CHAPTER 4

How Tolerogenic Dendritic Cells Induce Regulatory T Cells

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Contents

Abstract

Since their discovery by Steinman and Cohn in 1973, dendritic cells (DCs) have become increasingly recognized for their crucial role as regulators of innate and adaptive immunity. DCs are exquisitely adept at acquiring, processing, and presenting antigens to T cells. They also adjust the context (and hence the outcome) of antigen presentation in response to a plethora of environmental inputs that signal the occurrence of pathogens or tissue damage. Such signals generally boost DC maturation, which promotes their migration from peripheral tissues into and within secondary lymphoid organs

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and their capacity to induce and regulate effector T cell responses. Conversely, more recent observations indicate that DCs are also crucial to ensure immunological peace. Indeed, DCs constantly present innocuous self- and nonself-antigens in a fashion that promotes tolerance, at least in part, through the control of regulatory T cells (Tregs). Tregs are specialized T cells that exert their immunosuppressive function through a variety of mechanisms affecting both DCs and effector cells. Here, we review recent advances in our understanding of the relationship between tolerogenic DCs and Tregs.

1. INTRODUCTION

Dendritic cells (DCs) are a family of leukocytes that have mostly been studied as potent stimulators of adaptive immunity, but there is mounting evidence that DCs also establish and maintain immunological tolerance (Steinman *et al.*, 2003). Indeed, DCs can prevent, inhibit, or modulate T cell-mediated effector responses through a variety of mechanisms, ranging from the production of pleiotropic anti-inflammatory factors that exert broadly attenuating effects to the induction of antigen-specific T cell responses resulting in anergy, deletion, or instruction of regulatory T cells (Tregs; Fig. 4.1). Here, we will focus on the mechanisms by which DCs induce and control tolerance, particularly the function and differentiation of Tregs, which are crucial to contain autoimmunity and chronic inflammation. Failure of Treg function has been implicated in the development of many autoimmune processes, whereas cellular therapy by adoptive transfer of Tregs has shown efficacy in these disorders (Roncarolo and Battaglia, 2007). However, Treg-mediated suppressive activity can also contribute to the immune escape of pathogens or tumors. Indeed, elimination of Tregs in mice carrying malignancies can improve antitumor immune responses and survival (Zou, 2006). Therefore, understanding the role of DCs in Treg activation and differentiation is critical for the development of therapeutic strategies in many disease settings.

At steady-state, tissue-resident DCs are immature (henceforth called iDCs); these cells are poised to acquire antigenic material from their environment, but they are poorly immunogenic because they express only modest levels of MHC molecules and little or no costimulatory molecules and proinflammatory cytokines. iDCs sense the presence of infectious microbes using specific receptors that detect pathogen-associated molecular patterns (PAMPs) or damage associated molecular patterns (DAMPs) that are released within tissues as a consequence of cellular distress. These ‘‘danger’’ signals trigger signaling cascades in iDCs that result in their maturation, a profound phenotypic and functional metamorphosis driven by changes in gene expression (McIlroy

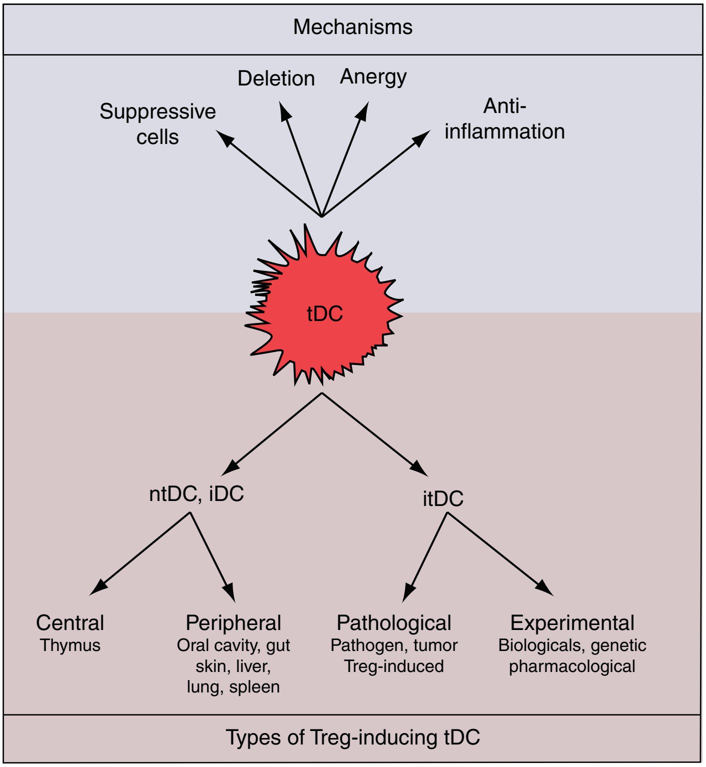

FIGURE 4.1 Types of tolerogenic DCs and their mechanisms of action. Tolerogenic DCs (tDCs) participate to the establishment of T cell tolerance by a variety of mechanisms, including the induction of anergy, deletion of antigen-reactive T cells, stimulation of suppressive regulatory T cells (Tregs) either by activation of existing Tregs or *de novo* differentiation of Tregs from Tns and production of anti-inflammatory cytokines and other factors. Depending on the differentiation state of the DC and the site of tolerogenic instruction, tDCs can be separated in natural tolerogenic DCs (ntDCs) and induced tolerogenic DCs (itDCs). The steady state environment instructs ntDCs (and includes iDCs) while itDCs arise during pathologies or after manipulation.

et al., 2005; Türeci *et al.*, 2003). During the maturation process, DCs lose their capacity to acquire soluble antigen but gain T cell stimulatory capacity due to increased antigen processing and upregulation of MHC, costimulatory molecules and cytokines (Banchereau *et al.*, 2000). Maturation signals also trigger in iDCs a profound change in their repertoire of traffic molecules, such as the upregulation of CCR7, a chemokine receptor that enables DCs in peripheral tissues to access local lymph vessels and migrate to the draining lymph nodes (Alvarez *et al.*, 2008). Here, the now fully mature DCs (mDCs) report the inflammatory and antigenic status of their source tissue to recirculating lymphocytes (Banchereau *et al.*, 2000).

Whereas newly generated mDCs are generally believed to possess primarily immunogenic functions, the role of iDCs is less well defined as they are not in a final differentiation state and can give rise to both immunogenic, proinflammatory mDCs as well as semimature DCs that share some phenotypic features of mDCs, such as CCR7 expression, but possess the capacity to establish and maintain tolerance.

Clues that iDCs themselves can either convert conventional naïve T cells (Tns) to assume a Treg phenotype and/or promote the function of existing Tregs have been gleaned from experiments in which antigen was administered to mice without a concomitant maturation signal (Apostolou and von Boehmer, 2004; de Heer *et al.*, 2004; Kretschmer *et al.*, 2005; Lambrecht and Hammad, 2009; Ostroukhova *et al.*, 2004; Tsuji and Kosaka, 2008; Vermaelen *et al.*, 2001). Under these conditions, antigen accumulated on DCs in secondary lymphoid organs (SLOs) and triggered the differentiation and/or proliferation of Tregs resulting in antigen-specific tolerance that could prevent or reverse autoimmune processes (Table 4.1). Animals that lack functional iDCs develop severe autoimmunity, possibly due, at least in part, to reduced numbers of circulating Tregs (Bar-On and Jung, 2010; Birnberg *et al.*, 2008; Darrasse-Jeze *et al.*, 2009; Ohnmacht *et al.*, 2009). Similarly, a DC-restricted genetic deficiency in $\alpha_v\beta_8$ integrin, which activates TGFβ, a key cytokine for the induction and maintenance of Tregs (Travis *et al.*, 2007), or disruption of DC-expressed TGFβ receptor (TGFβR) impairs the tolerogenic function of DCs and fosters autoimmunity (Laouar *et al.*, 2008). However, increased DC numbers are accompanied by a concomitant increase in Tregs, whereas elimination of Tregs elevates the number of DCs (Darrasse-Jeze *et al.*, 2009; Liu *et al.*, 2009; Lund *et al.*, 2008) suggesting that DCs and Tregs regulate each other's homeostasis.

It must be noted that neither iDCs nor mDCs are homogenous cell populations. Several distinct subsets that express discrete surface markers have been identified nearly two decades ago (Vremec *et al.*, 1992). The phenotypic diversity of the DC family is reflected in distinct functional properties that are rooted, in part, in the expression of different PAMP and DAMP receptors, divergent antigen presentation and crosspresentation capacities, as well as differential propensities to induce tolerance and Treg differentiation.

