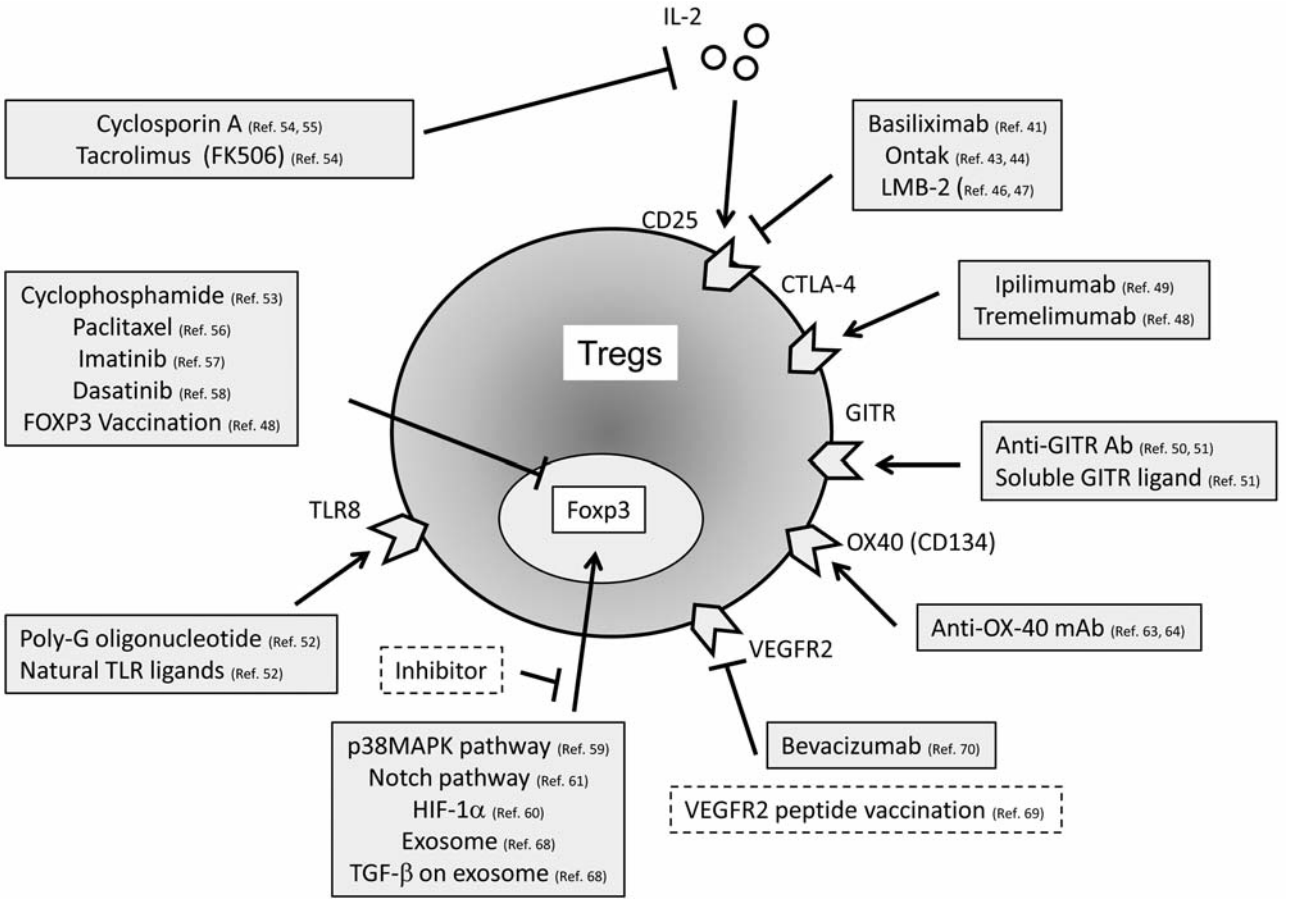

Figure 2. *A potential approach for eliminating and inhibiting regulatory T-cells (Tregs). Colored squares with solid lines show known approaches or factors. Empty squares with dotted lines show possible approaches. Toll-like receptor; TLR, Mitogen-activated protein kinase; MAPK, hypoxia inducible factor; HIF, transforming gowth factor; TGF, vascular endothelial growth factor receptor; VEGFR, Glucocorticoid-induced TNF-receptor; GITR.*

