Regulatory T Cells in Cancer

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At the present time, regulatory T cells (Tregs) are an integral part of immunology but the route from discovery of "suppressive" lymphocytes in the 1980s to the current established concept of Tregs almost 20 years later has been a rollercoaster ride. Tregs are essential for maintaining self-tolerance as defects in their compartment lead to severe autoimmune diseases. This vitally important function exists alongside the detrimental effects on tumor immunosurveillance and antitumor immunity. Beginning with the identification of $CD4^+CD25^+$ Tregs in 1995, the list of Treg subsets, suppressive mechanisms, and knowledge about their various origins is steadily growing. Increase in Tregs within tumors and circulation of cancer patients, observed in early studies, implied their involvement in pathogenesis and disease progression. Several mechanisms, ranging

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from proliferation to specific trafficking networks, have been identified to account for their systemic and/or local accumulation. Since various immunotherapeutic approaches are being utilized for cancer therapy, various strategies to overcome the antagonistic effects exerted by Tregs are being currently explored. An overview on the biology of Tregs present in cancer patients, their clinical impact, and methods for modulating them is given in this review. Despite the extensive studies on Tregs in cancer many questions still remain unanswered. Even the paradigm that Tregs generally are disadvantageous for the control of malignancies is now under scrutiny. Insight into the specific role of Tregs in different types of neoplasias is the key for targeting them in a way that is beneficial for the clinical outcome.

I. INTRODUCTION

A. Discovery and Fall

The current view on immunology can arguably be thought to begin with the discovery that adaptive immunity is composed of two major types of lymphocytes; the B (bone marrow-derived) and T (thymus-derived) cells (Miller, 1961; Mosier, 1967). Almost concurrently, anecdotal observations were already extending the role of T cells, beyond functioning as effectors and positive regulators, to suppressors of immunological responses. Pioneering studies by Gershon and Kondo in the early 1970s demonstrated for the first time that lymphocytes can suppress T cell responses in an antigen-specific manner (Gershon and Kondo, 1970) and that transfer of antigen-experienced T cells into naïve mice can lead to an antigen-specific tolerance by attenuating T cell activity (Gershon and Kondo, 1971). With great foresight, this cell population was named "suppressor cells" and fit perfectly into the dogma of homeostatic immunoregulation. It was hypothesized that by sustaining quantitatively and qualitatively optimal responses, the immune system facilitated an efficient elimination of pathogens and simultaneously prevented autoimmunity (Penhale *et al.*, 1973). Based on the observations that T cells from tumor bearing hosts were endowed with immunosuppressive capacities preventing the rejection of even highly immunogenic tumors by immunocompetent hosts, potential interconnections between "suppressor cells" and malignancies were presumed (Berendt and North, 1980; Fujimoto *et al.*, 1975). Despite the great significance of these findings, a growing skepticism led to a major loss of momentum and interest for almost 20 years. The main reasons for this were the failure to unequivocally define these cells together with a number of key misleading publications on MHC regions postulated as characteristic for "suppressor cells;" in particular, the illusory I-J locus as well as T-T suppressor hybridomas not transcribing T cell receptor (TCR) genes (Moller, 1988; Simpson, 2008).

B. Renaissance Through Steady Characterization

Finally, in 1995 Sakaguchi and colleagues initiated the renaissance of the "suppressive cells" (Sakaguchi *et al.*, 1995). In very elegant experiments they showed that transfer of thymic CD25-depleted T cells induced autoimmune diseases in athymic nude mice, while addition of a small proportion of $CD4^+CD25^+$ T cells was sufficient to maintain tolerance. Accordingly, the CD25 molecule was the first promising candidate for a phenotypic definition of "suppressive cells" that were named as thymus-derived naturally occurring regulatory T cells (nTregs). CD25 is the α-chain of the high-affinity receptor for interleukin-2 (IL-2R). Although nTregs do not produce IL-2 (Allan *et al.*, 2005) they are vitally dependent on IL-2 production by their environment. This is markedly illustrated by the development of a lethal lymphoproliferative disease in mice deficient for IL-2 or the IL-2Rβ, which resulted in dysregulated T cell activation and severe alterations within the nTreg compartment (Suzuki *et al.*, 1995). The constitutive expression of the IL-2R on nTregs may reflect this dependence on external IL-2. Several models to date have explored how IL-2 signaling contributes to suppressive function, thymic development, and homeostasis of Tregs (Bayer *et al.*, 2005; Furtado *et al.*, 2002; Setoguchi *et al.*, 2005). Interestingly, IL-2 is one of the primary cytokines secreted by effector T cells upon stimulation (Sojka *et al.*, 2004), and drives proliferation and clonal expansion of T cells (Morgan *et al.*, 1976). In parallel, IL-2 appears to be crucial for mechanisms involved in the termination of T cell responses, thereby forming a sophisticated negative feedback circuit.

Although CD25 was sufficient to characterize and further analyze a relatively homogeneous population of nTregs in mice, the same approach was rather challenging in humans. The reason is the limited specificity provided by CD25, whose intrinsic expression at varying levels can be noted in approximately 30% of the T cells and is further upregulated on effector T cells upon stimulation (Baecher-Allan *et al.*, 2004). Unlike mice kept under pathogen-free conditions, humans are continually exposed to immunogenic stimuli resulting in T cell activation and potential CD25 upregulation. In pathological conditions associated with ongoing inflammation this problem is even more pronounced. Consequently, studying Tregs in autoimmune and malignant diseases is complicated further. It may even be speculated that past studies describing $CD25^+$ Tregs as functionally defective may have been influenced by contamination of activated $CD25^+$ effector T cells (Dejaco *et al.*, 2006). In the steady effort to define Tregs more accurately, it was demonstrated that up to 5% of human peripheral $CD4^+$ T cells that express CD25 at high levels are endowed with strong immunosuppressive capacities. This observation narrowed the phenotype of human Tregs further

down to $CD4^+CD25^{high}$ T cells (Baecher-Allan *et al.*, 2001). Due to the lack of a standardized methodological cut off point for $CD25^{high}$ expression, comparability between clinical studies remained difficult and elevated levels of CD25 expression on effector T cells under conditions of severe inflammatory activity could not be excluded (Han *et al.*, 2008; Seddiki *et al.*, 2006).

Efforts to identify the genetic defects responsible for the severe autoimmune disorders in patients with the IPEX (Immunodysregulation, Polyendocrinopathy, Enteropathy, X-linked) syndrome led to the discovery of germline mutations resulting in a *FOXP3* gene deletion on the X-chromosome (Bennett *et al.*, 2001; Chatila *et al.*, 2000). The *FOXP3* gene encodes for a transcription factor (TF) of the forkhead-box/winged-helix family. Extensive studies in mice and humans revealed the critical importance of the FOXP3 TF as a master regulator of nTreg development and function. Late double-positive lymphocytes that already express FOXP3 at early thymic developmental stages appear to be destined for the nTreg lineage (Tai *et al.*, 2005; Zhou *et al.*, 2009). Ectopic expression of FOXP3 by retroviral gene transfer in $CD4^+CD25^-$ T cells has been shown *in vitro* and *in vivo* to result in phenotypic and functional suppressive cells demonstrating the plasticity of lymphocytes and the pivotal role of FOXP3 for nTregs (Fontenot *et al.*, 2003; Hori *et al.*, 2003). Concordant to the CD25 expression-based characterization of Tregs, the majority of $CD4^+FOXP3^+$ T cells were found to be $CD25^{high}$ (Baecher-Allan *et al.*, 2004; Roncador *et al.*, 2005). FOXP3 dimerizes with the nuclear factor of activated T cells (NF-AT) leading to suppression of IL-2, IL-4, and interferon-γ (IFN-γ) expression, while inducing CD25, cytotoxic T lymphocyte antigen 4 (CTLA-4), and gluco-corticoid-induced TNF receptor family-related gene/protein (GITR) (Lopes *et al.*, 2007; Wu *et al.*, 2006). Like CD25, both CTLA-4 and GITR are also upregulated on effector T cells upon activation (Ermann and Fathman, 2003; Roncador *et al.*, 2005; Tai *et al.*, 2005). Although FOXP3 is presently considered the most reliable (intracellular) phenotypic marker for nTregs, major concerns arose when it became evident that FOXP3 expression could be transiently induced in $CD4^+$ and $CD8^+$ effector T cells upon stimulation, albeit at lower levels (Gavin *et al.*, 2006; Roncador *et al.*, 2005; Roncarolo and Gregori, 2008; Walker *et al.*, 2003; Ziegler, 2007). Consequently, Zou and colleagues suggested a combination of FOXP3 and intracellular cytokine staining, especially for IL-2, IFN-γ, and tumor necrosis factor-α (TNF-α), as an accurate tool to identify nTregs based on the fact that activated $FOXP3^+$ conventional T cells express these polyfunctional cytokines in contrast to nTregs (Kryczek *et al.*, 2009). A promising approach to overcome these impediments can be initiated at the epigenetic level. A major criterion for the lineage commitment of nTregs is

the sustained, stable expression of FOXP3 as compared to the transient expression found in $FOXP3^+$ effector T cells. A static gene expression can be achieved stably through remodeling of the chromatin structure by epigenetic modifications like DNA methylation. In fact a specific methylation pattern, particularly a demethylated DNA sequence within the FOXP3 locus, associated with stable FOXP3 expression upon *in vitro* expansion, was identified as nTreg-specific and defined as a Treg-specific demethylated region (Baron *et al.*, 2007). This methodology has recently been further optimized allowing enumeration of nTregs in clinical samples such as peripheral blood (PB) and tissues (Wieczorek *et al.*, 2009). Furthermore, two studies have demonstrated that expression of the IL-7R α-chain (CD127) is a useful marker for discriminating between activated conventional T cells and nTregs (Liu *et al.*, 2006b; Seddiki *et al.*, 2006). Suppressive $CD4^+$ T cells are negative or weakly positive for CD127, which inversely correlates with the FOXP3 expression, regardless of the CD25 levels. Consequently, the following proposed phenotype of $CD4^+CD25^+CD127^{low/neg}FOXP3^+$ T cells corresponds to the majority of nTregs. Importantly, this phenotype allows a more homogeneous purification of viable $CD4^+CD25^+CD127^{low/neg}$ nTregs.

The characterization of "suppressive cells" based on CD25 expression heralded a new era of Treg research. More than 10 years later this process is still ongoing and has definitely gained momentum. One of the research areas with the strongest interest in Treg biology has traditionally been cancer research. The biology of human Tregs and their various subtypes, their complex role in cancer and translational approaches in modern cancer therapy are discussed in subsequent sections.

II. REGULATORY T CELL SUBSETS

Several studies have demonstrated that nTregs are primarily formed by high-avidity selection of CD4 single-positive thymocytes through major histocompatibility complex (MHC) class II-dependent TCR interactions (Apostolou *et al.*, 2002; Bensinger *et al.*, 2001; Fontenot *et al.*, 2005b; Jordan *et al.*, 2001; Larkin *et al.*, 2008; Modigliani *et al.*, 1996; Sakaguchi, 2001). However, other contributory mechanisms like selective survival rather than induced differentiation (van Santen *et al.*, 2004) or the expression of the TF AIRE (autoimmune regulator) by medullary thymic epithelial cells are also implicated (Liston *et al.*, 2003). In addition to sustaining self-tolerance, Tregs control a broad spectrum of immune responses including those against tumor cells, allergens, pathogenic microbes as well as allogeneic transplants and the fetus during pregnancy (Baecher-Allan and Anderson, 2006; Battaglia and Roncarolo, 2006;

Chatila, 2005; Mills, 2004; Zenclussen, 2006). Although Tregs could be integrated into an overall T cell population with suppressive properties there is an increasing number of reports on various Treg subsets with distinct development, phenotype and functions (Jiang and Chess, 2006) (summarized in Table 1). It has become apparent that under various conditions, Tregs that are termed adaptive or induced Tregs (iTregs) can be generated extrathymically. Suboptimal antigenic stimulation within specific cytokine milieus, particularly rich in transforming growth factor-β (TGF-β), can result *in vivo* and *in vitro* in the induction of iTregs from conventional T cells (Apostolou and von Boehmer, 2004; Kretschmer *et al.*, 2005; Roncarolo *et al.*, 2006). Physiologically, Treg induction in mesenteric lymph nodes (LNs) and the enteric lamina propria in response to gut flora and food antigens is a major mediator of oral tolerance (Coombes *et al.*, 2007; Mucida *et al.*, 2005; Sun *et al.*, 2007). Furthermore, iTregs are also found in chronically inflamed or transplanted tissues as well as tumors, all of which typically have an altered cytokine milieu (Cobbold *et al.*, 2004; Curotto de Lafaille *et al.*, 2008; Liu *et al.*, 2007). To date several phenotypically and functionally distinct iTreg subsets of both CD4 and CD8 lineage have been described. The most delineated populations include IL-10$^+$ T regulatory 1 (Tr1), TGF-β T helper (Th) 3, CD4$^+$CD25$^+$ nTreg-like, CD8$^+$CD25$^+$, and CD8$^+$CD28$^-$ cells.