It is thus apparent that DCs encompass a heterogeneous mix of antigen presenting cells that differ not only with regard to phenotype, differentiation, and maturation status but also with regard to tolerance-inducing capacity. For the purpose of this chapter, we will functionally (rather than phenotypically) define two subsets of DCs based on their net effect on T cells: one subset is represented by immunogenic DCs that induce effector responses, while the other subset induces or enhances tolerance (Fig. 4.2). We will refer to the former as stimulatory DCs (sDCs) and the latter as tolerogenic DCs (tDCs). tDCs not only comprise most iDCs but also include

TABLE 4.1 Natural tolerogenic DC

Mechanism of t-DC induction	Treg phenotype	Origin of DC	DC phenotype	Mechanism of Treg induction	Disease model	Reference
Central suppressive tolerance						
TSLP	CD4+CD25+Foxp3+	Thymus	mDC			Watanabe *et al.* (2005)
	CD4+CD25+Foxp3+	Thymus	pDC			Proietto *et al.* (2008, 2009)
Peripheral suppressive tolerance						
Dermal tolerance						
Retinoic acid	CD4+Foxp3+	Skin DC	CD103− iDC		IBD	Guilliams *et al.* (2010)
	CD4+CTLA4+Foxp3+ IL-10+TGFβ+	Skin LN	DEC-205+ iDC		T1D	Bruder *et al.* (2005)
	CD4+CD25+CTLA4+	Skin LN	DEC-205+ iDC			Mahnke *et al.* (2003)
Oral tolerance						
	CD4+CD25+*	Peyer's patches	CD11c+ CD11b+		CIA	Min *et al.* (2006)
	CD25+*	Peyer's patches	pDC-like-CD8α+			Bilsborough *et al.* (2003)
	CD25+IL-10+INFγ+*	Oral cavity	CD11c+			Mascarell *et al.* (2008)
	CD25+Foxp3+*	Peyer's patches	CD11c+ IDO+		CIA	Park *et al.* (2008)
	CD25+CD103+Foxp3+	LP		(RA, TGFβ)		Sun *et al.* (2007)

(*continued*)

TABLE 4.1 (*continued*)

Mechanism of t-DC induction	Treg phenotype	Origin of DC	DC phenotype	Mechanism of Treg induction	Disease model	Reference
	CD4+Foxp3+	MLN and LP	CD103+	(RA, TGFβ)		Coombes *et al.* (2007)
	CD4+Foxp3+	MLN	CD103+	IDO	IBD	Matteoli *et al.* (2010)
IEC secreting TGFβ, RA	CD4+CD25+Foxp3+*	BMDC or SpDC	CD103+		IBD	Iliev *et al.* (2009b)
IEC secreting TGFβ, RA	CD4+CD25+Foxp3+*	MLN	CD103+			Iliev *et al.* (2009a)
Systemic tolerance						
	CD4+IL-10+	Spleen	CD11clow CD45RB+			Wakkach *et al.* (2003)
	CD4+*	Spleen	pDCs			Martín *et al.* (2002)
	CD4+Foxp3+*	Spleen	CD8α+		EAE	Smith *et al.* (2010)
	CD4+CD25+Foxp3+*	Spleen	DEC-205+			Kretschmer *et al.* (2005)
	CD4+CD25−*	hu-PBMC-pDC	BDCA4+Lin-CD123+	IDO		Chen *et al.* (2008)
	CD4+CD25+Fopx3+ IL-10+TGFβ+	hu-PBMC-pDC	BDCA4+Lin-CD123+			Moseman *et al.* (2004)
	IL-10+*	hu-PBMC-pDC	BDCA4+Lin-CD123+	CD275		Ito *et al.* (2007)

	CCR4+CD25+Foxp3+*	Allograft draining LN	pDCs		HA	Ochando *et al.* (2006)
	CD4+CD25+Foxp3+	Spleen and LN	pDC CCR9+		aGVHD	Hadeiba *et al.* (2008)
Inhaled tolerance						
	CD4+IL-10+*	Lung LN		IL-10		Akbari *et al.* (2001)
	CD4+IL-10+*	Lung LN		IL-10 CD275	EA	Akbari *et al.* (2002)
***In vitro* immature**						
	CD4+CTLA-4+IL-10+*	huMoDC	CD83−			Jonuleit *et al.* (2000)
	CD4+IL-10+	huMoDC	CD83−			Dhodapkar *et al.* (2001)
	CD8+IL-10+*	huMoDC				Dhodapkar and Steinman (2002)
	CD4+IL-10+*	huMoDC	CD1a+CD83-ILT3+ILT4+	IL-10		Levings *et al.* (2005)
	CD4+IL-10+	huMoDC	iDC	CD275		Tuettenberg *et al.* (2009)
	CD4+CD25+Foxp3+ IL-10+TGFβ+	huMoDC				Cools *et al.* (2008)
	CD4+CD25+Foxp3+	BMDC			PA	Stepkowski *et al.* (2006)

aGVHD: acute Graft Versus Host Disease, CIA: Collagen-Induced Arthritis, EA: Experimental asthma, EAE: Experimental Autoimmune Encephalomyelitis, HA: Heart Allograft, IBD: Intestinal Bowel Disease, T1D: Type 1 Diabetes, PA: Pancreatic Allograft, * with suppressive activity.

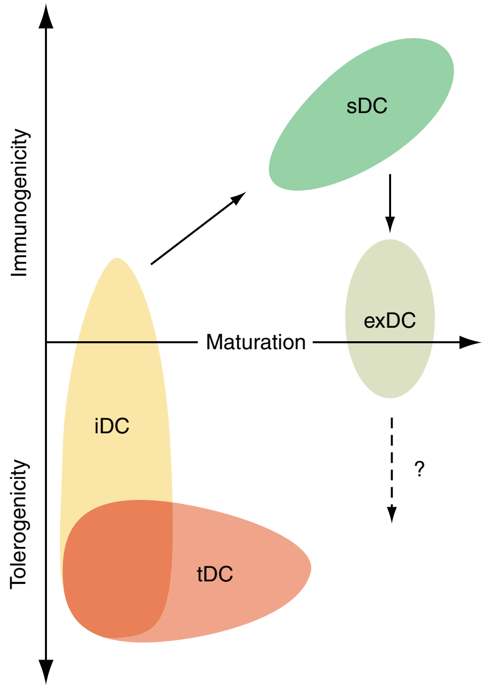

FIGURE 4.2 Relationship of maturation status, tolerogenicity, and immunogenicity among DC subsets. Immature DCs (iDCs) receive activation signals from microbial byproducts or tissue distress to acquire a mature phenotype, including the ability to migrate to lymph nodes and enhanced antigen presentation and costimulatory capacities. These mature DCs are highly stimulatory (sDC) and induce effector responses. Tolerogenic DCs (tDCs) include most iDCs but also comprise some cells with advanced maturation status. Only iDCs can give rise to mDCs. mDCs may lose their immunostimulatory capacity to become exhausted (exDC); however, their role in the induction of Tregs remains uncertain.

other DCs covering a spectrum of different maturation states. This review will summarize current knowledge of the origins and phenotypes of tDCs, the factors maintaining or inducing their tolerogenicity, and how these cells promote the expansion, function, or differentiation of Tregs.

2. WHAT IS THE ORIGIN OF TREG-INDUCING TDCS?

2.1. Tregs induction sites

Mammals, including humans, that lack functional Tregs succumb to fatal autoimmune disorders (Paust and Cantor, 2005), highlighting the importance of Tregs in controlling immune responses. In general, we discriminate between two major types of Tregs based on their origin (Bluestone and Abbas, 2003). Natural Tregs (nTregs) originate during thymic development and first appear in the fetal circulation (Lio and Hsieh, 2008; Min *et al.*, 2007; Mold *et al.*, 2008). The phenotype and suppressive program of CD4+ nTregs is controlled by the transcription factor Foxp3, which is upregulated in developing T cells upon recognition of self-antigens in the thymus (Bensinger *et al.*, 2001; Kim and Rudensky, 2006; Ribot *et al.*, 2006). Innocuous self- and nonself-antigens that appear postnatally (like hormones, food, and commensal flora) can drive the differentiation of additional Tregs (Vigouroux *et al.*, 2004). Some of these antigens may be transported into the thymus by migratory iDCs (Bonasio *et al.*, 2006) that may then induce new nTregs. In addition, conventional Tns can be converted to so-called adaptive Tregs (aTregs) in extrathymic sites such as SLOs. aTregs are phenotypically heterogeneous and include both CD4+ and CD8+ T cells, most (but not all) of which also express Foxp3 (Table 4.1). A common trait of all Tregs is the expression of one or more anti-inflammatory molecules, such as IL-10, TGFβ, or IL-35 and/or inhibitory receptors, such as cytotoxic T-lymphocyte antigen 4 (CTLA4), lymphocyte-activation gene-3 (LAG-3), glucocorticoid-induced tumor necrosis factor receptor (GITR), CD39, or CD73, among others (Tang and Bluestone, 2008; Vignali *et al.*, 2008).