IL-2 secretion and inhibition of IL-2 signaling (54, 55). Tregs are sensitive to paclitaxel (56) and imatinib suppresses FOXP3, a master gene of Tregs (57). Dasatinib suppresses inhibition of Tregs proliferation and reduces FOXP3 expression by inducing G_0/G_1 arrest (58).

Other possible molecular mechanism that may target Tregs. p38 mitogen-activated protein kinase (MAPK) pathway: The p38MAPK pathway in Tregs is activated more so than in typical $CD4^+$ T- cells. Recent study revealed that depletion of $CD25^+$ Tregs in combination with treatment with a p38 chemical inhibitor is necessary to completely block the immunosuppressive function of IL-10-producing anergic $CD25^-$ iTregs (59).

Hypoxia inducible factor-1α (HIF-1α): α): Ben-Shoshan showed that in vivo expression of HIF-1α induced FOXP3 expression and an increase in the number of functionally active $FOXP3^+CD4^+CD25^+$ Tregs (60).

Notch signaling pathway: Notch is a morphogen and contributes to cancer initiation and progression by regulating FOXP3 expression *via* interaction through the *FOXP3* promoter (61). Notch signaling, therefore, may be a target for regulating both cancer progression and antitumor immunity.

OX40. OX40 (CD134) is a co-stimulatory TNF receptor family molecule that is constitutively expressed on Tregs (62). OX40 activation inhibits *FOXP3* gene expression and limits Tregs suppression of effector T-cells (63). Furthermore, intratumoral injection of anti-OX40 monoclonal antibody strongly suppressed tumor growth (64). This result suggests the OX40 receptor may be a target for antitumor immunotherapy.

Exosome. Exosomes are endosome-derived organelles of 50-100 nm that are actively secreted through an exocytosis pathway by many cell types (65). They are very rigid and resistant to enzymatic degradation in blood, ascites, and effusions. (66). These biophysical properties allow exosomes

to play an important role in cell to cell communication, in particular communication between immune cells (67). In fact, exosomes express many cell–cell communication-related molecules, including MHC class I and II, CD86, tetraspanins, and heat-shock proteins (65, 66). Recently, tumor-derived exosomes were shown to contribute to maintaining Tregs numbers and suppressive function in malignant effusions. It appears that surface-bound TGF-β1 on tumor-derived exosomes mediates FOXP3 expression (68). Thus, elimination of malignant effusion derived exosome, or control of such exosomes expressing TGF-β1, may be new immunotherapy therapeutic strategies for advanced cancer with malignant effusions.

Vascular endothelial growth factor receptor 2 (VEGFR2) expression on Tregs. We identified VEGFR2 as a potential marker expressed on the surface of Tregs (69). VEGFR2 is expressed selectively on $CD4^+FOXP3^{high}$ cells and has strong immunosuppressive functions on allogeneic T-cells. We reported that anti-VEGF antibody (bevacizumab) inhibited Tregs expansion, suggesting that VEGF contributed to Treg induction (70). Taken together, VEGFR2 may be a useful target since it is expressed on the cell surface of only the $FOXP3^{high}$ population. Moreover, peptide vaccination using VEGFR2 is undergoing clinical investigation in a phase I study (71).

Conflict of Interest Statement

The Authors declare no conflict of interest.

Acknowledgements

This study was supported by General Scientific Research Grants (23591894) from the Ministry of Education, Culture, Sports, Science and Technology of Japan. We thank Ms. Kaori Nomiyama for her skilful technical assistance.