A. Naturally Occurring CD4$^+$ Regulatory T cells

As described in the previous sections, most CD4$^+$ nTregs produced by the normal thymus constitutively express CD25 and represent a functionally mature population. Development and function of nTregs depend on the expression of the FOXP3 TF. The *FOXP3* gene contains one AP-1 (Activator Protein-1) and six NF-AT binding sites (Mantel *et al.*, 2006). Previous studies have shown that FOXP3 is a repressor of the *Il2*, *Il4*, and *Ifng* gene transcription through direct interaction with NF-κB and NF-AT. Formation of NF-AT–FOXP3 complexes is essential for the suppressive activity (Bettelli *et al.*, 2005). At the same time this complex is involved in the upregulation of CD25, CTLA-4, and GITR expression (Wu *et al.*, 2006). One hallmark of nTregs is anergy manifested by their inability to proliferate and produce IL-2 upon TCR stimulation. IL-2 is a critically important cytokine for their generation and normal activity *in vivo* (Malek *et al.*, 2002; Suzuki *et al.*, 1995; Wolf *et al.*, 2001). In addition to IL-2, other γ-chain cytokines such as IL-4, IL-7, and IL-15 have also been reported to play a role in the development and suppressive capacity of nTregs (Cupedo *et al.*, 2005; Thornton *et al.*, 2004; Yates *et al.*, 2007). Early studies on TGF-β and TGF-βR

Table I Regulatory T Cell Subsets and Suppressive Mechanisms

Cell type	Origin	Phenotype	Suppressive mechanisms	References
Naturally occurring Tregs	Thymus			
CD4 nTregs		$CD4^+CD25^+FOXP3^+CD127^{-/low}$ $CTLA\text{-}4^+LAG\text{-}3^+GITR^+$	Contact, cytotoxicity, IL-10, TGF-β	Sakaguchi (2004)
CD8 nTregs		$CD8^+CD25^+FOXP3^+CTLA\text{-}4^+CD122^+$	Contact	Fontenot *et al.* (2005a), Rifa'i *et al.* (2004)
Adaptive/Induced Tregs	Periphery			
CD4 nTreg-like		$CD4^+CD25^+FOXP3^+CTLA\text{-}4^+GITR^+$	Contact (requires IL-2 and TGF-β)	Apostolou and von Boehmer (2004)
Tr1		$CD4^+CD25^{-/low}FOXP3^{-/low}$	IL-10	Groux *et al.* (1997)
Th3		$CD4^+CD25^+FOXP3^+$	TGF-β, IL-10 (to a lesser extent)	Chen *et al.* (1994)
CD8 iTregs		$CD8^+CD25^+FOXP3^+$	IL-10, TGF-β	Chaput *et al.* (2009), Wei *et al.* (2005)
CD8 iTregs		$CD8^+CD25^+CD28-FOXP3^+$ $CTLA\text{-}4^+GITR^+$	Contact, IL-10, ILT3, ILT4	Cortesini *et al.* (2001)

knockout mice did not indicate an involvement of the TGF-β pathway in the development of nTregs; findings were strengthened by recent observations that in the absence of TGF-β signaling IL-2 compensates for its effects (Liu *et al.*, 2008).

With regard to the function of nTregs, it is now established that nTregs suppress activation and expansion of cells from adaptive as well as innate immunity hampering cellular and humoral immune responses. Effector and memory T cells of both $CD4^+$ and $CD8^+$ compartments are efficiently suppressed by $CD4^+CD25^+FOXP3^+$ nTregs with regard to activation, proliferation, and function (Levings *et al.*, 2001; Piccirillo and Shevach, 2001; Takahashi *et al.*, 1998; Thornton and Shevach, 1998). Proliferation, immunoglobulin (Ig) production, and Ig class switch of B cells can be suppressed by nTregs, partly mediated by TGF-β secretion (Lim *et al.*, 2005; Nakamura *et al.*, 2004). Furthermore, nTregs have been shown to inhibit the function of natural killer (NK) cells and NKT cells as well as the function and maturation of dendritic cells (DCs) (Azuma *et al.*, 2003; Ghiringhelli *et al.*, 2005a; Misra *et al.*, 2004). Immature DCs, on the other hand, provide aberrant stimuli to naïve T cells and potentially transform them to iTregs, thereby forming a positive loop. Macrophages that are entering the tissues can switch between proinflammatory M1 and anti-inflammatory M2 phenotypes. A tolerogenic milieu, which is typically found in tumors, skews macrophages toward an M2 phenotype. In experiments performed *in vitro* nTregs induced an analogous immunosuppressive M2-like alternative activation phenotype in macrophages (Tiemessen *et al.*, 2007).

B. Induced (Adaptive) $CD4^+$ Regulatory T Cells

While nTregs play a critical role in regulating self-tolerance, iTregs are thought to be responsible for governing the immune response to a wide variety of microbial and tissue antigens. They develop in peripheral lymphoid tissues from naive T cells normally at very low frequencies in a steady state and endow the immune system with an extraordinary environmental adaptability. The physiological processes and environmental conditions driving their development are as yet incompletely determined. Up till now, tumor-induced Tregs are phenotypically indistinguishable from other iTregs and often also from nTregs. However, it remains to be further investigated whether tumor-associated iTregs acquire specific characteristics contributed by the tumor environment. A prerequisite for iTreg development is TCR triggering of naïve T cells by antigenic stimulation under conditions not optimal for the generation of effector T cells. The circumstances under which iTregs are induced are wideranging and may include among others the

presence of certain cytokines most notably high levels of IL-2, IL-10, or TGF-β, low dose of antigens and antigen presenting cells (APCs) exhibiting alterations in maturation and function (Curotto de Lafaille and Lafaille, 2009; Lohr *et al.*, 2006). It is obvious that the local microenvironment is the key to the generation of iTregs. Tumor cells can directly initiate the induction of Tregs through several factors including CD70, cyclooxygenase-2 (COX-2), indoleamine 2,3-dioxygenase (IDO), IL-10, Galectin-1, and TGF-β (Bergmann *et al.*, 2007; Curti *et al.*, 2007; Juszczynski *et al.*, 2007; Li *et al.*, 2007; Liu *et al.*, 2007; Yang *et al.*, 2007). In addition, neoplastic cells can modulate recruited or local APCs to become tolerogenic, which thereby strongly contribute to the induction of Tregs within the microenvironment or the local LNs.

Several subsets of $CD4^+$ iTregs have been described, which differ but also overlap with regard to their phenotype, function, and mechanisms of suppression. Well-established subsets of $CD4^+$ iTregs are the Th3, Tr1, and $CD25^+FOXP3^+$ nTreg-like cells. Th3 cells are defined by their production of large amounts of TGF-β that they utilize for direct suppression and the creation of a tolerogenic milieu and to a lesser extent IL-4 and IL-10 (Chen *et al.*, 1994). This subset is one of the earliest regulatory populations described *in vivo* following oral tolerance toward myelin basic protein (MBP) and suppressing the induction of MBP-specific experimental autoimmune encephalitis (Chen *et al.*, 1994). Th3 generation appears to be triggered in an antigen-dependent fashion but suppression is antigen-independent, leading to the term "bystander suppression". Tr1 cells were initially observed to develop *in vitro* in the presence of high dose of IL-10 and chronic antigenic stimulation. They produce high levels of IL-10 and negligible amounts of IL-2 and IL-4, if any (Groux *et al.*, 1997). In accordance to the *in vitro* results Tr1 cells could also be generated *in vivo* by multiple rounds of stimulation with immature DCs in presence of IL-10 (Levings *et al.*, 2005). In contrast to a minor proportion of Th3 cells, nTreg-like cells and nTregs, Tr1 cells express no or low levels of FOXP3 and CD25 (Bacchetta *et al.*, 2005; Foussat *et al.*, 2003; Levings *et al.*, 2002). Like Th3 cells, Tr1 cells require TCR ligation in order to acquire suppressive activity, and once activated Tr1 cells can mediate bystander suppression. Tr1 cells and their supernatants containing IL-10 directly suppress T cells but can also reduce the capacity of DCs to induce alloantigen-specific T cell responses. Cancer is often associated with complement activation. Stimulation via the CD46 molecule, which is a receptor for the complement factors CD3b and CD4b and widely expressed on lymphocytes can lead to the generation of $IL\text{-}10^+$ Tr1 cells when combined with TCR triggering (Kemper *et al.*, 2003). The highly suppressive $FOXP3^+$ iTregs called nTreg-like cells express CD25, CTLA-4, and GITR and to date several settings leading to their generation from naïve T cells have been described.

Antigenic stimulation in the presence of TGF-β or IL-2 can lead to the induction of this suppressive phenotype in naive T cells (Apostolou and von Boehmer, 2004). Studies in mice have suggested that conversion of $CD4^+CD25^-$ T cells to nTreg-like cells *in vivo* requires costimulation via B7 (CD80 and CD86) molecules (Liang *et al.*, 2005). Another rather antagonistic key cytokine is IL-6, which abolishes the conversion to suppressive iTregs and at the same time promotes the generation of Th17 cells. Cumulatively, the observations emphasize the role of soluble factors and cytokines in determining cell differentiation from tolerogenic to responsive subtypes and vice versa (Korn *et al.*, 2008).

C. Naturally Occurring and Induced $CD8^+$ Regulatory T Cells

Although, $CD4^+$ Tregs have been the focus of Treg research, $CD8^+$ Tregs are increasingly emerging as crucial components in the negative control of immune responses. Interestingly, $CD8^+$ suppressor cells were already described together with their $CD4^+$ counterparts in the early 1970s (Gershon and Kondo, 1970). Similar to $CD4^+$ Tregs, Tregs from the $CD8^+$ lineage may develop intrathymically as well as in peripheral tissue. $CD8^+CD25^+FOXP3^+CTLA\text{-}4^+$ nTregs have been identified in several studies in rodents and humans and act mainly in a cell-to-cell contact-dependent fashion (Cosmi *et al.*, 2003, 2004; Fontenot *et al.*, 2005a; Rifa'i *et al.*, 2004; Xystrakis *et al.*, 2004a,b). Peripherally induced $CD8^+$ iTregs are generated from naïve $CD8^+CD25^-$ T cells upon antigenic stimulation (Mills, 2004). $CD8^+$ Tregs described in humans with mycobacterial infections expressed lymphocyte-activation gene 3 (LAG-3) and suppressed T cell activation by CC chemokine ligand 4 secretion, which interferes with TCR signaling (Joosten *et al.*, 2007) whereas $CD8^+$ Tregs in systemic lupus erythematodes patients produced significant amounts of TGF-β (Zhang *et al.*, 2009). Recent reports also describe $CD8^+$ Tregs in cancer patients. In prostate cancer patients, $CD8^+$ Tregs were described to be $CD25^+CD122^+FOXP3^+$ and partly $GITR^+$. Their suppressive activity was mediated via cell-to-cell contact as well as through yet unidentified soluble factors other than IL-10 or TGF-β (Kiniwa *et al.*, 2007). $CD8^+CD25^+FOXP3^+$ Tregs in colorectal cancer were positive for TGF-β (Chaput *et al.*, 2009). Tumor plasmacytoid DCs (pDCs) from ovarian cancer patients induced $CD8^+$ iTregs *in vitro* which corroborates with the *ex vivo* data showing an accumulation of $CD8^+$ Tregs in ascites, draining LNs and PB of the patients (Wei *et al.*, 2005). In this particular setting suppression was mainly mediated by secreted IL-10 underlining the plasticity of the suppressive phenotype as well as its dependence on the shaping milieu. The proposed model of induction and activation of the $CD8^+$ Tregs at the tumor site is analogous to $CD4^+$ Tregs. $CD8^+$ Tregs

accumulate in tumor tissues (Chaput *et al.*, 2009; Kiniwa *et al.*, 2007; Wei *et al.*, 2005) and can be activated in a peptide-specific manner as recently shown in various types of tumors (Andersen *et al.*, 2009). Another type of $CD8^+$ iTregs is $CD8^+CD28^-$ iTregs, which was first described in the allogeneic setting induced through MHC class I peptide stimulation, but is also found in cancer patients (Cortesini *et al.*, 2001; Filaci and Suciu-Foca, 2002; Suciu-Foca *et al.*, 2005). $CD8^+CD28^-$ iTregs have been shown to be suppressive via contact-dependent mechanisms, IL-10 secretion as well as upregulation of inhibitory immunoglobulin-like transcript (ILT) receptors ILT3 and ILT4 on APCs (Filaci *et al.*, 2007; Suciu-Foca and Cortesini, 2007). Characterization and understanding of $CD8^+$ Tregs is at its inception and consequently subclassification and function is relatively tentative and will surely be modified and expanded in the future.

III. MECHANISMS MEDIATING THE SUPPRESSIVE FUNCTION

In the past decade extensive studies have been performed to further explore the underlying cellular and molecular mechanisms of Treg-mediated immunomodulation (summarized in Fig. 1), which has led to significant improvement in our understanding.