2.2. The phenotype of tDCs

The mechanisms by which tDCs exert their activity are varied and incompletely understood. As mentioned above, iDCs are typically tolerogenic (Steinman *et al.*, 2003), so the maturation status, or rather, the absence of maturation provides a hint for the tolerogenic capacity of DCs. However, iDCs comprise several different subsets that possess distinct abilities to present antigen, secrete cytokines, and induce tolerance (Ueno *et al.*, 2007). Thus, the various subsets of iDCs and mDCs do not fill a well-defined functional niche, but cover a spectrum of immunological properties, wherein iDCs primarily maintain tolerance, whereas mDCs initiate and control predominantly (but not exclusively) effector responses (Fig. 4.2).

2.2.1. Maturation phenotype

DCs receive maturation signals by a variety of inputs, including PAMP and DAMP receptors that sense certain microbial and tissue damage signatures. Such sensors include toll-like receptors (TLRs), NOD-like receptors (NLRs), RIG-I-like receptors (RLRs), and others (Barton and Medzhitov, 2003; Franchi *et al.*, 2010; Pétrilli *et al.*, 2007; Re and Strominger, 2004). Additionally, inflammatory cytokines (e.g., TNFα and IL-1β) or the ligation of surface-expressed activating receptors such as CD40 can trigger DC maturation (Aggarwal, 2003; Elgueta *et al.*, 2009;

Sims and Smith, 2010). One key consequence of DC recognition of ‘‘danger’’ signals is the activation of members of the nuclear factor kappa B (NFκB) and interferon responsive factor (IRF) families (Meylan and Tschopp, 2006; Re and Strominger, 2004; Salter and Watkins, 2009). Upon maturation, DCs upregulate a plethora of gene products involved in antigen presentation and costimulation including MHC-II, CD40, CD80, CD86, OX40L, and inducible T cell costimulator ligand (ICOSL or CD275), as well as cytokines that promote and modulate inflammation and effector cell functions, including IL-1β, IL-2, IL-6, IL-8, IL-12, and IL-18 (Banchereau *et al.*, 2000). These changes are necessary for DCs to initiate T cell responses because Tns require three concomitant inputs to differentiate into full-fledged effector cells (Teffs): signal 1 is the antigenic stimulus provided by MHC molecules displaying a cognate peptide; signal 2 is provided by costimulatory molecules; and signal 3 is provided by cytokines produced by DCs or other microenvironmental sources (Cronin and Penninger, 2007). Since many tDCs have an immature phenotype (Tables 4.1 and 4.2; Fig. 4.2), it has been suggested that a major mechanism of their tolerogenicity is a consequence of their presentation of an antigen (signal 1) to T cells without concomitant costimulation or cytokines (signals 2 and 3). However, when iDCs are subjected to certain *in vitro* manipulations, such as exposure to TNFα or IFNγ or inhibition of E-cadherin, they assume phenotypic features of mDCs, including high levels of MHC and costimulatory molecules (Reis and Sousa, 2006; Tisch, 2010, and our unpublished results). Nevertheless, Tns that are exposed to such treated DCs preferentially differentiate into aTregs (Table 4.3). Moreover, although CCR7 is usually considered an indicator of DC maturation, some iDCs in peripheral tissues can also upregulate CCR7, which allows them to migrate to lymph nodes without assuming a fully mature phenotype. These migratory DCs favor the induction of aTregs rather than effector cells (Hintzen *et al.*, 2006; Jang *et al.*, 2006; Ohl *et al.*, 2004; Worbs *et al.*, 2006). CCR7 deficiency impairs lymphatic migration of iDCs and compromises the induction of inhaled and oral tolerance (Förster *et al.*, 2008; Martin-Fontecha *et al.*, 2003).

Thus, while immaturity appears to be a good indicator of DC tolerogenicity, phenotypically mDCs do not always induce immunity but, depending upon prior exposure to certain differentiation signals, may retain their tolerogenic function. This suggests that tolerance is not always a mere consequence of T cells perceiving insufficient signal 2 or 3, but additional DC-derived tolerance-promoting factors are likely to play a role. A case in point are so-called exhausted DCs (exDCs), which were observed to arise *in vitro* following an extended interval after exposure to maturation signals, such as bacterial lipopolysaccharide (LPS). The term ‘‘exhaustion’’ was proposed because exDCs, unlike freshly activated mDCs, have lost their initial capacity to induce Tn differentiation into T helper (Th)-1 cells. Instead, exDCs secrete immunosuppressive IL-10 and

TABLE 4.2 Disease-induced tolerogenic DC

Mechanism of t-DC induction	Treg phenotype	Origin of DC	DC phenotype	Mechanism of Treg induction	Disease model	Reference
Pathogen-induced tolerogenic DC						
F. hepatica products	CD4+CD25+Foxp3+	BMDC	iDC			Falcón *et al.* (2010)
S. japonicum SJMHE1 peptide	CD4+CD25+*	BMDC	iDC		DTH	Wang *et al.* (2009)
C. albicans	CD4+Foxp3+IL-10+	BMDC			IBD	Bonifazi *et al.* (2009)
Monophosphoryl lipid A	CD4+Foxp3+ IL-10+TGFβ+	Oral cavity	Oral-m-LC			Allam *et al.* (2008)
LPS	CD4+CD25+Foxp3+	BMDC			EAU	Lau *et al.* (2008)
Cryptococcus neoformans glucuronoxylomannan	CD4+Foxp3+	BMDC				Liu *et al.* (2008)
Curcuma longa L. products (Curcumin)	CD4+CD25+ Foxp3+IL10+*	BMDC			IBD	Cong *et al.* (2009)
Yersinia virulence factor	CD4+IL-10+	BMDC				Depaolo *et al.* (2008)
Tumor-induced tolerogenic DC						
Pancreatic tumor-derived mucins	*	huMoDC				Monti *et al.* (2004)
B16 Melanoma	CD4+CD25+Foxp3-*	Spleen	iDC	TGFβ	TI	Ghiringhelli *et al.* (2005b)
P815 Mastocytoma	CD4+IL-10+	Tumor-infiltrating	CD4-CD8−		TI	Liu *et al.* (2005)
MO4 Carcinoma	CD4+IL-10+*	Spleen	CD4-CD8−		TI	Zhang *et al.* (2005)
Necrotic myeloma cells	CD4+IL-10+	huMoDC				Fiore *et al.* (2005)
Ovarian carcinoma			pDC		CP	Wei *et al.* (2005)

(*continued*)

TABLE 4.2 (*continued*)

Mechanism of t-DC induction	Treg phenotype	Origin of DC	DC phenotype	Mechanism of Treg induction	Disease model	Reference
	CD8+CCR7+ CD45RO+IL-10+*	Ovary ascite				
Lung carcinoma cells	CD4+CD25+Foxp3+*	huMoDC	iDC			Dumitriu *et al.* (2009)
Retrocontrol-induced tolerogenic DC						
CD8+CD28− suppressor	CD4+	huMoDC	ILT3+ILT4+			Chang *et al.* (2002)
CD8+CD28− suppressor	CD4+CD45RO+ CD25+	huMoDC	ILT3+ILT4+			Manavalan *et al.* (2003)
CD4+ Tregs	CD4+*	BMDC				Martin *et al.* (2003)

CP: Cancer-bearing patients, DTH: delayed-type hypersensitivity, EAU: Experimental Autoimmune Uveoretinitis, IBD: Intestinal Bowel Disease, TI: Tumor implantation,
* with suppressive activity.

TABLE 4.3 Experimentally induced tolerogenic DC

Mechanism of t-DC induction	Treg phenotype	Origin of DC	DC phenotype	Mechanism of Treg induction	Disease model	Reference
Biologically induced tolerogenic DC						
Galectin 1	CD4+IL-10+	BMDC		IL-27	EAE	Ilarregui *et al.* (2009)
CD40L+IL-3	CD4+CD25+Foxp3+ IL-10+TGFβ+	Thymus	pDC			Martín-Gayo *et al.* (2010)
IL-10+IFNα	CD8+CD28−	huMoDC				Qin *et al.* (2008)
Blocking CD200R	CD4+CD25+[a]	BMDC			SA	Gorczynski *et al.* (2004)
Thymosin α1+TLR9	CD4+CD25+[a]IL-10+[a]	BMDC				Romani *et al.* (2006)
In vivo induced GM-CSF DC	CD4+Foxp3+IL-10+[a]	Spleen	iCD8α−		EAT	Ganesh *et al.* (2009)
Vitamin D3	CD4+IL-10+[a] CD4+IL-10+	huMoDC huMoDC	smDC CCR7+	PD-L1		Unger *et al.* (2009)
Vitamin D3+ dexamethasone + LPS						Anderson *et al.* (2009)
Vitamin D3	CD4+Foxp3+[a]	huMoDC				Penna *et al.* (2005b)
Vitamin D3	CD4+CD25+ Foxp3+CD62L+	BMDC	iDC			Ureta *et al.* (2007)
P-selectin	CD4+CD25+CD25+ Foxp3+[a]	huMoDC				Urzainqui *et al.* (2007)
CTLA4–Ig fusion protein	CD4+CD25+Foxp3+[a]	Spleen		TGFβ	CIA	Ko *et al.* (2010)
Estrogen	CD28-[a]	Spleen			EAE	Pettersson *et al.* (2004)
VIP	CD4+TGFβ+IL-10+[a]	BMDC			IBD	Gonzalez-Rey and Delgado (2006)