References

1 Workman CJ, Szymczak-Workman AL, Collison LW, Pillai MR and Vignali DA: The development and functuin of regulatory T cells. Cell Mol Life Sci *66:* 2603-2622, 2009.
2 Sakaguchi S: Naturally arising $CD4^+$ regulatory T-cells for immunologic self-tolerance and negative control of immune response. Annu Rev Immunol *22*: 531-562, 2004.
3 Asano M, Toda M, Sakaguchi N and Sakaguchi S: Autoimmune disease as a consequence of developmental abnormality of a T cell subpopulation. J Exp Med *184*: 387-396, 1996.
4 Vukmanovic-Stejic M, Zhang Y, Cook JE, Fletcher JM, McQuaid A, Masters JE, Rustin MH, Taams LS, Beverley PC, Macallan DC and Akbar AN: Human $CD4^+$ $CD25^+$ $FOXP3^+$ regulatory T-cells are derived by rapid turnover of memory populations *in vivo*. J Clin Invest *116*: 2423-2433, 2006.
5 Akbar AN, Vukmanovic-Stejic M, Taams LS, Macallan DC: The dynamic co-evolution of memory and regulatory $CD4^+$ T-cells in the periphery. Nat Rev Immunol *7*: 231-237, 2007.
6 Vieira PL, Christensen JR, Minaee S, O'Neill EJ, Barrat FJ, Boonstra A, Barthlott T, Stockinger B, Wraith DC and O'Garra A: IL-10-secreting regulatory T cells do not express FOXP3 but have comparable regulatory function to naturally occurring $CD4^+CD25^+$ regulatory T-cells. J Immunol *172:* 5986-5993, 2004.
7 Weiner HL: Induction and mechanism of action of transforming growth factor-beta-secreting Th3 regulatory cells. Immunol Rev *182*: 207-214, 2001.
8 La Cava A, Van Kaer L and Fu-Dong-Shi: $CD4^+CD25^+$ Tregss and NKT cells: regulators regulating regulators. Trends Immunol *27*: 322-327, 2006.
9 Taniguchi M, Harada M, Kojo S, Nakayama T and Wakao H: The regulatory role of Valpha 14 NKT cells in innate and acquired immune response. Annu Rev Immunol *21*: 483-513, 2003.
10 Kronenberg M: Toward an understanding of NKT cell biology: progress and paradoxes. Annu Rev Immunol *23*: 877-900, 2005.
11 Liu Z, Tugulea R, Cortesini R and Suciu-Foca N: Specific suppression of T helper alloreactivity by allo-MHC classI-restricted $CD8^+CD28^-$ T-cells. Int Immunol *10*: 775-783, 1998.
12 Myers L, Croft M, Kwon BS, Mittler RS and Vella AT: Peptide-specific CD8 T-regsulatory cells use IFN-gamma to elaborate TGF-beta-based suppression. J Immunol *174*: 7625-7632, 2005.
13 Tsai S, Clemente-Casares X and Santamaria P: $CD8^+$ Tregs in autoimmunity: learning self-control from experience. Cell Mol Life Sci *68*: 3781-3795, 2011.
14 Odyniec A, Szczepanik M, Mycko MP, Stasiolek M, Raine CS and Selmaj KW: γδ-T-cells enhance the expression of experimental autoimmune encephalomyelitis by promoting antigen presentation and IL-12 production. J Immunol *173*: 682-694, 2004.
15 Hayday A and Geng L: γδ-T-cells regulate autoimmunity. Curr Opin Immunol *9*: 884-889, 1997.
16 Peng SL, Madaio MP, Hayday AC and Craft J: Propagation and regulation of systemic autoimmunity by γδ-T-cells. J Immunol *157*: 5689-5698, 1996.
17 Tsuji S, Char D, Bucy RP, Simonsen M, Chen CH and Cooper MD: γδ-T-cells are secondary participants in acute graft-*versus*-host reactions initiated by $CD4^+$ αβ-T-cells. Eur J Immunol *26*: 420-427, 1996.
18 Dieli F, Ptak W, Sireci G, Romano GC, Potestio M, Salerno A and Asherson GL: Cross talk between $V\beta8^+$ and γδ-T-lymphocytes in contact sensitivity. Immunology *93*: 469-477, 1998.
19 Sundrud MS and Rao A: New twists of T-cell fate: control of T-cell activation and tolerance by TGF-β and NFAT. Curr Opin Immunol *19*: 287-293, 2007.
20 Shevach EM: $CD4^+$ $CD25^+$ suppressor T-cells: more questions than answers. Nat Rev Immunol *2*: 389-400, 2002.
21 Wood KJ, Sakaguchi S: Regulatory T-cells in transplantation tolerance. Nat Rev Immunol *3*: 199-210, 2003.
22 von Herrath MG and Harrison LC: Antigen-induced regulatory T-cells in autoimmunity. Nat Rev Immunol *3*: 223-232, 2003.
23 Bach JF: Regulatory T-cells under scrutiny. Nat Rev Immunol *3*: 189-198, 2003.
24 Campbell DJ and Koch MA: Phenotypical and functional specialization of $FOXP3^+$ regulatory T- cells. Nat Rev Immunol *11*: 119-130, 2011.
25 Woo EY, Chu CS, Goletz TJ, Schlienger K, Yeh H, Coukos G, Rubin SC, Kaiser LR and June CH: Regulatory $CD4^+$ $CD25^+$ T-cells in tumors from patients with early-stage non-small cell lung cancer and late-stage ovarian cancer. Cancer Res *61*: 4766-4772, 2001.