Proliferation and cytokine production of conventional T cells can be inhibited upon TCR activation of Tregs (Takahashi *et al.*, 1998; Thornton and Shevach, 1998). This process is cell-to-cell contact dependent and leads to an inhibition of IL-2 production. Functional activity can be rescued by the administration of IL-2 and activating anti-CD28 antibodies, which implies a disruption of costimulatory signaling being involved. Furthermore, CTLA-4 and LAG-3 surface molecules constitutively expressed on nTregs contribute to the cell-to-cell-dependent suppressive mechanisms via interactions with CD80 and CD86 on APCs (Huang *et al.*, 2004; Sakaguchi, 2004). CTLA-4 is a ligand for CD80 and CD86, possessing a higher affinity than the CD28, thereby directly competing with the costimulatory signal transduction. Blockage of CTLA-4 *in vivo* results in the development of organ-specific autoimmune diseases (Sakaguchi, 2004). Another role of CTLA-4 could be that it directly exerts suppressive activity through induction of the enzyme IDO in DCs via interaction with their CD80 and CD86 (Fallarino *et al.*, 2006). IDO catalyzes the conversion of tryptophan into kynurenine, leading to (A) tryptophan depletion and (B) generation of immunosuppressive metabolites, both of which attenuate T cell function (Fallarino *et al.*, 2006). It has also been proposed that binding of CTLA-4 to CD80 and CD86 mediates their downregulation on DCs in a negative feedback manner (Misra *et al.*,

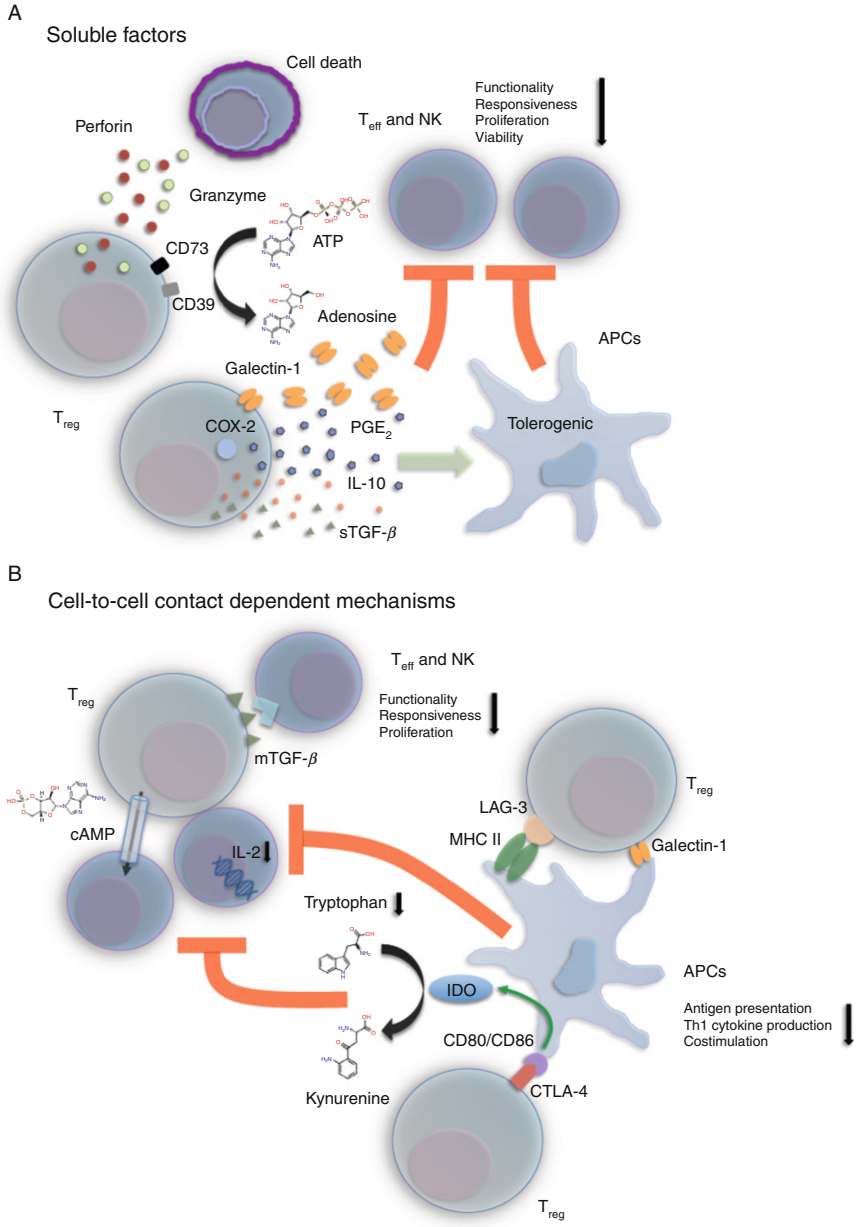

Fig. 1 (Continued)

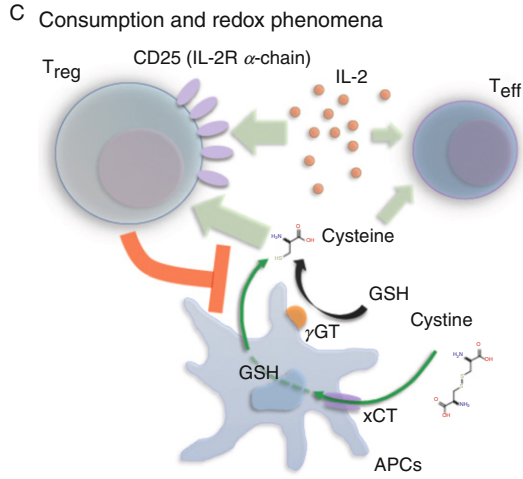

Fig. 1 Regulatory T cell-mediated immunosuppression. (A) Several soluble factors released by regulatory T cells (Tregs) (e.g., Galectin-1, Prostaglandin E_2 [PGE_2]) may directly suppress or induce cell death (e.g., Perforin, Granzyme) of effector T (T_{eff}) and NK cells. Ectoenzymes located on the cell membrane of Tregs (e.g., CD39, CD73) mediate the metabolization of ATP to Adenosine, a potential suppressant of T cells. Antigen presenting cells (APCs) are functionally modulated (e.g., by IL-10, soluble TGF-β [sTGF-β]) contributing to a tolerogenic tumor milieu. (B) Cell-to-cell contact between Tregs and immune cells is obligatory for certain direct and indirect suppressive pathways. Tregs weaken T_{eff} and NK cell responses by membrane-bound TGF-β (mTGF-β) as well as cAMP "injections." Close interaction with APCs (e.g., via LAG-3, Galectin) reduces their immunostimulatory capacity through attenuation of costimulation and antigen presentation, while increases their tolerizing potential, especially by a CTLA-4-mediated upregulation of the enzyme Indoleamine 2,3-dioxygenase (IDO). IDO activity leads to a depletion of tryptophan accompanied by an accumulation of kynurenine, both with a negative impact on T cells. (C) Proper function of Tregs depends on IL-2 produced by other cells. Tregs express high levels of CD25, a component of the IL-2 high-affinity receptor, enabling them to withdraw IL-2 from their local environment. Tregs alter the redox balance of T cells by inhibition of their supply of thiols provided by APCs mainly in form of cysteines, which are obligatory for an efficient activation.

2004). Consequently, further activation of T cells by the DCs is abrogated which leads to aberrant stimulation and generation of iTregs. LAG-3 is a CD4 homologue expressed on nTregs upon activation and on certain $CD8^+$ Tregs (Joosten *et al.*, 2007). The LAG-3 binds to MHC class II molecules expressed on several types of APCs and is required for maximal suppressive activity (Huang *et al.*, 2004). Unlike CTLA-4, mice deficient for LAG-3 do not develop severe autoimmunity. Recent studies suggest the involvement of LAG-3 in suppressing DC maturation and immunostimulatory capacity by recruitment of SH2-domain-containing protein tyrosine phosphatase 1 (Liang *et al.*, 2008). Gene expression analyses have shown that *GITR* transcription is under the control of the FOXP3 TF and is thus

highly, but not exclusively, expressed in Tregs (McHugh *et al.*, 2002; Shimizu *et al.*, 2002). Studies on T cells from GITR-deficient mice have revealed that ligation of GITR on naïve $CD4^+CD25^-$ T cells is involved in the inhibition of the Treg-mediated suppression (Stephens *et al.*, 2004). In addition to GITR there are several molecules found in Tregs that contribute to the control of Treg-mediated suppression including toll-like receptors (TLRs) like TLR2 and TLR8 (Peng *et al.*, 2005; Sutmuller *et al.*, 2006).

In contrast to the requirement for cell-to-cell contact for suppression by Tregs *in vitro*, there are numerous reports that indicate the need for soluble factors such as IL-10 and TGF-β for suppression *in vivo*. Several studies on rodents especially in models of autoimmune diseases, like colitis or asthma, have demonstrated the importance of IL-10 for Treg-mediated immunosuppression (Annacker *et al.*, 2001, 2003; Hawrylowicz and O'Garra, 2005; Tang *et al.*, 2004). However, *in vitro* experiments with human nTreg-clones did not show secretion of IL-10, but only of TGF-β (Roncarolo *et al.*, 2006). Similarly, IL-10 and TGF-β are rarely detectable in the supernatants from suppression assays with nTregs *in vitro* (Sakaguchi, 2004). In contrast, adaptive Tr1 cells and selected $CD8^+$ T cells produce and secrete substantial amounts of IL-10. Interestingly, membrane-bound TGF-β can be found on Tregs and is implicated in mediating nTreg suppression of T and NK cells in a cell-to-cell contact-dependent manner (Chen *et al.*, 2005; Ghiringhelli *et al.*, 2005a). In patients with gastrointestinal stromal tumors (GIST) an inverse correlation between NK cell activation and Treg expansion was observed. Subsequent analyses revealed that Tregs utilized membrane-bound TGF-β to attenuate the cytotoxic function of NK cells and downregulate the expression of the activating NKG2D receptor (Ghiringhelli *et al.*, 2005a). The controversy regarding the role of IL-10 and TGF-β for nTreg-mediated suppression is ongoing and inferences appear to strongly depend on the model studied. Another newly identified inhibitory cytokine belonging to the IL-12 heterodimeric family is IL-35, which is found in murine Tregs. IL-35 may contribute to the function of Tregs but is not constitutively expressed in human Tregs and warrants further investigation (Bardel *et al.*, 2008; Collison *et al.*, 2007). Galectin-1, a member of a highly conserved family of β-galactoside-binding proteins is preferentially expressed on human Tregs and upregulated upon TCR activation (Garin *et al.*, 2007). It is secreted as a homodimer and binds glycoproteins such as CD45, CD43, and CD7 leading to growth arrest, apoptosis as well as abrogation of proinflammatory cytokine production in activated T cells. Blocking galectin-1 clearly reduces the maximal intrinsic inhibitory efficacy of both mouse and human Tregs. However, it is still not clear whether galectin-1 works *in vivo* mainly as a soluble factor or exerts its suppressive effect via cell-to-cell contact. Induced Tregs secrete T cell suppressive prostaglandin (PG) E_2, which is generated by COX-2 (Mahic *et al.*, 2006) and $COX\text{-}2^+$

iTregs were noted in colorectal cancer patients in whom T cell function could be restored by the COX inhibitor indomethacin (Yaqub *et al.*, 2008).

As previously described, Tregs require IL-2 for a proper function, which they do not produce themselves and need conventional T cells as their main source *in vivo*. Accordingly, the nTregs express increased levels of the high-affinity heterotrimeric receptor for IL-2 composed of CD25, CD122, and CD132. Competitive depletion of available IL-2 by Tregs and the resultant starvation of activated, dividing T cells has been proposed as a minor suppressive mechanism at minimum within the tumor microenvironment (Pandiyan *et al.*, 2007; von Boehmer, 2005). Exhaustion of free thiol groups by a process similar to cytokine depletion can also produce a negative effect on activated T cells. Conventional T cells require thiols for efficient activation. Activated T cells need cysteine as they lack transporters for its oxidized form, cystine. It has been shown that DCs create a cysteine-rich milieu by intra- and extracellular redox reactions thereby providing cysteine to the T cells (Angelini *et al.*, 2002). Tregs interfere with this process with one very likely mechanism being competitive consumption of thiols including cysteine, as Tregs exhibit increased levels of intra- and extracellular thiols (Mougiakakos *et al.*, 2009; Yan *et al.*, 2009).

The perforin/granzyme pathway classically mediates cytolytic effects of $CD8^+$ T and NK cells. Perforins traffic granzymes into target cells, whereas granzyme A and B induce apoptosis by cleaving important substrates. Tregs utilize this system to initiate cytolysis of monocytes, B and T cells as well as DCs (Gondek *et al.*, 2005; Grossman *et al.*, 2004; Zhao *et al.*, 2006). Granzyme A expression by human Tregs has been established; however, the expression of granzyme B remains equivocal (Grossman *et al.*, 2004). One study demonstrated in a mouse tumor model that up to 30% of Tregs located at the tumor site utilize the perforin/granzyme B pathway to suppress antitumor responses suggesting a tumor-driven induction of cytolytic Tregs (Cao *et al.*, 2007). In another recent study, Wilms Tumor 1 (WT1)-specific Treg clones from leukemia patients, upregulated granzyme B upon peptide stimulation. These cells had an nTreg-like $CD4^+CD25^+CD127^{neg}$ $FOXP3^+GITR^+$ phenotype and induced cytolysis of APCs (Lehe *et al.*, 2008). Nevertheless, the role of cytolysis as a major suppressive mechanism *in vivo* remains unresolved and is further expanded by the addition of TRAIL/DR5 and galectin pathways as potential cytolytic mechanisms (Ren *et al.*, 2007; Toscano *et al.*, 2007). As described previously nTregs are anergic. In this context it has been observed that elevated cyclic adenosine monophosphate (cAMP) levels in Tregs contribute to their anergic state. Formation of gap junctions between Tregs and effector T cells permits diffusion of cAMP following the concentration gradient into effector T cells inducing suppression through the cAMP–protein kinase A type I–C-terminal Src kinase inhibitory pathway (Bopp *et al.*, 2007). Additionally, it has

recently been reported that Tregs express ectoenzymes like CD73 that cleave extracellular adenosine triphosphate (ATP) generating adenosine, which inhibits T cell function through the adenosine receptor 2A (Vignali *et al.*, 2008).