(*continued*)

TABLE 4.3 (*continued*)

Mechanism of t-DC induction	Treg phenotype	Origin of DC	DC phenotype	Mechanism of Treg induction	Disease model	Reference
VIP	CD4+TGFβ+IL-10+[a] CD8+CD28−[a]	huMoDC	iDC			Gonzalez-Rey *et al.* (2006)
VIP	CD4+IL-10+	BMDC	iDC IL-10+		DTH	Delgado *et al.* (2005)
VIP	CD4+TGFβ+IL-10+[a]	BMDC	iDC IL-10+		EAE RA	Chorny *et al.* (2005)
VIP	CD4+IL-10+[a]	BMDC	iDC		GVHD	Chorny *et al.* (2006)
BiP	CD4+CD25+CD27+[a]	huMoDC		IDO, IL-10		Corrigall *et al.* (2009)
HGF	CD4+CD25+Foxp3+ IL-10+	Spleen			EAE	Benkhoucha *et al.* (2010)
HGF	CD4+CD25+ Foxp3+IL-10+[a]	huMoDC		ILT3, IL-10		Rutella *et al.* (2006)
TSLP	CD25+Foxp3+[a]	BMDC	iDC		T1D	Besin *et al.* (2008)
HLA-G	CD25+CTLA-4+[a]	huMoDC				Ristich *et al.* (2005)
ILT3	CD8+CD28−[a]	BMDC				Vlad *et al.* (2010)
IL-10	CD4+CTLA-4+[a] CD8+[a]	huMoDC				Steinbrink *et al.* (2002)
IL-10	CD25+Foxp3+ LAG3+CTLA4+[a]	huMoDC	iDC ILT2+ IL-10+			Li *et al.* (2010)
IL-10	IL-10+Vα24+iNKT[a]	huMoDC	smDC			Yamaura *et al.* (2008)

IL-10	CD4+CD25+IL-10+[a]	huMoDC	iDC		xGVHD	Sato *et al.* (2003a)
IL-10	CD4+IL-10+	PBMC	DC-10	ILT4		Gregori *et al.* (2010)
IL-10	CD4+[a]	huMoDC				Pacciani *et al.* (2010)
IL-10	CD4+[a]	huMoDC	iDC IL-10+			Torres-Aguilar *et al.* (2010)
IL-10	CD4+IL-10+	BMDC	CD11clow CD45RB+			Wakkach *et al.* (2003)
IL-10	CD4+[a]	huMoDC				Kubsch *et al.* (2003)
IL-10+TGFβ	CD4+CD25+Foxp3+	BMDC	CD200R3+ CD49+		cGVHD	Sato *et al.* (2009)
IL-10+TGFβ	CD4+CD25+Foxp3+[a]	BMDC	iDC			Fujita *et al.* (2007)
IL-10+TGFβ	CD4+[a]	huMoDC	iDC IL-10+			Torres-Aguilar *et al.* (2010)
TGFβ	CD4+CD25+CTLA-4+[a]	BMDC	iDC		aGVHD	Sato *et al.* (2003b)
TNFα	CD4+CD25+[a]	BMDC	smDC		SA	Fu *et al.* (2010)
TNFα	CD4+CD25+[a]	BMDC	smDC IL-10+		SA	Fu *et al.* (2009)
TNFα	CD4+Foxp3+[a]	BMDC	smDC		EAE	Zozulya *et al.* (2009)
TNFα	CD4+CD25+ IL-10+CTLA4+ GITR+Foxp3+*	BMDC	smDC		EAT	Verginis *et al.* (2005)
TNFα	CD4+IL-10+	BMDC	smDC		EAE	Menges *et al.* (2002)
IFNγ	CD4+Foxp3+[a]	huMoDC	smDC			Eljaafari *et al.* (2009)

(*continued*)

TABLE 4.3 (*continued*)

Mechanism of t-DC induction	Treg phenotype	Origin of DC	DC phenotype	Mechanism of Treg induction	Disease model	Reference
Anti-CD45RB+LF150195	CD25+[a]	Spleen	iDC		HA	Min *et al.* (2003)
E-cadherin	CD4+IL-10+	BMDC	mDC		EAE	Jiang *et al.* (2007)
Pharmacologically induced tolerogenic DC						
Aspirin	CD25+Foxp3+[a]	huMoDC	iDC			Buckland *et al.* (2006b)
Dexamethasone	CD4+IL-10+[a]	huMoDC	smDC			Unger *et al.* (2009)
Dexamethasone	CD4+IL-10+[a]	huMoDC	smDC			Anderson *et al.* (2008)
Resveratrol	CD4+IL-10+	huMoDC	iDC			Svajger *et al.* (2010)
Rosiglitazone (NFkB inhibitor)	Foxp3+	BMDC	iDC		EAE	Iruretagoyena *et al.* (2006)
LF 15-0195 (IKK inhibitor)	CD4+CD25+ CTLA4+Foxp3+[a]	BMDC	iDC		HA	Zhang *et al.* (2008)
Curcumin	CD4+CD25+ Foxp3+IL10+[a]	BMDC		IL-10, TGFβ, RA	IBD	Cong *et al.* (2009)
Prednisolone	[a]	huMoDC	iDC		MG	Luther *et al.* (2009)
Genetically induced tolerogenic DC						
SOCS3KO	CD25+Foxp3+[a]	BMDC	iDC	TGFβ	EAE	Matsumura *et al.* (2007)
Dominant negative IKK2 transduction	[a]	BMDC	iDC			Tomasoni *et al.* (2005)

Foxp3 transduction	CD25+[a]	huMoDC		TGFβ		Lipscomb *et al.* (2010)
IL-10 transduced	CD4+CD25+ Foxp3+IL-10+[a]	BMDC	smDC	IL-10	EA	Henry *et al.* (2008)
CD40/80/86 KD	[a]	BMDC			CIA	Zheng *et al.* (2010)
RelB KD	Foxp3+	BMDC			EAMG	Yang *et al.* (2010)
RelBKO	CD4+IL-10+[a]	BMDC	iDC			Martin *et al.* (2003)
RelB KD	CD4+Foxp3+		iDC			Zhang *et al.* (2009a)
CD40 KD	IL-10+[a]	BMDC		IL-10	EAMG	Martin *et al.* (2003)

aGVHD: acute Graft Versus Host Disease, CIA: Collagen-Induced Arthritis, cGVHD: chronic Graft Versus Host Disease, DTH: delayed-type hypersensitivity, EA: Experimental asthma, EAE: Experimental Autoimmune Encephalomyelitis, EAMG: Experimental Autoimmune Myasthenia Gravis, EAT: Experimental Autoimmune Thyroiditis,GVHD: Graft Versus Host Disease, HA: Heart Allograft, IBD: Intestinal Bowel Disease, MG: Myasthenia Gravis, RA: Rheumatoid Arthritis, SA: Skin Allograft, T1D: Type 1 Diabetes,xGVHD: xenogeneic graft-versus-host disease,*with suppressive activity.

elicit nonpolarized memory cells and/or Th2 responses (Langenkamp *et al.*, 2000, 2002). Whether exDCs can also induce Tregs *in vivo* remains to be determined.

2.2.2. tDC subsets

In mice, at least seven different DC subpopulations can be identified, which are distinguishable by both surface and intracellular markers that govern their function (Coquerelle and Moser, 2010; Liu and Nussenzweig, 2010; Milling *et al.*, 2010; Pulendran *et al.*, 2008; Shortman and Heath, 2010; Siddiqui and Powrie, 2008; Steinman and Idoyaga, 2010; Swiecki and Colonna, 2010; Ueno *et al.*, 2007). Murine lymphoid tissue-resident DC subsets include CD8α+, CD4+, CD8α−,CD4− (DN), and plasmacytoid DCs (pDCs). Migratory DCs that carry antigen from peripheral organs to SLOs include CD103+ DCs that have been identified in the lung, the gastrointestinal tract, and the skin, CD11b+ "myeloid DCs" and epidermal Langerhans cells (LCs). *In vitro* assays suggest that there may be a hierarchy of tolerogenic potential that is highest for pDCs followed by CD103+ DCs and CD8α+ DCs with CD11b+ DCs having low activity in most assays.