26 Liyanage UK, Moore TT, Joo HG, Tanaka Y, Herrmann V, Doherty G, Drebin JA, Strasberg SM, Eberlein TJ, Goedegebuure PS and Linehan DC: Prevalence of regulatory T cells is increased in peripheral blood and tumor microenvironment of patients with pancreas or breast adenocarcinoma. J Immunol *169*: 2756-2761, 2002.

27 Wolf AM, Wolf D, Steurer M, Gastl G, Gunsilius E and Grubeck-Loebenstein B: Increase of regulatory T-cells in the peripheral blood of cancer patients. Clin Cancer Res *9*: 606-612, 2003.

28 Sasada T, Kimura M, Yoshida Y, Kanai M and Takabayashi A: CD4+ CD25+ regulatory T-cells in patients with gastrointestinal malignancies: possible involvement of regulatory T-cells in disease progression. Cancer *98*: 1089-1099, 2003.

29 Woo EY, Yeh H, Chu CS, Schlienger K, Carroll RG, Riley JL, Kaiser LR and June CH: Regulatory T cells from lung cancer patients directly inhibit autologous T-cell proliferation. J Immunol *168*: 4272-4276, 2002.

30 Nizar S, Meyer B, Galustian C, Kumar D and Dalgleish A: T-regsulatory cells, the evolution of targeted immunotherapy. Biochim Biophys Acta *1806*: 7-17, 2010.

31 Sakaguchi S, Yamaguchi T, Nomura T and Ono M: Regulatory T-cells and immune tolerance. Cell *133*: 775-787, 2008.

32 Curiel TJ, Coukos G, Zou L, Alvarez X, Cheng P, Mottram P, Evdemon-Hogan M, Conejo-Garcia JR, Zhang L, Burow M, Zhu Y, Wei S, Kryczek I, Daniel B, Gordon A, Myers L, Lackner A, Disis ML, Knutson KL, Chen L and Zou W: Specific recruitment of regulatory T-cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. Nat Med *10*: 942-949, 2004.