IV. REGULATORY T CELLS IN CANCER

The role of the immune system in cancerogenesis and tumor progression has been the subject of much controversy since the 1950s when Burnet and Thomas formulated their concept of "tumor immunosurveillance"; a process through which the immune system recognizes and (ideally) eliminates self-cells that have undergone malignant transformation (Burnet, 1957). Numerous observations in clinical and experimental settings have fortified this concept that was further advanced by the model of "immune editing." According to this theory, multiple factors generated by the oncogenic process counteract the immune system cumulatively hampering an efficient immune response and facilitating the "tumor escape" (Dunn *et al.*, 2002). Tregs as regulatory elements have the ability to actively suppress immune responses and represent a predominant tolerance-inducing modality (Sakaguchi *et al.*, 2008). Already in the early days of the discovery of the suppressor cells, observations from tumor mouse models indicated a central (negative) role of Tregs in immunosurveillance; namely hindering an efficient tumor eradication. Tumor cells, in particular methylcholanthrene-induced fibrosarcomas, elicited measureable T cell responses that were not sufficient to eradicate the tumors due to the development of tumor-induced suppressor T cell activity within the $CD4^+$ T cell population (Berendt and North, 1980; Dye and North, 1981). In the following part of the review, we have focused mainly on the impact of Tregs in patients with solid tumors and hematological malignancies. The underlying biological mechanisms and targeted therapeutic interventions are discussed.

A. Regulatory T Cells in Solid Malignancies

The vast majority of the studies on Tregs in cancer are performed on patients with solid malignancies. It is obligatory to take into consideration that virtually all of these studies were carried out during the period when the phenotype of Tregs was being refined thereby complicating direct comparisons between studies. Shortly after the publication on the existence of $CD4^+CD25^{high}$ Tregs in the PB of healthy individuals (Baecher-Allan *et al.*, 2001) the group

of Carl June was the first to provide direct evidence that patients with epithelial malignancies, in particular ovarian and non-small-cell lung cancer (NSCLC) displayed increased levels of $CD4^+CD25^{high}$ Tregs in the circulation and within the tumor infiltrating lymphocytes (TILs). These cells constitutively expressed CTLA-4 and exhibited suppressive effects by inhibiting the proliferation of conventional T cells and IFN-γ production. The suppressive activity was partly mediated by TGF-β (Woo *et al.*, 2001, 2002). In patients with pancreatic and breast cancer, increased levels of cells with similar phenotype were found in the PB, LNs, and tumor tissue. These cells were positive for IL-10, TGF-β, and CTLA-4 (Liyanage *et al.*, 2002). Furthermore, results from these initial studies strongly indicated a tropism of Tregs toward tumor sites as their proportion in draining LNs and TILs was higher than that expected theoretically, based on their frequencies in PB. In addition, the first Treg cell lines derived from autologous cocultures of tumor cells and lymphocytes from colorectal cancer patients were generated. These cells displayed tumor-dependent expansion and suppressed both allogeneic and autologous T cell responses independent of cell-to-cell contact via TGF-β (Somasundaram *et al.*, 2002). One of the first proposed mechanisms underlying the activation and induction of Tregs was heavy-chain Ferritin (H-Ferritin), which is produced in large amounts by melanoma cells. Melanoma patients exhibited a significant positive correlation between serum levels of H-Ferritin and increased Treg frequencies and activation (Gray *et al.*, 2003; Javia and Rosenberg, 2003; Viguier *et al.*, 2004). Several studies on gastro-esophageal cancers also reported that increased frequencies of IL-10-producing $CD4^+CD25^{high}$ Tregs can be found in PB, TILs, draining LNs, and ascites fluid, which were strongly associated to disease stage (Ichihara *et al.*, 2003; Kawaida *et al.*, 2005; Kono *et al.*, 2006; Sasada *et al.*, 2003). Importantly, the proportion of Tregs was significantly reduced in patients to almost physiological levels upon curative surgery. Furthermore, the level of Tregs rebounded at the timepoint of postoperative recurrent disease, strongly indicating an interconnection between tumor burden and Treg accumulation (Kono *et al.*, 2006). It has been shown that $CD4^+CD25^+$ Tregs are capable of suppressing NK cell-mediated cytotoxicity in patients with various types of epithelial tumors including lung, breast, and colorectal cancer (Wolf *et al.*, 2003). Upon identification of FOXP3 as a more reliable marker for Tregs and potentially as a surrogate measure for their suppressive function, an increasing number of subsequent studies included FOXP3 in their staining panels such as the pivotal work carried out by Tyler J. Curiel and colleagues on ovarian cancer patients (Curiel *et al.*, 2004). In this comprehensive study it was convincingly demonstrated that $CD4^+CD25^+FOXP3^+$ Tregs were present in PB, malignant ascites, tumoral tissue, and draining LNs. Interestingly, Treg levels in tumor-draining LNs were lower as compared to control LNs and tonsils and decreased with increasing disease stage. One of the proposed mechanisms underlying this phenomenon

was the presence of the chemokine CCL22. Secreted by ovarian cancer cells and tumor-associated macrophages (TAMs), a concentration gradient of CCL22, which binds to CCR4 expressed on Tregs, is generated and thereby mediates migration of Tregs away from the draining LNs toward the CCL22-rich tumor microenvironment. It is worth mentioning that physiologically CCL22 facilitates the encounter between DCs and activated antigen-specific T cells suggesting that tumors elegantly capture this process in order to efficiently suppress activated effector cells (Tang and Cyster, 1999). Similar findings regarding Treg trafficking and redistribution have been largely made in various types of malignancies (Gobert *et al.*, 2009; Haas *et al.*, 2008; Olkhanud *et al.*, 2009; Qin *et al.*, 2009; Shevach, 2004), pointing toward the need for examining the distribution of Tregs in multiple tissue compartments since quantification of Tregs in PB alone may not accurately portray Treg frequency or trafficking.

Analysis of subset frequency for effector cells such as NK and T cells together with Tregs revealed that a shift of the Treg/effector T cell ratio was often linked to the tumor burden and disease course (Gao *et al.*, 2007; Leffers *et al.*, 2009; Sato *et al.*, 2005). The global interest in Tregs resulted in several analogous studies on Treg (-subsets) in different types of malignancies including melanoma (Viguier *et al.*, 2004), hepato-cellular carcinoma (HCC) (Kobayashi *et al.*, 2007; Ormandy *et al.*, 2005), Ewing sarcoma (Brinkrolf *et al.*, 2009), head-and-neck (Schaefer *et al.*, 2005), prostate (Kiniwa *et al.*, 2007; Miller *et al.*, 2006), ovarian (Kryczek *et al.*, 2005; Wolf *et al.*, 2005), breast (Leong *et al.*, 2006), colorectal (Chaput *et al.*, 2009; Ling *et al.*, 2007), and pancreatic cancer (Liyanage *et al.*, 2002). Despite the fact that the preponderance of results indicated a negative impact of Tregs in carcinogenesis and disease progression, some findings raised doubts with regard to this "simplification". The presence of Tregs was in fact correlated to positive prognosis in head-and-neck as well as gastric cancer (Badoual *et al.*, 2006; Haas *et al.*, 2009). These *prima facie* contradictory findings gained further credibility from studies in animal models of colorectal and gastric cancer providing further evidence for the plasticity of Tregs and their rather complex role in immunoregulation (Erdman *et al.*, 2003, 2005, 2009; Gounaris *et al.*, 2009). It must be emphasized that these anecdotal exceptions do not negate the perception that Tregs hamper "immune surveillance" but rather they present a more holistic view of their functional repertoire. Tregs are *per se* associated with immunosuppression and anti-inflammatory activity. Consequently, by counteracting inflammatory processes Tregs may mediate an anticarcinogenic effect given that inflammation-initiated carcinogenesis and tumor progression is a well-established model (Colotta *et al.*, 2009; Marshall *et al.*, 2004). Under certain proinflammatory conditions characterized by elevated levels of IL-6, IL-1β, IL-23, and lactic acid, Tregs can convert from anti- to proinflammatory,

IL-17$^+$ cells. Thus, Treg populations with contradictory functions can coexist at elevated levels in tumor tissue. One speculation is that functionally reversed Tregs may contribute at an early stage to the escalation of cancer-associated inflammation and subsequently during the course of disease inhibitory Tregs suppress tumor-specific responses as implied by most studies.

B. Regulatory T Cells in Hematologic Malignancies

Various studies on the role of Tregs in hematologic diseases have been reported providing a more complex mosaic of diverse observations. In Hodgkin's lymphoma (HL), the draining LNs, rich in infiltrating B and T cells as well as macrophages, showed the presence of Tregs, which suppressed T cells via CTLA-4 and IL-10, thus contributing to an ineffective clearance of Hodgkin's disease-associated Sternberg Reed cells (Marshall *et al.*, 2004). Results from studies on immune effector cells indicated that a more immunoreactive environment is associated with a worse outcome in HL. In accordance, the presence of FOXP3$^+$ Tregs cells appeared to have a positive impact on event-free and disease-free survival in HL, especially when noted together with low infiltration of cytotoxic TIA-1$^+$CD8$^+$ T cells (Alvaro *et al.*, 2005). In chronic lymphocytic leukemia (CLL), increased levels of circulating CD4$^+$CD25^{high} Tregs have been observed and mediate T cell suppression through CTLA-4 (Beyer *et al.*, 2005; Motta *et al.*, 2005). Interestingly, CLL, a chronic B cell-derived leukemia, is associated with hypoglobulinemia that has been found to inversely correlate with the Treg frequency. This observation indicates a direct suppressive effect of Tregs on Ig production; an observation that has been further bolstered by basic studies on the suppressive effects of Tregs on B cells (Lim *et al.*, 2005). In addition, patients with CLL treated with the nucleoside analogue Fludarabine showed a selective reduction of Tregs (Beyer *et al.*, 2005). In B cell-derived non-Hodgkin lymphomas (B-NHLs) as well as acute myeloid leukemia (AML), Tregs were also overrepresented (Wang *et al.*, 2005b; Yang *et al.*, 2006a,b). In AML, the proportion of apoptotic (7-AAD$^+$) and proliferating (Ki67$^+$) cells among Tregs was higher in patients as compared to healthy controls. It was later demonstrated in independent studies that Tregs can have a rapid turnover rate and may be generated from rapidly dividing, highly differentiated memory CD4$^+$ T cells. They are also relatively susceptible to apoptotic stimuli partly due to critically short telomeres and reduced telomerase activity (Vukmanovic-Stejic *et al.*, 2006). The cumulative evidence indicates that accumulation of Tregs associated with malignancies may result from the proliferation of a preexisting pool, rather than blockade in senescence. Myelodysplastic syndrome (MDS) is often regarded as the antecedent condition for AML. Parallel to AML, MDS

patients exhibit increased Treg frequencies and a skewed $CD8^+$ T cell/Treg ratio toward Tregs. Furthermore, high-risk subgroups of MDS and disease progression to more aggressive MDS subtypes were accompanied by an increase of Treg levels, suggesting a direct role of Tregs in progression and malignant transformation (Hamdi *et al.*, 2009; Kordasti *et al.*, 2007). Some hematologic malignancies display quantitative and functional deficits of the Treg compartment, for example, cutaneous T cell lymphoma (Tiemessen *et al.*, 2006) and multiple myeloma (Prabhala *et al.*, 2006). There is an ongoing discussion how the inflammatory component of the disease, manifested for example by high levels of IL-6 in multiple myeloma, may impact the Treg compartment and whether functional Tregs may have a direct suppressive effect on malignant clones.

C. Regulatory T Cells as Biomarkers

As it became increasingly evident that levels of Tregs often correlate with tumor burden and disease progression, their role as predictors of disease prognosis was explored. In gastric cancer, patients with higher frequencies of circulating Tregs had a worse survival (Kono *et al.*, 2006; Sasada *et al.*, 2003). Interestingly, an evaluation of primary gastric cancer material revealed that merely increased presence of Tregs did not strongly correlate with prognosis but in fact the pattern of localization predicted the outcome. In particular, a diffuse intratumoral distribution predicted a shortened survival as compared to a peritumoral pattern (Mizukami *et al.*, 2008b). A persistent Treg infiltration in tumors that were radically resected was also associated with a worse prognosis (Perrone *et al.*, 2008). The significance of the topological distribution of Tregs at the tumor site was also observed by our group in patients with uveal melanoma, where only intratumoral localization of Tregs was an independent negative prognostic factor in contrast to peritumoral formation (Mougiakakos *et al.*, in press). An increased number of circulating Tregs is associated with high mortality and reduced survival in patients with HCC (Fu *et al.*, 2007). However, only Tregs in the center of advanced HCC and not at the noncancerous margins were of negative impact (Gao *et al.*, 2007; Kobayashi *et al.*, 2007). Obviously, the evidence is far from conclusive since Treg localization has been assessed in only a minority of reported studies. In patients with ovarian cancer, reduced survival correlated with increasing Treg numbers (Curiel *et al.*, 2004). Immunohistochemical (IHC) analysis of tumor specimens from 117 patients with epithelial ovarian cancer demonstrated that a skewing of the $CD8^+$ T cell/Treg ratio toward Tregs correlated with a poor prognosis (Sato *et al.*, 2005). A similar study in cervical cancer evaluated the $CD8^+$ T cell/Treg ratio as well as the MHC class I expression (Jordanova *et al.*, 2008). Other studies in NSCLC examined the $CD3^+$ T cell/Treg ratio (Petersen *et al.*, 2006) while in

HCC ratio of activated Granzyme B^+ $CD8^+$ T cell/Treg was measured (Gao *et al.*, 2007). Thus, the relative proportion of negative regulators like Tregs to effector T cells in the tumor infiltrate may be of greater significance for prognosis than absolute numbers of Tregs in itself. Consistent with these findings, results from breast cancer patients suggest that Tregs negatively affect overall and relapse-free survival (Bates *et al.*, 2006; Gobert *et al.*, 2009). Increased levels of tumor infiltrating Tregs define a new high-risk subgroup within the cohort of breast cancer patients positive for estrogen receptors, serving as a predictive marker for late relapse (Bates *et al.*, 2006). In order to better understand and define the role of infiltrating Tregs in breast cancer, Tregs were assessed in two different locations: within the tumor tissue and the surrounding lymphoid aggregates. The Tregs within the lymphoid infiltrates were identified as the ones with the leading negative impact on disease course and outcome, suggesting that at this site they counteract the recruited effector lymphocytes by abrogating their reactivation (Gobert *et al.*, 2009). In ovarian cancer, a prominent colocalization of Tregs and $CD8^+$ T cells within the tumor tissue has also been observed (Curiel *et al.*, 2004). Patients with breast cancer who show complete responses to chemotherapy have a persistence of $CD8^+$ TILs and a total disappearance of Tregs, indicating that immune responses released from negative regulation may cofacilitate chemotherapy-mediated complete regression of tumor cells (Ladoire *et al.*, 2008). Studies linking the presence of Tregs to a worse outcome have been performed in various other malignancies as well including colorectal, pancreatic, and renal cancer (Griffiths *et al.*, 2007; Hiraoka *et al.*, 2006; Ling *et al.*, 2007).