It should be cautioned, however, that the tolerogenicity of DC subsets is context dependent. For instance, CD8α+ DCs preferentially promote aTreg differentiation in the presence of TGFβ (Shortman and Heath, 2010; Yamazaki *et al.*, 2008), although it should be noted that addition of TGFβ to activated Tns induces aTreg differentiation even in absence of DCs (Chen *et al.*, 2003). pDCs are key participants in the establishment of oral and transplant tolerance (Goubier *et al.*, 2008; Ochando *et al.*, 2006; Swiecki and Colonna, 2010), presumably owing to their expression of indoleamine 2,3-dioxygenase (IDO), an enzyme that inhibits effector T cell proliferation (Puccetti and Grohmann, 2007). Intestinal CD103+ DCs also express IDO and secrete all-trans retinoic acid (RA), which promotes Tn differentiation into aTreg (Matteoli *et al.*, 2010; Siddiqui and Powrie, 2008). Some skin-derived CD103− DCs and other DCs can also produce RA (Guilliams *et al.*, 2010), while IDO expression is inducible in DCs by a variety of signals, including TGFβ, interferons (Belladonna *et al.*, 2008; Guilliams *et al.*, 2010; Matteoli *et al.*, 2010; Puccetti and Grohmann, 2007), and engagement of GITR (Grohmann *et al.*, 2007), among others. Therefore, although DCs subpopulations have different tolerogenic capacities *a priori*, they can adapt their function according to environmental inputs.

3. INSTRUCTIVE SIGNALS FOR TREG-INDUCING TDCS

In addition to the fact that immature tDCs present little or no signals 2 and 3 (see above), they can receive tolerance-promoting molecular "reminders" that counteract sDC differentiation in response to maturation stimuli (Fig. 4.3). These signals can be mimicked *in vitro* to induce tDCs

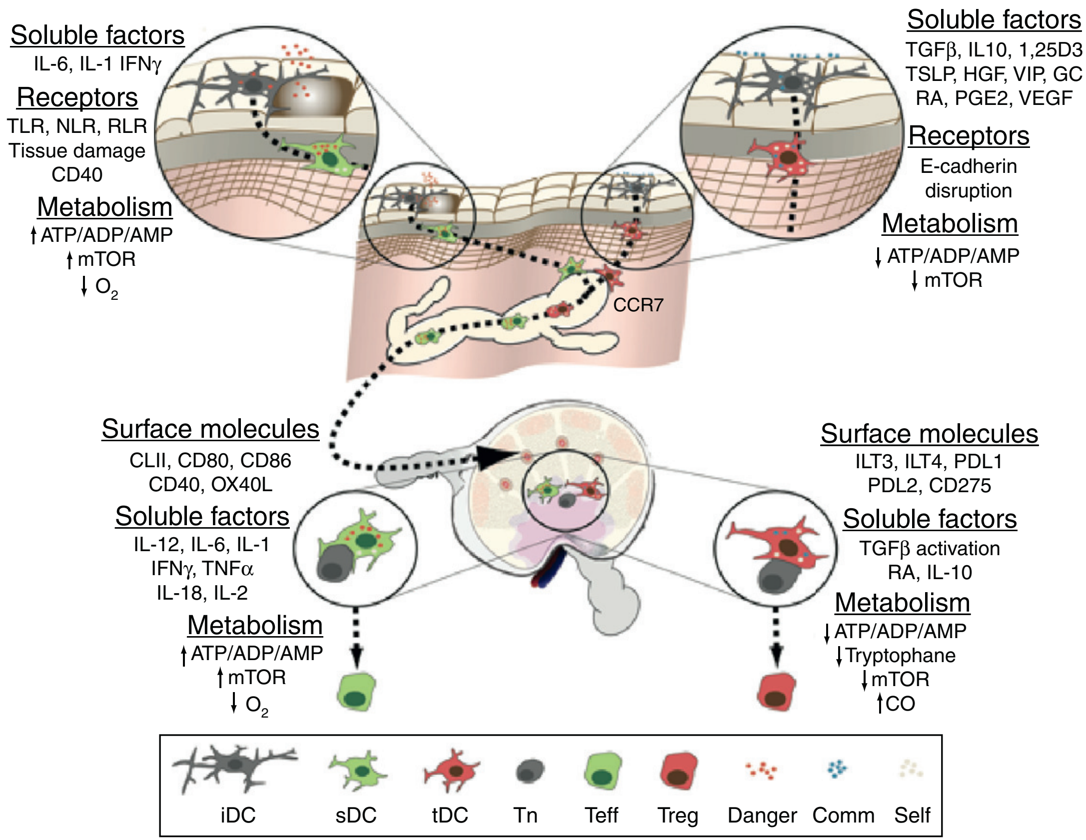

FIGURE 4.3 Education of immunogenic or tolerogenic DCs by environmental signals. Immature DCs (iDCs) perceive a myriad of inputs leading to their differentiation into sDCs or tDCs. Upon engagement of danger signal receptors by microbes or cellular distress, the presence of activating cytokines or changes in the abundance of certain metabolites, these cells mature and become sDCs that migrate to the draining

under tissue culture conditions. Thus, we can differentiate between tDCs that arise naturally from hematopoietic precursors, and tDCs that have received instructive signals that may cement or modulate their tolerogenic phenotype. To facilitate discussion, we will refer to natural versus induced tDCs as ntDCs and itDCs, respectively (Fig. 4.1). While ntDCs maintain tolerance constitutively within a steady-state environment, itDCs have received inputs from their environment, such as experimental or pharmacological interventions, infectious agents or other pathophysiological conditions. It should be emphasized that this terminology is merely meant to offer a conceptual frame of reference and does not imply that ntDCs and itDCs are strictly separate populations. Both subsets overlap and likely coexist and cooperate within tissues, making a real-life distinction between them often difficult.

3.1. Natural tolerogenic DCs

As discussed above, nTreg and aTreg originate from different anatomic compartments and in response to distinct immunological processes. The rules governing the function of tDCs in the thymus, where central tolerance is established by selection of Tns and generation of nTregs, and in peripheral tissues, where tDCs convert Tns into aTregs, are only beginning to be understood.

3.1.1. Central suppressive tolerance

Although thymic epithelial cells contribute to self-antigen-reactive nTreg commitment (Aschenbrenner *et al.*, 2007; Bensinger *et al.*, 2001; Liston *et al.*, 2008), thymic DCs and, in particular, thymic pDCs also promote the induction of Foxp3+ nTreg (Table 4.1; Atibalentja *et al.*, 2009; Martín-Gayo *et al.*, 2010; Proietto *et al.*, 2008, 2009). The mechanism(s) by which

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secondary lymphoid organs (SLOs) using CCR7. Through presentation of cognate antigen and costimulatory surface receptors as well as production of cytokines and the regulation of metabolites, sDCs coerce naïve T cells (Tns) to become effector cells (Teffs). However, at steady state, commensals and structural cells produce anti-inflammatory cytokines that in combination with regular levels of metabolites and minute quantities of danger signals imprint tDCs to migrate to SLOs using CCR7. Upon contact with antigen-specific cells, tDCs induce the differentiation of regulatory T cells (Tregs) through a variety of mechanisms. Toll-like receptors (TLR), NOD-like receptors (NLR), RIG-I-like receptors (RLR), mammalian target of rapamycin (mTOR), 1,25-dihydroxyvitamin D3 (1,25D3), thymic stromal lymphoietin (TSLP), hepatocyte growth factor (HGF), vasoactive intestinal peptide (VIP), glucocorticoid (GC), all-trans retinoic acid (RA), prostaglandin E2 (PGE2), vascular endothelial growth factor (VEGF), programmed death-1 ligand (PDL), carbon monoxide (CO), and commensal (Comm).

the thymic environment promotes this capacity on DCs involves IL-7-related thymic stromal lymphoietin (TSLP) produced by Hassall's corpuscles in the thymic medulla (Besin *et al.*, 2008; Liu *et al.*, 2007; Mazzucchelli *et al.*, 2008; Wang and Xing, 2008; Watanabe *et al.*, 2005). By contrast, in extrathymic sites, such as the lung and skin (Ziegler and Artis, 2010), TSLP biases DCs and Tns toward a Th2 response, suggesting that other, as yet unknown, factors may contribute to tDC instruction or function in the thymus.