33 Wolf D, Wolf AM, Rumpold H, Fiegl H, Zeimet AG, Muller-Holzner E, Deibl M, Gastl G, Gunsilius E and Marth C: The expression of the regulatory T cell-specific fork-head box transcription factor FOXP3 is associated with poor prognosis in ovarian cancer. Clin Cancer Res *11*: 8326-8331, 2005.

34 Hiraoka N, Onozato K, Kosuge T and Hirohashi S: Prevalence of FOXP3+ regulatory T-cells increases during the progression of pancreatic ductal adenocarcinoma and its premalignant lesions. Clin Cancer Res *12*: 5423-5434, 2006.

35 Bates GJ, Fox SB, Han C, Leek RD, Garcia JF, Harris AL and Banham AH: Quantification of regulatory T cells enables the identification of high-risk breast cancer patients and those at risk of late relapse. J Clin Oncol *24*: 5373-5380, 2006.

36 Gao Q, Qiu SJ, Fan J, Zhou J, Wang XY, Xiao YS, Xu Y, Li YW and Tang ZY: Intratumoral balance of regulatory and cytotoxic T-cells is associated with prognosis of hepatocellular carcinoma after resection. J Clin Oncol *25*: 2586-2593, 2007.

37 Tzankov A, Meier C, Hirschmann P, Went P, Pileri SA and Dirnhofer S: Correlation of high numbers of intratumoral FOXP3+ regulatory T-cells with improved survival in germinal center-like diffuse large B-cell lymphoma, follicular lymphoma and classical Hodgkin's lymphoma. Haematologica *93*: 193-200, 2008.

38 Sasada T, Kimura M, Yoshida Y, Kanai M and Takabayashi A: CD4+ CD25+ regulatory T-cells in patients with gastrointestinal malignancies: possible involvement of regulatory T-cells in disease progression. Cancer *98*: 1089-1099, 2003.

39 Sato E, Olson SH, Ahn J, Bundy B, Nishikawa H, Qian F, Jungbluth AA, Frosina D, Gnjatic S, Ambrosone C, Kepner J, Odunsi T, Ritter G, Lele S, Chen YT, Ohtani H, Old LJ and Odunsi K: Intraepithelial CD8+ tumor-infiltrating lymphocytes and a high CD8+/regulatory T-cell ratio are associated with favorable prognosis in ovarian cancer. Proc Natl Acad Sci USA *102*: 18538-18543, 2005.

40 Suzuki H, Chikazawa N, Tasaka T, Wada J, Yamasaki A, Kitaura Y, Sozaki M, Tanaka M, Onishi H, Morisaki T and Katano M: Intratumoral CD8+ T/FOXP3+ cell ratio is a predictive marker for survival in patients with colorectal cancer. Cancer Immunol Immunother *59:* 653-661, 2010.

41 Bluestone JA, Liu W, Yabu JM, Laszik ZG, Putnam A, Belingheri M, Gross DM, Townsend RM, and Vincenti F: The effect of costimulatory and interleukin 2 receptor blockade on regulatory T cells in renal transplantation. Am J Transplant *8*: 2086-96, 2008.

42 Foss FM: DAB(389)IL-2 (denileukin diftitox, ONTAK): a new fusion protein technology. Clin Lymphoma *1*: S27-31, 2000.

43 Rasku MA, Clem AL, Telang S, Taft B, Gettings K, Gragg H, Cramer D, Lear SC, McMasters KM, Miller DM, and Chesney J: Transient T-cell depletion causes regression of melanoma metastases. J Transl Med *6*: 1-18, 2008.

44 Telang S, Rasku MA, Clem AL, Carter K, Klarer AC, Badger WR, Milam RA, Rai SN, Pan J, Gragg H, Clem BF, McMasters KM, Miller DM and Chesney J: Phase II trial of the regulatory T cell-sepleting agent, denileukin diftitox, in patients with unresectable stage IV melanoma. BMC Cancer In press, 2011.