Although most studies link Tregs to a poor disease course and outcome, data from other investigations show the opposite. In patients with follicular, Hodgkin's, and cutaneous T cell lymphoma, head-and-neck as well as colorectal cancer high numbers of intratumoral Tregs are associated with longer disease-free and event-free survival (Alvaro *et al.*, 2005; Badoual *et al.*, 2006; Carreras *et al.*, 2006; Gjerdrum *et al.*, 2007; Klemke *et al.*, 2006; Lee *et al.*, 2008; Salama *et al.*, 2009; Tiemessen *et al.*, 2006; Tzankov *et al.*, 2008). The role of Tregs in cancer is complex as it is not identical for all types of cancers and even differs at distinct phases of disease course for the same type of malignancy. This can clearly be exemplified by observations in ovarian cancer, where presence of Tregs is an unfavorable predictor for an unselected group of patients (Curiel *et al.*, 2004) but a positive factor for overall survival in a subgroup of patients with advanced disease (Leffers *et al.*, 2009). It has been shown in murine tumor models that elimination of Tregs before tumor establishment was beneficial for the survival in contrast to established tumors as Tregs dominated multiple immune evasion mechanisms early on but not during late phases of tumor development (Elpek *et al.*, 2007). Compelling studies showing that Tregs can have anticancerous effects through their anti-inflammatory role have also been described (Erdman *et al.*, 2003, 2005,

2009; Gounaris *et al.*, 2009). Malignancies characterized by massive infiltration of proinflammatory cells that drive the neoplastic process, may actually benefit from Treg infiltration. It has been demonstrated that Tregs can exert an anti-inflammatory effect not only on cells of the adaptive immunity but also on the innate immunity, which is strongly involved in the inflammatory responses (Tiemessen *et al.*, 2007; Venet *et al.*, 2006). A possible scenario in hematological malignancies may be that Tregs directly suppress the malignant clone and may thereby have antineoplastic effects. For instance, it has been shown that Tregs can kill B cells and potentially malignant B cell clones too may be targeted (Lim *et al.*, 2005; Zhao *et al.*, 2006). The same applies to T cell and myeloid-derived malignancies, where nonmalignant counterparts are known to be under the control of Tregs.

V. ACCUMULATION OF REGULATORY T CELLS

A. Compartmental Redistribution

Increasing evidence confirms the hypothesis that Tregs selectively migrate to the site where regulation is required (Fig. 2A). This system, relying on interactions between chemokines/chemokine-receptors and integrins/integrin-receptors (Wei *et al.*, 2006), is often usurped by tumors. Curiel and colleagues were the first to show in ovarian cancer a CCL22-orchestrated migration of CCR4-expressing Tregs toward tumor tissue and malignant ascites (Curiel *et al.*, 2004). In addition to tumor cells, bystander cells especially of myeloid origin including TAMs are sources of CCL22. Expression of CCL22 can be upregulated in myeloid cells *in vitro* upon addition of tumor cells and/or tumor supernatant. To date, a CCL22-mediated Treg attraction has been observed in several types of neoplastic diseases including breast, prostate cancer, and B-NHLs (Gobert *et al.*, 2009; Miller *et al.*, 2006; Qin *et al.*, 2009; Yang *et al.*, 2006a). Decreased expression of CD62L (L-selectin) and CCR7 on infiltrating Tregs as compared to circulating counterparts substantiates active recruitment of these cells to the site of action. In regional LNs, the majority of the Tregs express CD62L (80%) and CCR7 (50%) (Huehn and Hamann, 2005). Tregs internalize CCR4 upon binding of CCL22 which accounts for the varying levels of CCR4 on Tregs found in the circulation, draining LNs, and tumor microenvironment (Gobert *et al.*, 2009). CCL17 is another ligand for CCR4 and has been shown to be involved in Treg trafficking in gastric cancer and HL (Ishida *et al.*, 2006; Mizukami *et al.*, 2008a). Supporting these observations, major CCL17 and CCL22 sources like tolerogenic DCs, immature myeloid cells, and TAMs can be found in different tumor microenvironments (Penna *et al.*, 2002). In pancreatic

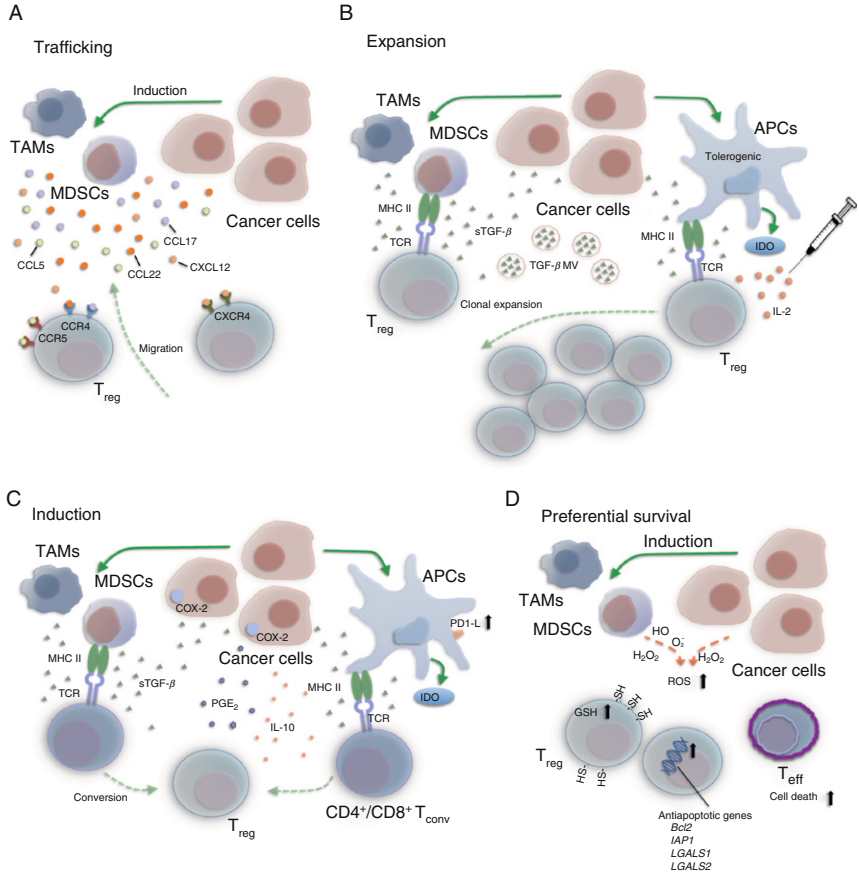

Fig. 2 Accumulation of regulatory T cells in cancer. (A) Tregs may be attracted by various chemokines (CCL5, CCL17, CCL22, CXCL12) to the tumor site. Cancerous cells and/or bystanding tumor-associated macrophages (TAMs) and myeloid-derived suppressor cells (MDSCs) secrete these chemokines of which Tregs possess the corresponding receptors (CCR4, CCR5, CXCR4). (B) Preexisting Tregs expand upon (suboptimal) antigen stimulation provided by APCs, TAMs, and MDSCs within an overall tolerizing environment. TGF-β directly secreted or carried in microvesicles (MV) as well as IDO play a central role in this process. Administration of IL-2 as a component of therapeutic schemes in malignancies may drive such a Treg expansion. (C) Tregs can be generated *de novo* from conventional $CD4^+$ and $CD8^+$ T cells (T_{conv}). Several factors, among others TGF-β, Prostaglandin E_2 (PGE_2), IL-10, and IDO in conjunction with (suboptimal) T cell activation have been identified to favor this induction of Tregs. (D) In the tumor microenvironment, reactive oxygen species (ROS) produced mainly by cancer and myeloid cells (e.g., TAMs, MDSCs) are responsible for high levels of oxidative stress, which is harmful for immune cells. Tregs depict a better protection against oxidative stress as compared to conventional effector T cells (T_{eff}), as they possess higher amounts of intracellular glutathione (GSH) and surface thiols (–SH). Furthermore, Tregs in cancer patients show a higher expression of antiapoptotic genes (*Bcl2*, *IAP1*, *LGALS1*, *LGALS2*) as compared to their counterparts from healthy donors indicating an increased resistance toward apoptotic stimuli.

adenocarcinoma, the migration of Tregs is partly driven by CCR5 chemotaxis. Tregs from these patients express CCR5 and tumors secrete the cognate ligand CCL5. In a murine tumor model of pancreatic cancer, tumor growth was significantly inhibited by reducing CCL5 production by tumor cells or by systemic administration of a CCR5 antagonist (Tan *et al.*, 2009). Interestingly IL-2, which is utilized as an immunologic adjuvant in cancer therapies, modifies Treg trafficking. IL-2 can lead to an upregulation of CCR4 expression on Tregs and thereby potentially drive migration toward the tumor site. In addition, Tregs have been observed to exhibit increased CXCR4 levels upon IL-2 treatment in patients with ovarian carcinoma (Wei *et al.*, 2007). CXCR4 is the receptor for CXCL12, also known as stromal-derived-factor (SDF-1), which is strongly associated with the regulation of organ-specific metastases in various cancers (Kryczek *et al.*, 2005). A recent report on cervical cancer showed that expression of CXCL12 in the tumor tissue positively correlates with the tumor infiltration of $FOXP3^+$ Tregs and cancer progression (Jaafar *et al.*, 2009). Dependent on activation status and tissue localization, Tregs can express a plethora of chemokine receptors including CCR2, CCR4, CCR5, CCR7, CCR8, CXCR4, and CXCR5 and thus are responsive to a variety of ligands. An interesting aspect is the role of the cancer-related inflammatory component for Treg recruitment. Indeed, it has been shown that Tregs migrate toward sites of inflammation. This process is mediated partly by the integrin CD103 ($\alpha_E\beta_7$), which interacts with E-cadherin, and CCR2 (Wei *et al.*, 2006), though it remains to be elucidated whether it contributes to Treg migration in cancer patients. A comprehensive analysis of the cytokine pattern in tumors combined with a characterization of chemokine receptors expressed on tumor infiltrating Tregs may help to address some of the unanswered questions.

B. Expansion

Much evidence directly or indirectly suggest that cancers not only attract but also facilitate proliferation of different Treg subsets as they appear to be highly activated and underwent proliferation when investigated in tumor patients (Fig. 2B). Physiologically, Tregs have been observed to exhibit high turnover rates (Vukmanovic-Stejic *et al.*, 2006). Tregs isolated from cancer patients depict a decreased content of TCR excision circles (TRECs) as compared to Tregs from healthy donors, which points toward proliferation rather than a mere redistribution (Wolf *et al.*, 2006). An increased proportion of proliferating $Ki67^+$ Tregs has also been shown in various types of cancers including breast cancer and AML (Gobert *et al.*, 2009; Wang *et al.*, 2005b). TGF-β, an autonomous regulator of tumor initiation, progression, immune escape, and metastasis in epithelial cells has been observed to play a central role for

peripheral expansion of Tregs (Huber *et al.*, 2004; Yamagiwa *et al.*, 2001). Tumor cells are capable of producing TGF-β, and in addition can modulate myeloid-derived suppressor cells (MDSCs) (Filipazzi *et al.*, 2007), and immature DCs (Ghiringhelli *et al.*, 2005b) to become major sources of TGF-β. Several studies have also shown that Tregs, especially the Th3 Treg subtype, produce TGF-β in its membrane-bound or secreted form, which besides mediating suppression may also act as an autocrine pathway of stimulating self-expansion (Nakamura *et al.*, 2004). Both MDSCs and immature DCs express MHC II and costimulatory molecules at low levels, which may be sufficient to elicit Treg but not effector T cell responses since weak or diminished TCR signaling (e.g., by rapamycin) can favor Treg expansion (Battaglia *et al.*, 2006). Self- and non-self antigens can drive Treg activation and proliferation manifested by a skewed TCR repertoire and further implicated by the importance of APC presence at the site of inflammation and/or cancer in such a process (Belkaid and Oldenhove, 2008; Kumar, 2004). Several studies in mouse models have provided evidence to support these observations (Walker, 2004). Mature APCs are now also being implicated in the expansion of Tregs, in contrast to earlier thought that only immature or aberrant APCs promote Treg expansion (Lundqvist *et al.*, 2005). IDO is a key immunomodulatory enzyme found in the tumor tissue or in APCs of the draining LNs and is linked to tumor-associated immunosuppression and tumor-induced tolerance (Munn and Mellor, 2007). It was recently shown that IDO expressed by APCs could directly activate Tregs and promote their proliferation (Baban *et al.*, 2009; Chung *et al.*, 2009). Ligation of CD80 and CD86 by CTLA-4, constitutively expressed on Tregs increases the functional activity of IDO forming a positive feedback loop (Fallarino *et al.*, 2003). TLRs have been increasingly demonstrated to have roles beyond mere antimicrobial surveillance to multiple physiologic functions as they are also regulated by several intrinsic ligands. TLRs can be found in Tregs and are of significance to their function (van Maren *et al.*, 2008). Activation of TLR2, in particular by heat shock protein 60 (Hsp60), leads to proliferation of Tregs and an increased production of IL-10 and TGF-β (Caramalho *et al.*, 2003; Liu *et al.*, 2006a). Members of the Hsp-family released by (dying) tumor cells within the tumor microenvironment can serve either as immunostimulatory signals or be immunosuppressive as in the case of Hsp60. TLR4 and TLR5 stimulation by lipopolysaccharide and flagellin, respectively, can lead to Treg activation and proliferation, although their exact role warrants further investigation (Caramalho *et al.*, 2003). Cumulatively, these findings suggest that activation of certain TLRs by proinflammatory bacterial by-products can promote Treg proliferation in the absence of APCs. Tregs are found to express higher levels of TLRs as compared to conventional T cells which is suggesting a greater degree of environmental control. Tumor-derived microvesicles (MVs) constitute a potent mechanism by which malignancies transform the host microenvironment. Tumor cells