3.1.2. Peripheral suppressive tolerance

Oral intake of antigenic material, such as food and commensal microorganisms, efficiently generates antigen-specific systemic tolerance (Tsuji and Kosaka, 2008). Recent reviews have summarized the current knowledge of intestinal tract-associated Tregs and DCs and their role in oral tolerance (Belkaid and Oldenhove, 2008; Coombes and Powrie, 2008; Milling *et al.*, 2010; Siddiqui and Powrie, 2008). DCs within the intestinal mucosa directly sample the lumen of the intestinal tract (Chieppa *et al.*, 2006) and transport antigen to mesenteric lymph nodes (MLNs) in a CCR7-dependent manner. Here, the antigen-laden DCs promote the differentiation of Tns into Foxp3+ aTregs (Coombes *et al.*, 2007; Hultkrantz *et al.*, 2005; Miyamoto *et al.*, 2005; Zhang *et al.*, 2001). DCs from the lamina propria (LP) are also thought to induce Foxp3+ aTregs (Sun *et al.*, 2007). This tolerogenic ability of intestinal DCs is presumably controlled by the mucosal environment, which is rich in anti-inflammatory factors such as TGFβ, RA, IL-10, vasoactive intestinal peptide (VIP), TSLP, and hepatocyte growth factor (HGF). When these agents are added to iDCs *in vitro*, they promote the differentiation of itDC, which elicit more efficient Tn-to-aTreg conversion than iDCs (Table 4.3; Göke *et al.*, 1998; Grider and Rivier, 1990; Iwata, 2009; Nilsen *et al.*, 1998; Taylor *et al.*, 2009a). Intestinal tDCs with the most potent aTreg inductive capacity express CD103 (alpha-E), an integrin chain, whose expression is regulated by TGFβ signaling (Robinson *et al.*, 2001). In addition, TGFβ and RA also act directly on activated Tns and promote aTreg differentiation, even in the absence of DCs (Chen *et al.*, 2003; Mucida *et al.*, 2009; Nolting *et al.*, 2009).

Intestinal epithelial cells (IECs) are central for the local milieu that fosters tolerogenic responses by both DCs and activated T cells. Not only are IECs a rich source of TSLP, TGFβ, and RA (Dignass and Podolsky, 1993; Iliev *et al.*, 2009a,b; Rimoldi *et al.*, 2005; Shale and Ghosh, 2009) but also IEC-derived RA induces in DCs the expression of retinal dehydrogenases (RALDH). This presumably enables intestinal DCs to metabolize food-derived vitamin A to produce RA by themselves. However, RA-and/or TGFβ-conditioned splenic DCs fail to promote significant Foxp3+ aTreg differentiation *in vitro* (our unpublished results), suggesting that other instructive elements are necessary for full-fledged tDC induction in the intestine.

Like intestinal DCs, lung DCs, which capture antigens from the airways, are tasked with balancing immune responses to pathogens with those to the regular microbial flora and harmless inhaled antigens (Lambrecht and Hammad, 2009). Pulmonary DCs traffic continuously from the lungs to the draining mediastinal and peribronchial LNs, but to do so they are thought to require subtle maturation signals presumably from the local flora (Jakubzick *et al.*, 2008). Thus, DCs surveilling the airways acquire a semimature phenotype, whereby they upregulate CCR7, which enables their migration to lymph nodes (Hintzen *et al.*, 2006) and induction of aTregs that control pulmonary tolerance and homeostasis (Bakocević *et al.*, 2010; Curotto de Lafaille *et al.*, 2008; Lloyd and Hawrylowicz, 2009; Ostroukhova *et al.*, 2004). Similar to IECs, resting pulmonary stromal cells promote TGFβ-dependent differentiation of tDCs that promote the differentiation of Tregs *in vitro* (Li *et al.*, 2008). However, upon exposure to TLR ligands, lung stroma cells are critical initiators of inflammatory responses to infections by generating cytokines that instruct immunogenic sDCs (Hammad *et al.*, 2009).

In the skin, DCs function is influenced by vitamin D3, which is activated by ultraviolet radiation and then enzymatically converted to 1,25-dihydroxyvitamin D3 (1,25D3). *Ex vivo* treatment of DCs with vitamin D receptor agonists elicits Treg-inducing tDC (Adorini and Penna, 2009; Anderson *et al.*, 2008, 2009; Farquhar *et al.*, 2010; Mora *et al.*, 2008; Penna *et al.*, 2005a, 2007; Unger *et al.*, 2009; Ureta *et al.*, 2007). Of note, vitamin D signaling appears to engage an autonomous transcriptional program in DCs that is distinct and independent from the transcriptional pathways that underlie DC maturation (Griffin *et al.*, 2001; Széles *et al.*, 2009). Some DCs in skin-draining lymph nodes induce Foxp3+ aTregs through the production of RA (Guilliams *et al.*, 2010), but dermal lymph nodes contain much fewer RA-producing DCs (which are CD103−) than the intestinal tract (Iwata *et al.*, 2004).

The liver arguably provides the quintessential tolerogenic environment for T cells and DCs (Tiegs and Lohse, 2010). Thus, liver allografts typically require much less immunosuppression for long-term survival (Crispe *et al.*, 2006), and targeted expression of antigens in the liver can establish tolerance by inducing antigen-specific Foxp3+ Tregs (Cao *et al.*, 2007; Lüth *et al.*, 2008; Martino *et al.*, 2009). Although the liver is a major reservoir for RA, vitamin D3, and TSLP (Friedman *et al.*, 1992), the role of these factors in hepatic tDC function is unclear. Liver sinusoidal endothelial cells elicit tolerogenic functions in cocultured DCs *in vitro* (Schildberg *et al.*, 2008), and they are also implicated in the conversion of adoptively transferred DC precursors into hepatic tDCs *in vivo* (Xia *et al.*, 2008). Hepatic DCs can induce both T cell anergy and deletional tolerance (Goubier *et al.*, 2008).

They also regulate inflammatory processes during liver fibrosis and hepatic ischemia by producing cytokines, such as TNFα or IL-10 (Bamboat *et al.*, 2009, 2010; Connolly *et al.*, 2009; Goddard *et al.*, 2004).

In summary, while the factors implicated in DC instruction to promote Treg differentiation seem to possess organ-specific flavors, TGFβ, RA, and vitamin D3 appear to play a major role. Moreover, the balance of tDCs and sDCs in peripheral organs is the result of continuous intimate cross-talk between iDCs and their local surroundings. Stromal, epithelial, and endothelial cells are particularly well positioned to perceive homeostatic changes at body surfaces, the extracellular environment, and the blood stream. Therefore, it makes sense that these cells communicate with DCs through cytokines and direct contact and apparently contribute to the regulation of DC function and tolerance.

3.2. Induced tolerogenic DCs

A variety of inputs have been implicated in the induction of tDCs, including pathological conditions and specific molecular manipulations of iDCs or DC precursors. For example, many pathogens and tumors can mimic or produce tolerogenic factors and instruct tDCs as an immune escape mechanism. Preexisting Tregs can also educate iDCs to become tolerogenic and induce more Tregs, a phenomenon termed ''infectious tolerance.'' The tolerogenic potential of DCs has also been harnessed by modifying their biology using compounds and introducing genetic alterations.

3.2.1. Disease-induced tolerogenic DC

3.2.1.1. Pathogen-induced tolerogenic DC Certain pathogens have evolved immune escape mechanisms that exploit Tregs (Belkaid, 2007; Grainger *et al.*, 2010; Mills and McGuirk, 2004). In most cases, the contribution of tDCs to these infectious settings is still unclear, although different modalities have been described by which pathogens can modify DCs. For example, products from *Fasciola hepatica*, *Candida albicans*, *Schistosoma japonicum*, *Schistosoma mansoni*, *Bordetella pertussis*, and *Vibrio cholerae* all promote DC tolerogenicity and induce Treg differentiation (Table 4.2), but the molecular basis for their recognition and signaling remains largely unknown. One mechanism involves microbial and parasite byproducts or toxins that prompt DCs to produce anti-inflammatory cytokines, like IL-10 and TGFβ. Examples for these compounds include cyclosporin, FK506 (Tacrolimus), FK520, ISA247 (voclosporin), and rapamycin (Sirolimus), which have been harnessed as immunosuppressive drugs to treat immune disorders and transplant rejection (Cooper and Wiseman, 2010; Korom *et al.*, 2009). Cholera toxin (CTx), an exotoxin secreted by *V. cholerae*, is a multimeric complex of six protein subunits recognized and internalized by membrane-bound gangliosides. Within the cell it increases cytosolic cyclic AMP levels (Fishman and Orlandi, 2003). DC treatment with CTx B subunit (CTB) inhibits their maturation and production of IL-12 while increasing IL-10 secretion and aTreg differentiation (D'Ambrosio *et al.*, 2008; Lavelle *et al.*, 2003). Other pathogens, such as helminths, also release factors that mimic immunosuppressive molecules like TGFβ and promote itDCs, thereby staging a permissive microenvironment. Helminth infection *in vivo* is associated with increased numbers of Tregs whose depletion enhances parasite clearance (Gomez-Escobar *et al.*, 2000; Grainger *et al.*, 2010b). However, whether and how helminth-derived products act on DCs to induce Tregs has not been determined. Similarly, some viruses encode analogs of IL-10 that are produced by infected cells (Fleming *et al.*, 2000; Hsu *et al.*, 1990; Kotenko *et al.*, 2000) and attenuate DC's immunogenicity (Chang *et al.*, 2004, 2009); however, a direct effect on Treg differentiation remains to be demonstrated.