45 Kreitman RJ, Batra JK, Seetharam S, Chaudhary VK, FitzGerald DJ and Pastan I: Single-chain immunotoxin fusions between anti-Tac and *Pseudomonas* exotoxin: relative importance of the two toxin disulfide bonds. Bioconjug Chem *4*: 112-120, 1993.

46 Kreitman RJ, Wilson WH, White JD, Stetler-Stevenson M, Jaffe ES, Giardina S, Waldmann TA and Pastan I: Phase I trial of recombinant immunotoxin anti-Tac(Fv)-PE38 (LMB-2) in patients with hematologic malignancies. J Clin Oncol *18*: 1622-1636, 2000.

47 Kreitman RJ, Wilson WH, Robbins D, Margulies I, Stetler-Stevenson M, Waldmann TA and Pastan I: Responses in refractory hairy cell leukemia to a recombinant immunotoxin. Blood *94*: 3340-3348, 1999.

48 de Rezende LC, Silva IV, Rangel LB and Guimarães MC: Regulatory T-cell as a target for cancer therapy. Arch Immunol Ther Exp *58*: 179-190, 2010.

49 Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, Gonzalez R, Robert C, Schadendorf D, Hassel JC, Akerley W, van den Eertwegh AJ, Lutzky J, Lorigan P, Vaubel JM, Linette GP, Hogg D, Ottensmeier CH, Lebbé C, Peschel C, Quirt I, Clark JI, Wolchok JD, Weber JS, Tian J, Yellin MJ, Nichol GM, Hoos A and Urba WJ: Improved survival with ipilimumab in patients with metastatic melanoma. N Engl J Med *363*: 711-23, 2010.

50 McHugh RS, Whitters MJ, Piccirillo CA, Young DA, Shevach EM, Collins M and Byrne MC: CD4+CD25+ immunoregulatory T-cells: gene expression analysis reveals a functional role for the glucocorticoid-induced TNF receptor. Immunity *16*: 311-323, 2002.

51 Schaer DA, Murphy JT and Wolchok JD: Modulation of GITR for cancer immunotherapy. Curr Opin Immunol In press, 2012.

52 Peng G, Guo Z, Kiniwa Y, Voo KS, Peng W, Fu T, Wang DY, Li Y, Wang HY and Wang RF: Toll-like receptor 8-mediated reversal of CD4+ regulatory T-cell function. Science *309*: 1380-1384, 2005.

53 Lutsiak ME, Semnani RT, De Pascalis R, Kashmiri SV, Schlom J and Sabzevari H: Inhibition of CD4(+)25+ T-regsulatory cell function implicated in enhanced immune response by low-dose cyclophosphamide. Blood *105*: 2862-2868, 2005.