actively release these endosome-derived 50–100 nm organelles (exosomes) that systemically exert protumorigenic effects as they can be found in virtually all body fluids. MVs carrying membrane-bound TGF-β, which skews $CD4^+$ T cell responses in favor of Tregs and deter cytotoxic cells have been identified in tumor patients (Clayton *et al.*, 2007). Recently, MVs isolated directly from patient's sera were shown to induce Treg proliferation (Wieckowski *et al.*, 2009). As described previously, IL-2 plays a major role *in vivo* for Treg maintenance and expansion via STAT-dependent mechanisms (Zorn *et al.*, 2006). STAT3 and STAT5 bind to a highly conserved binding site located in the first intron of the *FOXP3* gene. Consequently, patients with a STAT5b deficiency have been observed to have decreased numbers of $CD4^+CD25^{high}$ T cells, which display low FOXP3 levels and diminished suppressive function (Cohen *et al.*, 2006). Treatment with IL-2 commonly used for patients with renal cancer and melanoma may result in an increase of Treg frequency and suppressive activity in patients; IL-2-based therapy of cancer thus requires a more judicious appraisal, an outlook supported by recent reports on melanoma and renal cell carcinoma patients treated with IL-2. Discussions about the substitution of IL-2 with other immunostimulatory cytokines sharing the γc receptor such as IL-7, IL-15, and IL-21 are currently ongoing (Ahmadzadeh and Rosenberg, 2006; Jensen *et al.*, 2009; van der Vliet *et al.*, 2007).

C. D*e* N*ovo* Generation

Tregs can amass at tumor sites by *de novo* generation from naïve and memory $CD4^+$ and $CD8^+$ T cells as recently shown in B-NHL (Ai *et al.*, 2009) (Fig. 2C). Intensive efforts have been undertaken to determine exactly the tumor-derived factors promoting such a Treg *de novo* generation in order to explore avenues of potential intervention. It is apparent that malignant cells as well as other cells of the tumor microenvironment are involved in this process utilizing various mechanisms. In contrast to the intrathymic Treg generation, TGF-β holds a crucial role in peripheral development of induced Tregs. Antigen-mediated stimulation of the TCR in the presence of TGF-β induces Tregs; a mechanism that has been explored in multiple models (Chen *et al.*, 2003; Liu *et al.*, 2007; Yamagiwa *et al.*, 2001). Is should be pointed out that the promoter region of the *FOXP3* gene in these iTregs depicts more methylated nucleotides as compared to nTregs, indicating a less stable suppressive phenotype (Zhou *et al.*, 2009). Activin A, a member of the TGF-β family induced by inflammatory signals, was recently found to promote peripheral Treg conversion, suggesting a redundancy within the members of the TGF-β family (Huber *et al.*, 2009). The fact that TGF-β is associated with diverse cancer types emphasizes the significance of this pathway. Tumor cells not only produce and secrete significant amounts of TGF-β, but also modulate cells of the tumor

microenvironment, especially APCs, turning them into additional sources of soluble or even membrane-bound TGF-β (Filipazzi *et al.*, 2007; Ghiringhelli *et al.*, 2005b). Interestingly, TGF-β fuels an autoreactive loop by upregulating FOXP3, which downregulates SMAD7 and thereby leads to an increased TGF-β expression (Fantini *et al.*, 2004). Akin to TGF-β, IL-10 is the second most prominent cytokine involved in Treg induction. IL-10 is also associated with various types of cancers. Early on during tumor growth, antigenic stimulation of conventional T cells in the presence of IL-10 led to the generation of Tr1 cells in a B16 melanoma model (Seo *et al.*, 2001). Hemeoxygenase (HO)-1, inducible by inflammation and oxidative stress, may be involved in this process as it maintains DCs in an immature stage and promotes IL-10 production (Chauveau *et al.*, 2005). APCs are the interface of innate and adaptive immunity orchestrating numerous immunological responses. The net direction of adaptive immunity toward anergy or reactivity strongly depends on APCs; their developmental stage, activation, and costimulatory potential. Malignancies regularly suppress APC differentiation in the tumor microenvironment and thereby potentially drive Treg conversion. Minute antigen presentation in combination with weak costimulation, also termed subimmunogenic conditions, can convert conventional T cells to Tregs even in the absence of TGF-β (Kretschmer *et al.*, 2005). Observations from single injection of immature DCs pulsed with influenza matrix peptide and keyhole limpet hemocyanin in two healthy individuals provides evidence for this pathway of Treg induction (Dhodapkar *et al.*, 2001). Tregs that arose in this manner were capable of responding subsequently to optimal antigen presentation and expanding without losing their suppressive functions. This observation partly explains how functionally mature DCs that typically stimulate effector T cells can facilitate the expansion of available Tregs (Banerjee *et al.*, 2006; Lundqvist *et al.*, 2005). MDSCs are a new emerging population of suppressive cells that have yet not been thoroughly characterized. MDSCs are increased in cancer patients and potentially can induce Tregs. Hoechst and colleagues demonstrated that MDSCs from patients with HCC, characterized as $CD14^+HLA\text{-}DR^-$ cells, induced two suppressive populations including nTreg-like $CD4^+CD25^+FOXP3^+$ and $IL10^+$ Tr1-like cells (Hoechst *et al.*, 2008). In ovarian cancer patients pDCs can directly induce $CD8^+IL\text{-}10^+$ Tregs (Wei *et al.*, 2005). Subsequent investigations revealed that IDO is essential for this pDC-mediated Treg induction (Chen *et al.*, 2008), and appears to be strongly involved in cancer-related Treg conversion (Baban *et al.*, 2009; Liu *et al.*, 2007; Munn *et al.*, 2004). In melanoma patients, increased levels of H-Ferritin have been associated to increased levels of $CD4^+CD25^+$ iTregs. This Tr1 induction was mediated by a modulation of DCs by means of increased expression of CD86 and programmed death 1 ligand (PD1-L) (Gray *et al.*, 2003). Upregulation of CD86 and PD1-L have also been observed upon combined administration of vaccines and TLR3 agonists leading to

attenuated $CD8^+$ T cell responses (Pulko *et al.*, 2009). Coinhibitory signaling by PD1-L is important for TGF-β-mediated Treg induction (Wang *et al.*, 2008) and is significant for the suppression noted in T-lymphoproliferative diseases, promoting Treg induction among other effects (Wilcox *et al.*, 2009). A profound expression of COX-2, mediating the production of PGE_2, can be found in numerous inflammatory and malignant processes. PGE_2 can directly induce and expand Tr1 as shown in glioma, head-and-neck and lung cancer (Akasaki *et al.*, 2004; Bergmann *et al.*, 2007; Sharma *et al.*, 2005). Additionally, PGE_2 can indirectly increase immunosuppression by facilitating the generation of aberrant or immature myeloid cells. Like TGF-β, a positive feedback loop seems to be present as COX-2 utilized by iTregs for suppressive activity may concurrently drive their own generation (Mahic *et al.*, 2006). Recent clinical studies on HCC (Gao *et al.*, 2009), uveal melanoma (Mougiakakos *et al.*, in press), and renal cancer (Li *et al.*, 2009) have linked COX-2 expression to Treg infiltration and clinical prognosis. Additional cross-talk between cancer-related APCs and Tregs involving the inhibitory molecules B7.H3 and B7.H4 is under current investigation (Kryczek *et al.*, 2007; Mahnke *et al.*, 2007a). ICOS is an activation marker, which binds to the stimulatory molecule B7.H2 on APCs, and is expressed on Tregs in breast cancer and melanoma patients. The subset of $ICOS^{high}$ Tregs represents a hyperactivated population with increased suppressive properties and the ability to induce surrounding clusters of Tr1 cells (Gobert *et al.*, 2009; Strauss *et al.*, 2008). Of course several counteracting mechanisms do exist, explaining how there can even exist a paucity of Treg conversion in inflammatory milieus as exemplified by IL-6 possessing a prominent role by abolishing Treg induction and generating Th17 effector cells instead (Korn *et al.*, 2008). The balance of these factors consequently determines the extent of Treg induction and expansion.

D. Preferential Survival

In addition to redistribution, expansion, and conversion, a fourth mechanism may contribute to the accumulation of Tregs in cancer patients (Fig. 2D). We have demonstrated that nTregs are more resistant toward oxidative stress-mediated cell death compared to conventional $CD4^+$ T cells from healthy individuals (Mougiakakos *et al.*, 2009) as well as advanced melanoma patients (unpublished data). Moreover, nTregs maintained their suppressive properties at hydrogen peroxide levels that were lethal for 50% (LD_{50}) of conventional $CD4^+$ T cells. Increase in cell surface thiol groups (–SH) and intracellular glutathione content (the main thiol-containing redox buffer) appears to be the major mediators of these protective effects of nTregs (unpublished data). Oxidative stress is known to be

increased in several tumor types and can negatively affect cellular immunity (Mehrotra *et al.*, 2009). Both tumor cells and bystander myeloid cells contribute to increased oxidative stress. The expression of the enzyme HO-1, which has anti-inflammatory and antioxidative function adds to the suppressive function of nTregs and may partly contribute to the observed resistance toward oxidative stress (Brusko *et al.*, 2005). Furthermore, a recent study suggests that nTregs themselves contribute to a pro-oxidative local milieu, potentially by consumption of free thiols as a part of their suppressive repertoire (Yan *et al.*, 2009). Cumulatively, these results sustain recent observations that in comparison to healthy individuals, Tregs from patients with several types of epithelial cancers were less affected by apoptosis-inducing stimuli than other lymphocyte subsets examined (Stanzer *et al.*, 2008). In CLL, it has been shown that nTregs express higher levels of the antiapoptotic *Bcl2* and *IAP1* genes as compared to Tregs from healthy donors, indicating a switch toward increased survival in tumor-associated Tregs, reflected by *in vitro* assays depicting a reduced sensitivity toward CD95 ligation and p53-dependent (Fludarabine) and -independent (Roscovitine) apoptosis (Jak *et al.*, 2009). Similarly, gene expression analysis on Tregs from renal cancer patients revealed 49 genes to be differentially expressed. The most prominent genes observed to be overexpressed were *LGALS1* and *LGALS3*; both are galectin genes involved in control of apoptosis as well as implicated in the downregulation of the proapoptotic genes *BAX* and *TNFRSF25* leading to a shifted balance toward survival and fitness of Tregs (Jeron *et al.*, 2009).