3.2.1.2. Tumor-induced tolerogenic DC Cancer cells as well as the associated tumor stroma can confer tolerogenic properties on DCs resulting in differentiation and accumulation of aTregs within the tumor mass and in the draining lymph nodes (Table 4.2; Dumitriu *et al.*, 2009; Fiore *et al.*, 2005; Gabrilovich, 2004; Ghiringhelli *et al.*, 2005; Liu *et al.*, 2005; Wei *et al.*, 2005; Zhang *et al.*, 2005). Remarkably, the presence of DCs is crucial for the vascularization of some tumors, and DC depletion can enhance the elimination of malignant cells in animal models (Fainaru *et al.*, 2008, 2010). The mechanisms by which tumors instruct DCs to become itDCs involve the production of IL-10, vascular endothelial growth factor (VEGF), prostaglandin E2, TGFβ, and other tolerogenic factors by cancerous cells (Bernabeu *et al.*, 2009; Bierie and Moses, 2010; Gabrilovich, 2004; Ikushima and Miyazono, 2010; Kelly and Morris, 2010; Yigit *et al.*, 2010).

3.2.1.3. Treg-induced tolerogenic DC Even immune challenges that induce a potent effector response can trigger concomitant differentiation of aTregs (Bilenki *et al.*, 2010; Curotto de Lafaille *et al.*, 2008; Lanteri *et al.*, 2009; Lund *et al.*, 2008). The role of these inflammation-induced aTregs remains unclear but might limit immunopathology, suppress autoaggressive responses, and/or promote restitution of tissue homeostasis (via TGFβ) or T and B cell memory generation (via IL-10). Antigen-specific Tregs, either activated nTregs that expand when exposed to cognate antigen (Fisson *et al.*, 2003) or newly converted aTregs, can spread their tolerance-promoting message to local DCs and Tns through a mechanism termed ''infectious tolerance.'' This has been elegantly demonstrated by Waldmann and colleagues who transferred CD4+ T cells from tolerized animals to new recipients which, in turn, developed tolerance. Tregs contributed directly to Tn differentiation into aTreg by producing IL-10 and TGFβ and retained this capacity during multiple transfers to successive hosts (Andersson *et al.*, 2008; Belladonna *et al.*, 2009; Jonuleit *et al.*, 2002; Mekala *et al.*, 2005; Waldmann *et al.*, 2006). Similarly, McGuirk *et al.* (2002) showed that conditioning of DCs by Tregs confers them the ability to induce Tregs in an IL-10-dependent manner, suggesting that tDCs may be key players during Treg-induced ''infectious tolerance.''

3.2.2. Experimentally induced tolerogenic DC

Given their potent activity, researchers have attempted to emulate the conditions leading to tDC differentiation and function in order to understand the underlying biology and to utilize tDCs for immune therapy (Hackstein and Thomson, 2004; Morelli and Thomson, 2003, 2007; Steinman *et al.*, 2003). Indeed, tDCs can be induced *in vitro* by (1) anti-inflammatory biologicals, (2) pharmacologic agents, and (3) genetic modification (Table 4.3). Reports on this subject are dominated by work with murine or human DCs that were differentiated *in vitro* from blood or bone marrow progenitors (Inaba *et al.*, 1992) or blood monocytes (Sallusto and Lanzavecchia, 1994), respectively.

3.2.2.1. Induction of tolerogenic DCs using biologics A number of biomolecules that are physiologically encountered in tolerogenic situations can induce tDC differentiation *in vitro* (Fig. 4.4). For example, incubation of murine splenic or bone marrow-derived DCs (BMDCs), or of human monocyte-derived DCs (huMoDC) or rat BMDC with IL-10 alone or in combination with other cytokines confers a certain capacity to induce suppressive lymphocytes, including CD4+CD25+, CD8+, and Valpha24+ invariant natural killer T (iNKT). The suppressive capacity of these cells has been extensively tested in models of allograft rejection, allergies, and xenogeneic, acute, and chronic allogeneic graft-versus-host disease (Table 4.3). Signaling through the IL-10 receptor (IL10R) maintains iDCs in their immature state even in the presence of maturation signals (Lang *et al.*, 2002; Moore *et al.*, 2001). IL10R ligation triggers janus kinases (JAK)-mediated phosphorylation of signal transducer and activator of transcription 3 (Stat3; Murray, 2006). Activated phospho-Stat3 is translocated to the nucleus, where it represses genes associated with DC maturation and immunogenicity (Moore *et al.*, 2001; Murray, 2005). A few genes are specifically induced by IL-10, including suppressor of cytokine signaling 3 (SOCS3) and signaling lymphocytic activation molecule (SLAM; Perrier *et al.*, 2004). SOCS3 negatively regulates Stat-dependent signaling of inflammatory cytokines (Croker *et al.*, 2003), particularly IL-6, which can inhibit Tregs-mediated suppression (Pasare and Medzhitov, 2003). SLAM signaling activates src homology 2 domain-containing protein tyrosine phosphatase-1 (SHP-1), which inactivates costimulatory receptors by dephosphorylating their cytoplasmic tail (Akdis and

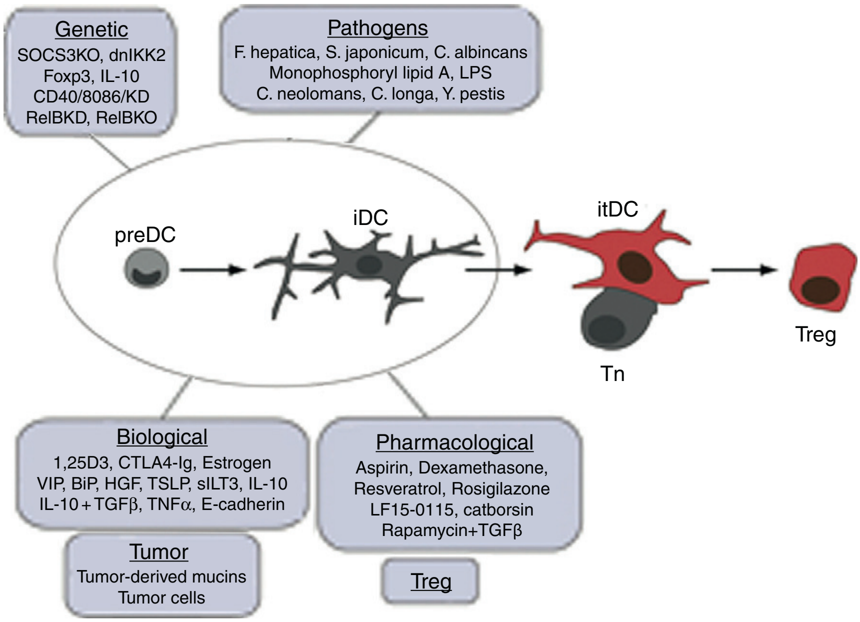

FIGURE 4.4 Induced-tolerogenic DCs. DCs progenitors (preDCs) and immature DCs (iDCs) from multiple sources are susceptible to tolerogenic instruction by multiple strategies. These cells can be used as therapeutic tools for the induction of antigen-specific tolerance.

Blaser, 2001; Veillette and Latour, 2003). More studies will be necessary to elucidate the effects of IL-10 on DCs *in vivo*.

TGFβ, a cytokine produced by Tregs and other sources in many tissues, has also profound effects on DCs *in vitro*. Using animals that express a dominant negative form of the TGFβR complex (dnTGFβR) specifically on DCs, the Flavell group has shown that the action of TGFβ allows DCs to attenuate the neuropathology associated with experimental autoimmune encephalomyelitis (EAE; Laouar *et al.*, 2008). Functional TGFβR (and TGFβ-producing Tregs; Feuerer *et al.*, 2009) is also required on NK cells to restrain their proinflammatory activity (Laouar *et al.*, 2005). Thus, the TGFβ pathway is a major mechanism by which Tregs control both NK cells and DCs. Ligation of TGFβR leads to heterodimerization of Smad2 and Smad4, which regulate gene expression in the nucleus (Miyazono, 2000; Rubtsov and Rudensky, 2007). The downstream consequences appear similar to those of IL-10 and include inhibition of DC the maturation through blockade of NFκB signaling. However, in contrast to IL-10, TGFβ signaling induces a much larger set of genes in DCs (Karlsson *et al.*, 2005). The TGFβ-induced transcriptional program in tDCs includes TGFβ production itself as well as TGFβR, CXCL14, IL-18, the transcription factors peroxisome proliferator-activated receptor γ (PPARγ) and plasminogen activator inhibitor 1 (Fainaru *et al.*, 2007; Sargent *et al.*, 2010). The specific role of each of these factors in tDC function remains to be analyzed.