54 Shibutani S, Inoue F, Aramaki O, Akiyama Y, Matsumoto K, Shimazu M, Kitajima M, Ikeda Y, Shirasugi N and Niimi M: Effects of immunosuppressants on induction of regulatory cells after intratracheal delivery of alloantigen. Transplantation *79*: 904-913, 2005.
55 Kawai M, Kitade H, Mathieu C, Waer M and Pirenne J: Inhibitory and stimulatory effects of cyclosporine A on the development of regulatory T-cells *in vivo*. Transplantation *79*: 1073-1077, 2005.
56 Zhang L, Dermawan K, Jin M, Liu R, Zheng H, Xu L, Zhang Y, Cai Y, Chu Y and Xiong S: Differential impairment of regulatory T-cells rather than effector T-cells by paclitaxel-based chemotherapy. Clin Immunol *129*: 219-229, 2008.
57 Larmonier N, Janikashvili N, LaCasse CJ, Larmonier CB, Cantrell J, Situ E, Lundeen T, Bonnotte B and Katsanis E: Imatinib mesylate inhibits CD4$^+$ CD25$^+$ regulatory T cell activity and enhances active immunotherapy against BCR–ABL$^-$ tumors. J Immunol *181*: 6955-6963, 2008.
58 Fei F, Yu Y, Schmitt A, Rojewski MT, Chen B, Götz M, Döhner H, Bunjes D and Schmitt M: Dasatinib inhibits the proliferation and function of CD4$^+$CD25$^+$ regulatory T-cells. Br J Haematol *144*: 195-205, 2009.
59 Ohkusu-Tsukada K, Toda M, Udono H, Kawakami Y, Takahashi K: Targeted inhibition of IL-10-secreting CD25- Treg via p38 MAPK suppression in cancer immunotherapy. Eur J Immunol *40*: 1011-1021, 2010.
60 Ben-Shoshan J, Maysel-Auslender S, Mor A, Keren G, George J: Hypoxia controls CD4$^+$CD25$^+$ regulatory T-cell homeostasis *via* hypoxia-inducible factor-1α. Eur J Immunol *38*: 2412-2418, 2008.
61 Ou-Yang HF, Zhang HW, Wu CG, Zhang P Zhang J, Li JC, Hou LH, He F, Ti XY, Song LQ, Zhang SZ, Feng L, Qi HW and Han H: Notch signaling regulates the FOXP3 promoter through RBP-J- and HES1-dependent mechanisms. Mol Cell Biochem *320*: 109-114, 2009.
62 Sagamura K, Ishii N and Weinberg AD: Therapeutic targeting of the effector T-cell co-stimulatory molecule OX40. Nat Rev Immunol *4*: 420-431, 2004.
63 Vu MD, Xiao X, Gao W, Degauque N, Chen M, Kroemer A, Killeen N, Ishii N and Li XC: OX40 co-stimulation turns off FOXP3$^+$ Treg. Blood *110*: 2501-2510, 2007.
64 Piconese S, Valzasina B and Colombo MP: OX40 triggering blocks suppression by regulatory T- cells and facilitates tumor rejection. J Exp Med *205*: 825-39, 2008.
65 Mignot G, Roux S, Thery C, Ségura E and Zitvogel L: Prospects for exosomes in immunotherapy of cancer. J Cell Mol Med *10*: 376-388, 2006.
66 Laulagnier K, Motta C, Hamdi S, Roy S, Fauvelle F, Pageaux JF, Kobayashi T, Salles JP, Perret B, Bonnerot C and Record M: Mast cell-and dendritic cell-derived exosomes display a specific lipid composition and an unusual membrane organization. Biochem J *380*: 161-171, 2004.
67 Ohno H: Overview: membrane traffic in multicellular systems: more than just a housekeeper. J Biochem *139*: 941-942, 2006.
68 Wada J, Onishi H, Suzuki H, Yamasaki A, Nagai S, Morisaki T and Katano M: Surface-bound TGF-β1 on effusion-derived exosomes participates in maintenance of number and suppressive function of regulatory T-cells in malignant effusions. Anticancer Res *30*: 3747-3757, 2010.
69 Suzuki H, Onishi H, Wada J, Yamasaki A, Tanaka H, Nakano K, Morisaki T and Katano M: VEGFR2 is selectively expressed by FOXP3^{high}CD4$^+$ Tregs. Eur J Immunol *40*: 197-203, 2010.
70 Wada J, Yamasaki A, Nagai S, Yanai K, Fuchino K, Kameda C, Tanaka H, Koga K, Nakashima H, Nakamura M, Tanaka M, Katano M and Morisaki T: Regulatory T-cells are possible effect prediction markers of immunotherapy for cancer patients. Anticancer Res *28*: 2401-2408, 2008.
71 Miyazawa M, Ohsawa R, Tsunoda T, Hirono S, Kawai M, Tani M, Nakamura Y and Yamaue H: Phase I clinical trial using peptide vaccine for human vascular endothelial growth factor receptor 2 in combination with gemcitabine for patients with advanced pancreatic cancer. Cancer Sci *101*: 433-439, 2010.

Received January 4, 2012
Revised February 3, 2012
Accepted February 6, 2012