VI. ANTIGEN SPECIFICITY OF TREGS IN CANCER

As nTregs, like conventional T cells are educated in the thymus, possess somatically rearranged TCRs and recognize self-Ags they should in theory be able to recognize tumor-associated antigens (TAAs). Mouse studies show that antigen-specific Tregs may be more suppressive compared to nonspecific Tregs. Wang and colleagues were the first ones to generate Treg clones specific for the LAGE1 cancer testis antigen from TILs of melanoma patients. These Treg clones required antigen-specific activation for an efficient suppressive activity (Wang *et al.*, 2004). The same group identified Tregs in melanoma patients specific for the tumor-specific ARTC-1 (Antigen Recognized by T Cells 1) antigen (Wang *et al.*, 2005a). The Tregs specific for LAGE1 and ARTC-1 were similar to thymic-derived nTregs in terms of FOXP3, GITR, CTLA-4, and CD25 expression as well as cytokine production. Circulating IL-10^{+} Tregs, reactive against gp100, TRP1 (melanoma tissue differentiation antigens), NY-ESO-1 (cancer/testis antigen), and

survivin (member of the inhibitor of apoptosis protein family) have subsequently been identified in metastatic melanoma patients (Vence *et al.*, 2007). A common feature of suppression exerted by these cells detected in melanoma patients was the need for cell-to-cell contact. In addition to the findings in melanoma, Tregs specific for WT1, an antigen overexpressed by several human leukemias were identified in AML patients. These cells displayed an nTreg-like phenotype and suppressed T cell responses *in vitro* independent of cell-to-cell contact (Lehe *et al.*, 2008). Tregs specific for telomerase, CEA, EGFR, Mucin-1, and HER2/neu have been detected in colorectal cancer patients, suggesting that these Tregs control TAA-specific effector cell responses in an antigen-selective manner (Bonertz *et al.*, 2009). Human papilloma virus (HPV) is the major risk factor for cervical cancers as it is directly involved in the process of carcinogenesis. High Treg frequency in the PB correlates with persistence of premalignant lesions caused by HPV infection (Molling *et al.*, 2007). Tumor cells express the HPV-encoded oncoproteins E6 and E7. In malignant tissue as well as draining LNs, E6- and E7-specific Tregs can be detected, which links viral antigen-specific Tregs to local immunosuppression in patients without a generalized immunodeficiency (van der Burg *et al.*, 2007). Theoretically, a vaccination against HPV may lead to an induction, activation, or expansion of such preexisting viral antigen-specific Tregs. In two out of six patients who received vaccination with E6/E7 long peptides, an expansion of suppressive antigen-specific Tregs that reached levels as high as those observed for effector $CD4^+$ T cells was reported (Welters *et al.*, 2008). Taken together, these results convincingly demonstrate that Tregs specific for self as well as foreign peptides expressed by tumor cells do exist. Furthermore, antigen-specific Tregs are not restricted to the $CD4^+$ T cell compartment as patients with melanoma, renal and breast cancer show significantly increased frequencies of circulating suppressive $CD8^+$ Tregs specific for HO-1 (Andersen *et al.*, 2009). These HO-1-specific $CD8^+$ Tregs exhibited a stronger suppressive function than $CD4^+CD25^+$ nTregs and not only hampered effector T cells directly but also protected directly the tumor target cells from an efficient CTL recognition by yet unidentified mechanisms. HO-1 is a late phase anti-inflammatory enzyme that promotes tolerogenic DCs producing IL-10. Cancer-mediated inflammation can theoretically lead to an increased HO-1 production in the tumor microenvironment, and thereby result in activation and expansion of HO-1-specific Tregs. These findings together with results from mouse models (Zhou *et al.*, 2006) raise concerns regarding the potential adverse effects of vaccination strategies that utilize self or foreign proteins expressed by tumors in order to elicit efficient $CD4^+$ and $CD8^+$ T cell responses. It seems possible or even likely that immunization with certain antigens may expand suppressive Tregs antagonizing the positive effects on effector T cells.

VII. CANCER VACCINES AND REGULATORY T CELLS

Abundant evidence exists that clinical responses to cancer vaccines are influenced by the disease stage at the time of vaccination. Tumor burden and Treg levels typically tend to go hand-in-hand. For example, patients with advanced melanoma have significantly higher circulating Tregs than those with minimal residual disease (Nicholaou *et al.*, 2009). Tregs may be induced or expanded by cancer vaccines as illustrated in studies with melanoma patients, where immunological and clinical responses pre- and/or postvaccination with either NY-ESO-1 protein or DCs pulsed with allogeneic cell lysate (TRIMEL) were reported to be associated with the presence of $CD4^+CD25^+FOXP3^+CD127^-$ and TGF-β-producing Th3 cells (Lopez *et al.*, 2009; Nicholaou *et al.*, 2009). Patients vaccinated with a NY-ESO-1 DNA vaccine had measurable T cell responses, which were clearly suppressed by $CD4^+CD25^+FOXP3^+$ Tregs (Gnjatic *et al.*, 2009). B-CLL patients who received autologous DCs loaded with tumor lysates had specific $CD8^+$ T cell responses against the TAAs RHAMM or fibromodulin, which correlated positively with levels of IL-12 in serum and inversely with $CD4^+CD25^+FOXP3^+$ Treg frequency (Hus *et al.*, 2008). As Tregs obviously represent a major obstacle for efficient cancer immunotherapies, Treg depletion has emerged as an adjuvant therapy that effectively synergizes with different cancer vaccine approaches in animal models. However, the effect of Treg depletion in these models was greatest when done immediately before or after tumor inoculation (Knutson, 2006; Onizuka, 1999; Sutmuller, 2001). Depletion of Tregs after the establishment of tumors often fails to significantly improve the therapeutic outcome (Elpek, 2007) implying a tumor stage-dependent impact of Tregs in cancer control. Several ongoing clinical studies in cancer are aimed at exploring Treg depletion in combination with different immunotherapeutic approaches. In renal cancer patients, elimination of Tregs using an immunotoxin, followed by vaccination with tumor RNA-transfected DCs significantly improved the induction of tumor-specific T cell responses (Dannull *et al.*, 2005). Similar results could be obtained with DC or peptide-based vaccination strategies combined with Treg depletion in colorectal and breast cancer as well as melanoma patients (Mahnke *et al.*, 2007b; Morse *et al.*, 2008; Rech and Vonderheide, 2009). Data from animal models support the concept that tumor-associated Tregs can be expanded in response to therapeutic vaccination and suppress the concomitantly generated effector T cells (Zhou *et al.*, 2006). In melanoma patients vaccinated either with specific peptides or APCs loaded with tumor lysates a significant increase of IL-10-producing Tr1 cells was noted in PB postvaccination (Chakraborty *et al.*, 2004). Melanoma patients who received MAGE-A3 peptide vaccines had an increased specific $CD4^+$ T cell response as detected by HLA class II tetramers.

After flow cytometric enrichment of MAGE-A3-specific $CD4^+$ T cells a substantial proportion of the subsequently generated clones showed phenotypic and functional characteristics of nTregs and Th3 cells, which exhibited suppressive activity *in vitro* upon peptide stimulation (Francois *et al.*, 2009). Thus therapeutic vaccination in cancer may potentially be a double-edged sword and expansion of Tregs and resultant immune suppression may ensue rather than boosting of effector T cell activity. Consequently, strategies to disarm Tregs should be considered as essential components of immunotherapeutical approaches and are a feature of virtually all prominent vaccine trials in recent times. In this scenario, a combination of vaccines with agents modulating Tregs is one additional option already under clinical evaluation. The microenvironment where T cells encounter the antigen and get primed by the local APCs plays a major role for the balance between tolerogenesis and immunogenesis. Features such as antigen availability (Turner *et al.*, 2009) and a Th1-biasing cytokine milieu (Nishikawa *et al.*, 2005) are considered as critical variables determining the resulting polarization of T cells. Vaccine adjuvants modulating this milieu by, for example, increasing the production of type-1 interferons may be a promising strategy to interfere with the induction of antigen-specific Tregs. The studies discussed in this section raise major concerns regarding the design of cancer vaccines and can explain at least partially the low objective responses observed in many clinical studies. In addition, these observations strongly suggest that monitoring of the Treg compartment is as important as the evaluation of the effector cell arm in patients receiving immunotherapies.

VIII. TARGETING REGULATORY T CELLS IN CANCER THERAPY

Taken together Tregs regardless origin, impede tumor surveillance and appear in many cases to be directly linked to the disease pathogenesis. In studies dating back to the 1980s performed by Robert North and colleagues, Treg depletion was shown to be an elegant approach for increasing immune reactivity against cancer. Especially to date, where various forms of immunotherapies find their way into cancer treatment it appears inevitable to counteract the suppressive effects of Tregs. Nevertheless, the impact of modulating Tregs is not trivial as it may result in unwanted side effects most notably autoimmunological phenomena. Furthermore, targeting of Tregs has to be restricted to malignancies, where Tregs have been shown to be undoubtedly linked to deleterious effects. Different strategies aimed to deplete Tregs or to functionally inactivate Tregs are currently under development or in clinical evaluation (selected studies are summarized in Table 2).

Table 2 Clinical Studies Using Different Strategies to Deplete Tregs in Cancer Patients

Type(s) of malignancy	No.	Depletion regimen	Treatment responses	References
Metastatic melanoma	13	CPM (60 mg/kg/d 2d) + Flu (25 mg/m^2/d 5d) prior ACT	Objective responses in 6 pts, AID in 5 pts	Dudley *et al.* (2002)
Metastatic melanoma	35	CPM (60 mg/kg/d 2d) + Flu (25 mg/m^2/d 5d) prior ACT	Objective responses in 18 pts, AID in 13 pts	Dudley *et al.* (2005)
Metastatic melanoma	93	CPM (60 mg/kg/d 2d) + Flu (25 mg/m^2/d 3d) + TBI (2 Gy or 12 Gy) prior ACT	Objective responses in 50–70% of pts, 4 CRs	Dudley *et al.* (2008)
Various types of metastatic solid tumors	9	Metronomic CPM (50 mg p.o., 2d/1 w)	PD in 3 pts, SD (2–3 months) in 4 pts	Ghiringhelli *et al.* (2007)
Chronic lymphocytic leukemia	73	Fludarabine-containing therapies	Reduced Treg frequency/function	Beyer *et al.* (2005)
Metastatic breast cancer (ongoing study)	3	Daclizumab (1 mg/m^2; single dose) 1 w prior peptide vaccination	Improved responses to vaccination in all pts	Rech and Vonderheide (2009)
Metastatic melanoma and renal carcinoma	13	ONTAK (9 or 18 μg/kg; successive doses)	No objective responses, no Treg depletion	Attia *et al.* (2005)
Metastatic renal cell carcinoma	10	ONTAK (18 μg/kg; single dose) + tumor RNA-transfected DC vaccine	Improved CTL responses, reduced Treg levels	Dannull *et al.* (2005)
Metastatic melanoma	7	ONTAK (5 or 18 μg/kg; successive doses) prior peptide vaccination	Peptide-specific CTLs in 5/6 pts, PD in 5 pts	Mahnke *et al.* (2007b)
Metastatic melanoma, renal cell cancer	15	Ipilumimab (1–9 mg/kg; successive doses)	Objective responses in 8 pts, AID in 5 pts	Maker *et al.* (2005)
B cell non-Hodgkin lymphoma	18	Ipilumimab (1–3 mg/kg; successive doses)	1 CR and 1 PR	Ansell *et al.* (2009)
Metastatic melanoma	14	Ipilumimab (3 mg/kg; successive doses) + peptide vaccination	2 CRs and 1 PR, AID in 6 pts	Phan *et al.* (2003)
Metastatic melanoma, ovarian cancer	20	Ipilumimab (3 mg/kg; successive doses) upon tumor cell vaccination (GVAX)	SD in 8 pts, PR in 4 pts, PD in 8 pts	Hodi *et al.* (2008)

Abbreviations: n, number of patients; CPM, cyclophosphamide; Flu, fludarabine; ACT, adoptive cell transfer; pts, patients; AID, autoimmune disease; TBI, total body irradiation; Gy, gray; PD, progressive disease; SD, stable disease; CTL, cytotoxic T lymphocyte; CR, complete remission; PR, partial remission.

Notes: Daclizumab, humanized anti-CD25 antibody (Zenapax); ONTAK, diphtheria toxin-interleukin-2 fusion protein (Denileukin diftitox); Ipilumimab, human anti-CTLA-4 antibody (MDX-010).

A. Depletion of Regulatory T Cells

The concept of "suppressing the suppressors" goes back to the 1980s beginning with the revolutionary studies by Robert North, who hypothesized that the antitumor effect of cyclophosphamide (CPM) in murine experimental cancer models was due to the depletion of by that time unidentified suppressor T cells (North, 1982). CPM, a DNA alkylating drug, is a standard chemotherapeutic agent utilized in numerous chemotherapy regimens since the 1950s. Observations in patients with autoimmune and malignant diseases treated with CPM revealed that while the drug was immunosuppressive at high dosages, low-dose CPM had an immunostimulatory effect. Studies in mice indicated that the immunostimulatory effects of low-dose CPM were due to selected depletion of Tregs (Ercolini *et al.*, 2005; Lutsiak *et al.*, 2005). Low-dose CPM has been shown to significantly reduce $CD4^+CD25^+$ Tregs but not the total T cell population (Lutsiak *et al.*, 2005). In a Her2/neu transgenic breast cancer mouse model, combination of peptide vaccination with CPM led to a decreased number of circulating Tregs and a parallel boost in tumor-specific, high-avidity $CD8^+$ T cells increasing tumor protection (Ercolini *et al.*, 2005). Potential mechanisms of action include induction of apoptosis, decrease of homeostatic proliferation as well as attenuation of suppressive function (Taieb *et al.*, 2006). Dudley and colleagues have performed clinical trials on patients with therapy-refractory metastatic melanoma by adoptively transferring autologous T cells after preconditioning with the Treg depleting agents CPM and fludarabine. Objective clinical responses were noted in an astonishing 50–70% of the patients (Dudley *et al.*, 2002, 2005, 2008). Low-dose "metronomic" CPM administration in end-stage cancer patients selectively depletes $CD4^+CD25^+FOXP3^+$ Tregs and restores function of T cells and NK cells (Ghiringhelli *et al.*, 2007). However, Treg depletion with low dose of CPM is short-lived, lasting only for 5–6 days. As mentioned previously, fludarabine a cytotoxic purine analog used in hematologic malignancies has been shown to decrease Treg frequencies and abolish their suppressive activity in CLL patients (Beyer *et al.*, 2005).

CD25, the high-affinity IL-2Rα, is constitutively expressed on major subsets of Tregs, especially nTregs. It is also transiently expressed on effector T cells initially during their activation, complicating a CD25-based Treg targeting strategy. Nevertheless, the anti-CD25 monoclonal antibody PC61, originally identified as a monoclonal antibody against the murine IL-2R, has been shown to abrogate suppressive function of $CD4^+CD25^+$ Tregs enhancing tumor rejection in mouse cancer models (Onizuka *et al.*, 1999; Shimizu *et al.*, 1999; Tanaka *et al.*, 2002). Whether PC61 in fact depletes Tregs or rather inactivates Tregs is still unclear (Kohm *et al.*, 2006). Two different antihuman CD25 antibodies, basiliximab (Zenapax) and daclizumab (Simulect) have been

approved for transplantation, autoimmune diseases, and cancer. In metastatic breast cancer patients, treatment with Simulect resulted in a marked depletion of circulating Tregs and peptide vaccination against TAAs generated an effective CTL response (Rech and Vonderheide, 2009). The development of the recombinant IL-2 diphtheria toxin conjugate called denileukin diftitox DAB_{389}IL-2 (ONTAK) was considered a breakthrough for some types of malignancies. It is Food and Drug Administration (FDA) approved for treatment of cutaneous T cell leukemia/lymphoma (Olsen *et al.*, 2001). ONTAK has a short half-life of 60 min and is designed to target cells expressing the high-affinity IL-2R. Upon internalization via endocytosis diphtheria toxin inhibits protein synthesis leading to apoptotic cell death (Figgitt *et al.*, 2000). Based on promising results in initial studies, ONTAK has been used in combination with other therapies in the treatment of diseases like B- and T-NHLs (Dang *et al.*, 2004; Foss *et al.*, 2005; Frankel *et al.*, 2006). There are conflicting reports whether ONTAK depletes Tregs or rather inhibits their function (Attia *et al.*, 2005; Dannull *et al.*, 2005; Vaclavkova *et al.*, 2006). In a study on melanoma patients it was shown *in vitro* and *in vivo* that ONTAK treatment resulted in both decreased numbers and a reduced function of Tregs (Mahnke *et al.*, 2007b). However, several facts need consideration when incorporating ONTAK into therapeutic regimens. In addition to Tregs, ONTAK may also target $CD25^+$ effector T cells. Moreover, Treg homeostasis is very robust and Treg levels recover rapidly following depletion to pretreatment levels or even exceed them. In order to achieve an optimal treatment efficacy, different application schemes and dosage protocols have to be carefully evaluated aiming for an ideal balance between depletion of Tregs and enhancement of effector T cell response.

B. Targeting Function of Regulatory T Cells

Another target molecule on Tregs is CTLA-4, which is involved in mediating suppression as described previously. Like CD25, CTLA-4 can also be expressed on activated $CD4^+$ and $CD8^+$ T cells (Egen *et al.*, 2002). This potential blocking of CTLA-4 function on many levels, including Tregs as well as effector T cells may be responsible for a superior efficacy (Egen *et al.*, 2002). However, it remains still to be elucidated, which is the predominant mechanism mediating the observed anti-CTLA-4 effects. Currently, two humanized anti-CTLA-4 antibodies, Ipilimumab (MDX-010) and Tremelimumab (CP-675206), have been used in phase I/II clinical trials. Results from a study on patients with advanced stage metastatic melanoma and renal cancer imply that antitumor effects are due to a direct enhancement of $CD4^+$ and $CD8^+$ T cell activity rather than inhibition or depletion of Tregs

(Maker *et al.*, 2005). In a mouse model expressing human instead of mouse CTLA-4 it was elegantly demonstrated that CTLA-4 blockade of Tregs alone failed to enhance antitumor responses (Peggs *et al.*, 2009). In contrast, concomitant blockade on both effector T cells and Tregs leads to a synergistic effect with maximal antitumor activity. In several phase I trials including mostly melanoma patients, but also ovarian and prostate cancer as well as B-NHL, blockage of CTLA-4 resulted in tumor regression, but in some cases it also generated severe autoimmune adverse effects (Ansell *et al.*, 2009; Dranoff, 2005; Maker *et al.*, 2005; Phan *et al.*, 2003). Autoimmunity may be minimized by altering the schedule of administration, dose and nature of the therapeutic antibody as well as the concomitant treatment, such as vaccines against certain TAAs. In a recent study on patients with metastatic melanoma and ovarian cancer, periodic infusion of anti-CTLA-4 antibodies after vaccination with autologous tumor cells secreting GM-CSF generated clinical antitumor immunity, and importantly, did not induce any grade 3 or grade 4 toxicity (Hodi *et al.*, 2008). Therapy-induced tumor necrosis correlated with intratumoral $CD8^+$ effector T cell/Treg ratio detected in post-treatment biopsies.

GITR, a molecule constitutively expressed on nTregs but also at lower levels on activated conventional T cells has also been considered a target for Treg depletion and functional inhibition (Nocentini and Riccardi, 2005; Shimizu *et al.*, 2002). *In vitro* stimulation of GITR in murine Tregs resulted in reduced suppressive activity, but this could not be reproduced in human Tregs (Kanamaru *et al.*, 2004; Levings *et al.*, 2002; Shimizu *et al.*, 2002). Tumor-bearing mice treated with the agonistic anti-GITR antibody DTA-1, or a GITR ligand showed decreased intratumoral Treg recruitment together with the generation of a potent specific antitumor response (Ko *et al.*, 2005; Levings *et al.*, 2002). Future studies are obligatory in order to evaluate the feasibility of such an approach for the treatment of cancer patients.

TLRs are widely expressed on multiple human cells and represent the first line of immunological defense through recognition of various pathogen-associated molecular patterns. TLRs are involved in DC maturation and activation of TLR pathways in DCs has been shown to prevent conversion of conventional T cells into Tregs (Iwasaki and Medzhitov, 2004). As described previously, Tregs express various TLRs, and thereby TLR ligands may have direct (positive or negative) effects on Tregs. Activation of TLR8 by natural or synthetic ligands independently of presence of DCs has been shown to reverse Treg function and augments *in vivo* tumor immunity in mouse models (Peng *et al.*, 2005; Sutmuller *et al.*, 2006). Stimulation of TLR signaling may be of particular importance for vaccination strategies, since appropriate TLR stimulation may overturn Treg-mediated tolerance (Yang *et al.*, 2004). Specific adjuvants providing vaccines with such properties are currently under investigation.

C. Disrupting Intratumoral Homing of Regulatory T Cells

As described in previous sections, chemokine/chemokine receptor interactions are vital to the migration of Tregs into the tumor microenvironment. One of the most important interplay is the one between CCL22 secreted by tumor and tumor conditioned myeloid cells, and CCR4, which is highly expressed on Tregs. Blocking of CCL22 significantly reduces the migration of Tregs into ovarian tumors as demonstrated in a preclinical murine xenograft model (Curiel *et al.*, 2004). In addition, CCL5–CCR5 interaction is crucial for Treg attraction in pancreatic adenocarcinoma (Tan *et al.*, 2009). Disrupting the CCL5–CCR5 signaling reduces Treg migration into the tumor bed also leading to significant tumor reduction. Both CCL5 and CCL22 are also involved in trafficking of effector T cells; a fact that needs to be taken into account during development of potential targeting strategies. Altogether, interfering with Treg trafficking represents a promising and very elegant potential approach in the treatment of cancer. However, it needs to be determined to what extent blocking of chemokine–chemokine receptor signaling will affect other cell types obligatory for an efficient immune response.

D. Modulation of Regulatory T Cell Proliferation/Conversion

As described in previous sections, DCs, regardless of maturation status, are involved in activation and induction of Tregs. One central molecule in that process is the enzyme IDO, which is highly expressed in tolerogenic myeloid and pDCs (Chen *et al.*, 2008; Chung *et al.*, 2009). Binding of CTLA-4 on CD80 and/or CD86 triggers IDO activity in DCs (Fallarino *et al.*, 2003), thus aforementioned anti-CTLA-4 treatment may interfere with the IDO pathway. Phase I clinical trials treating patients with relapsed or refractory solid tumor with the IDO inhibitor 1-methyl-D-tryptophan (D-1MT) are currently ongoing. In addition, animal studies have demonstrated that IDO-mediated immunosuppression can be reversed by celecoxib treatment (Lee *et al.*, 2009). Celecoxib is a specific inhibitor of the PGE_2-producing enzyme COX-2. The production of PGE_2 directly stimulates Treg expansion (Akasaki *et al.*, 2004) or indirectly facilitates Treg recruitment by promoting tolerogenic APCs (Bergmann *et al.*, 2007). Furthermore, Tregs themselves can suppress immune responses through PGE_2 secretion (Mahic *et al.*, 2006; Yaqub *et al.*, 2008) which further supports the evaluation of COX-2 inhibitors in the treatment of malignancies known to show high COX-2 and Treg levels such as HCC and renal cancer (Gao *et al.*, 2009;

Li *et al.*, 2009). PGE_2 stimulates expression of the enzyme aromatase through a cAMP-dependent pathway. Aromatase inhibitors, in particular letrozole (Femara) used to treat breast cancer patients have been shown to reduce the number of circulating Tregs, potentially by disrupting the PGE_2–aromatase pathway (Generali *et al.*, 2009).

In ovarian cancer patients, pDCs directly induce IL-10-producing $CD8^+$ Tregs (Wei *et al.*, 2005; Zou *et al.*, 2001). Tumor cells can produce CXCL12 and thereby attract pDCs expressing the specific receptor CXCR4. Blocking of the CXCL12–CXCR4 interaction induces apoptosis of tumor-related pDCs and abrogates their chemotaxis (Zou *et al.*, 2001). Furthermore, Tregs may upregulate CXCR4 upon IL-2 treatment (Wei *et al.*, 2006) or hypoxic conditions (Schioppa *et al.*, 2003), often noted in cancer. Therefore, agents like AMD-3100 used in HIV patients that antagonize the CXCR4 function may also be useful in the treatment of cancer.

Coinhibitory signaling through PD1-L is involved in the induction of Tregs (Gray *et al.*, 2003; Krupnick *et al.*, 2005; Wang *et al.*, 2008). Blockade of PD1-L on Tregs (Wang *et al.*, 2009) augments human tumor-specific T cell responses (Curiel *et al.*, 2003). An anti-PD-1 monoclonal IgG4 antibody, MDX-1106 (Ono-4538) is currently in a phase II trial for various types of cancer including melanoma, colon and lung cancer. Impact on clinical course, toxicities, and T cell subsets remains to be seen (Brahmer *et al.*, 2009).

Antiangiogenic treatment of colorectal cancer patients with the humanized anti-VEGF antibody bevacizumab (Avastin) induced a decrease in the levels of Tregs. The observations correlate with animal studies demonstrating a direct and positive correlation between VEGF expression and Treg levels (Li *et al.*, 2006). Expression of VEGF receptor-2 (VEGFR-2) within the T cell compartment is restricted to Tregs (Suzuki *et al.*, 2009). However, it is presently unclear whether the observed effects result directly from inhibiting VEGFR-2 or via an unknown intermediary mechanism (Wada *et al.*, 2009).

Two main cytokines involved in Treg induction and function are IL-10 and TGF-β. Disrupting their pathways may be useful for reducing the frequency and function of Tregs. Inhibitors of TGF-β for clinical use are currently under development and include anti-TGF-β antibodies, soluble TGF-β receptors as well as the antisense oligonucleotide, AP-12009, which blocks TGF-β expression and is currently being tested in phase I/II clinical trials.

E. Targeting the Antioxidative Capacity of Regulatory T Cells

As described previously, malignant diseases result in increased levels of oxidative stress mediated by reactive oxygen species (ROS) (Kusmartsev *et al.*, 2004; Szatrowski and Nathan, 1991). The detrimental effect of ROS

on effector cells of the immune system is well established and described in malignant and chronic inflammatory diseases (Gringhuis *et al.*, 2000; Li *et al.*, 2008; Malmberg *et al.*, 2001; Schmielau and Finn, 2001). Paradoxically, Treg levels are often increased in this hostile (for lymphocytes) milieu as described recently (Mougiakakos *et al.*, 2009). The mechanism underlying the increased resistance of Tregs toward oxidative stress is currently unclear but appears to be linked to the increased intracellular and surface thiol content. Nevertheless, the identification of this mechanistic pathway could provide yet another means for targeting Tregs in order to restore a "balance of power" between Tregs and conventional T cells as regards to oxidative stress susceptibility.

IX. CONCLUDING REMARKS

Tregs efficiently suppress innate and adaptive immunity. Despite the extensive research that has been carried out, many aspects of Treg biology in cancer remain to be explored. Vast majority of preclinical and clinical studies have linked the presence of Tregs to an increased risk for development as well as progression of cancer. This paradigm is currently under scrutiny as it has been convincingly shown that Tregs can act in a beneficial fashion in inflammatory driven malignancies, explaining controversial reports on some types of cancers, where Tregs were actually associated with a better disease course and outcome. In the context of controversial data regarding the impact of Tregs in cancer, it is important to point out the lack of comparability between distinct studies as differences in methodologies, enumeration strategies, Treg characterization as well as inclusion criteria for selection of patient groups have been substantial. The identification of Tregs specific for self and non-self TAAs is already leading to a major reevaluation of vaccine designs. Vaccination with tumor-specific peptides comprises the risk of boosting and/or inducing peptide-specific Tregs, which could thereby hamper the potential antitumor response. Strategies to incorporate adjuvants counteracting this process such as local induction of high IL-6 levels at site of antigen encountering or triggering of Treg-inhibiting TLRs are currently undertaken and evaluated in preclinical models. Altogether, the Treg population in cancer patients constitutes a very dynamic system as regards to subsets, origins, modes of suppression, and mechanisms leading to their accumulation. Interestingly, Tregs appear to generate a self-amplifying system by the production of cytokines that act in a positive feedback fashion and indirectly by promoting tolerogenic APCs. This complex system is at the same time a boon and a bane. On the one hand, it demands extensive efforts in order to decrypt all its building blocks, and on the other hand in-depth insight will allow us more specific and elegant

interventions into this web of tumor-associated immunosuppression. New technological achievements, like nanoparticles used as vehicles for a loco-regional delivery of Treg-targeting molecules may be very useful in our attempts to modulate Tregs at the site of their action in order to strengthen host surveillance and/or promote vaccine-induced immunity whenever it is considered beneficial for the clinical course of the particular type of cancer in question.

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