Other bioderivatives instructing itDCs are HGF and the vitamin D3 metabolite, 1,25D3. When treated *in vitro* with these compounds, DCs initiate the expression of gene products that have been implicated immune tolerance, including IDO, C5R1, CCL2, IL-10, TGFβ, TRAIL, inhibin, and the inhibitory receptors CD300LF and CYP24A1 (Rutella *et al.*, 2006; Széles *et al.*, 2009). Several other factors, such as estrogen, VIP, binding immunoglobulin protein (BiP), TSLP, GM-CSF, G-CSF, IFNα/β/γ, IL-6, PGE2, and TNFα, may also promote Treg-inducing capacities on tDCs.

Antibodies and synthetic soluble ligands of specific surface receptors have also been used to produce itDCs. For example, human MoDC treated with HLA-G, a nonclassical histocompatibility molecule associated with tolerance, induced suppressive autologous T cells that expressed CD25 and CTLA4, two markers commonly found on Tregs (Liang *et al.*, 2008; Ristich *et al.*, 2005). Similarly, the antibody-mediated activation of the suppressive receptor CD200R boosts the tolerogenicity of mouse BMDCs by activating Tregs *in vivo* (Gorczynski, 2006; Gorczynski *et al.*, 2004, 2005, 2008).

3.2.2.2. Pharmacologically induced tolerogenic DCs The use of immunosuppressive drugs has been crucial for the treatment of many diseases. Not surprisingly, immunosuppressants frequently affect DC immunogenicity often by intervening with their maturation, although the specific contribution of such drug effects on DCs relative to their influence over other target cells is not known. Nevertheless, immunosuppressive compounds have been successfully employed to manipulate DC function in many disease models (Hackstein and Thomson, 2004).

Glucocorticoids (GCs) were the first immunosuppressants to be used in a clinical setting (Leung and Bloom, 2003). Treatment of human MoDC or mouse BMDC with prednisolone or dexamethasone conditions these cells for tolerogenic instruction of aTregs (Table 4.3). GC binding to the glucocorticoid receptor (GR) regulates DC activation through nuclear glucocorticoid response elements (GRE) that negatively regulate promoters for members of the canonical NFκB pathway, inflammatory cytokines, chemokines, their receptors, and antigen presentation molecules (Leung and Bloom, 2003). In addition to repressing DC maturation, dexamethasone also induces a discrete set of anti-inflammatory gene products and chemoattractants, including IL-10, GITRL, IDO, CCL2 (MCP-1), CCL8 (MCP-2), CCR2, CCL9 (MIP-1c), and CCLl2 (MIP-2) (Grohmann *et al.*, 2007; Roca *et al.*, 2007). This impairs the DCs' ability to migrate and provokes them to assume a tolerogenic phenotype capable of instructing Tns to express CD25, Foxp3, and IL-10.

Many maturation signals for DCs induce phosphorylation and proteolysis of the inhibitor of NFκBα (IκBα) by the inhibitor kinase-β (IKKβ), thereby releasing Rel-A (or p65; a subunit of NFκB) for nuclear translocation. In contrast, the noncanonical pathway operational during tolerogenic instruction activates NFκB-inducing kinase (NIK) and IKKα resulting in the formation of Rel-B dimers (Bonizzi and Karin, 2004; Puccetti and Grohmann, 2007). The inhibitory effect of GCs on the canonical NFκB pathway likely plays a key role in the conversion of DCs to itDCs. Accordingly, inhibition of NFκB or IKKβ by small molecule antagonists produces itDCs with the capacity to stimulate Foxp3+CD25+ aTregs that alleviate disease symptoms in EAE, heart allograft rejection, and intestinal bowel disease (IBD; Buckland and Lombardi, 2009; Buckland *et al.*, 2006a,b; Cong *et al.*, 2009; Iruretagoyena *et al.*, 2006; Zhang *et al.*, 2008).

Recent observations suggest that cellular metabolism also plays a role in DC immunogenicity. For example, treatment of human MoDCs with resveratrol induces tDCs that stimulate IL-10-secreting aTregs (Kim *et al.*, 2004; Svajger *et al.*, 2010). Resveratrol activates sirtuin 1 (SIRT-1) and PPARγ coactivator (PGC)-1α, which are involved in energy metabolism (Pervaiz and Holme, 2009). Another pathway affecting metabolism and DC immunogenicity is represented by the serine/threonine kinase mammalian target of rapamycin (mTOR). This kinase forms signaling complexes that sense oxygen supply, free amino acids, ATP levels, growth factors, cytokines, and cellular stress (Hay, 2004). Inhibition of mTOR by rapamycin, a macrolide from *Streptomyces hygroscopicus*, exerts immunosuppressive effects in humans and animals (Augustine *et al.*, 2007) and has shown efficacy in both clinical and preclinical settings of autoimmunity and inflammatory disease (Battaglia *et al.*, 2006; Esposito *et al.*, 2010; Fu *et al.*, 2010; Ge *et al.*, 2009; Massey *et al.*, 2008; Monti *et al.*, 2008; Raimondi *et al.*, 2010; Valle *et al.*, 2009; Zang *et al.*, 2008). Treatment of DCs with rapamycin stimulates Treg expansion *in vivo* and *in vitro* (Battaglia *et al.*, 2005; Horibe *et al.*, 2008; Ohtani *et al.*, 2008; Thomson *et al.*, 2009; Turnquist *et al.*, 2007). We will further discuss this subject in Section 4.3 below.

3.2.2.3. Genetically induced tolerogenic DCs Various genetic manipulations have been used, including gene knock-out, knock-down, and transgenic overexpression of active or dominant negative mutants of molecules involved in DC maturation to enhance or inhibit DC tolerogenicity (Morelli and Thomson, 2007). Genetically induced tDCs can induce hyporesponsiveness and prolong allograft survival when transferred to transplant recipients, but a mechanistic role for tDC-induced Treg differentiation has only been established in a few cases. For instance, RelB deficient DCs induce CD40+ Tregs that suppressed delayed-type hypersensitivity (DTH) and experimental autoimmune myasthenia gravis (EAMG; Martin *et al.*, 2003; Yang *et al.*, 2010; Zhang *et al.*, 2009a). This provides yet another example for the importance of NFκB (and presumably CD40) activation in a DC's decision on whether to exert immunogenic or Treg-inducing effects. Similarly, BMDCs that overexpressed dominant negative IKKβ were refractory to maturation and prone to induce Tregs that enhanced kidney allograft survival (Tomasoni *et al.*, 2005). Another approach to target NFκB-dependent effects in maturing DCs is to eliminate the expression of downstream target genes. Silencing of IL-12, CD80, CD86, and/or CD40 results in DCs that stimulate Treg differentiation and alleviates disease symptoms in collagen-induced arthritis (CIA) and EAMG (Martin *et al.*, 2003; Zheng *et al.*, 2010).

An alternative approach to silencing immunogenic molecules is the forced expression of tolerogenic factors. For example, treatment with IL-10-transduced DCs prevents the development of experimental asthma (EA) by boosting CD4+CD25+Foxp3+, IL-10 secreting Tregs that effectively transfer tolerance to naïve animals. IL-10 produced by recipient cells is required to establish this infectious tolerance demonstrating that Tregs require other supporting cell populations to suppress immune responses (Henry *et al.*, 2008). Remarkably, transduction of DCs with ectopic Foxp3 also results in itDCs that stimulate CD4+Foxp3+ aTregs (Lipscomb *et al.*, 2010). The mechanism by which Foxp3 controls the tolerogenic potential of DCs remains unknown but likely involves pathways similar to those that induce Tregs (Kim and Rudensky, 2006).

4. HOW ARE TDCS INDUCING TREGS?

tDCs can induce Tregs by several different pathways that may act either alone or in combination. As discussed above (Section 2.2), a relatively simple Treg-promoting condition involves presentation of modest levels of a cognate antigen in the absence of signals 2 and 3, which is thought to be employed by iDCs but probably applies also to tDCs (Fig. 4.2). In addition, tDCs can produce anti-inflammatory molecules that may be secreted, membrane bound, or both. Such signals may act directly on T cells and/or modify environmental conditions, such as the metabolic state of a tissue to fine-tune T cell differentiation.

4.1. Influence of the maturation status of DC in the induction of Tregs

Studies by several laboratories have shown that presentation of very low levels of antigen in the absence of other stimuli promotes Treg differentiation *in vitro* and *in vivo* (Apostolou and von Boehmer, 2004; Hermann-

Kleiter and Baier, 2010; Kretschmer *et al.*, 2005, 2006; Picca *et al.*, 2006). Another key factor for efficient differentiation of aTregs and function of nTregs is a milieu containing little or no inflammatory cytokines, such as IL-6 and IL-12, or costimulatory membrane receptors (CD80/86/40), which counteract the tolerogenic effect of iDCs and enhance effector differentiation of Tns (King and Segal, 2005; Pandiyan *et al.*, 2007; Pasare and Medzhitov, 2003). TCR signals in conjunction with costimulation precipitate a signaling cascade resulting in intracellular calcium (Ca^2+) flux and the activation of the transcription factors nuclear factor of activated T cells (NFAT), activator protein 1 (AP-1), and NFκB that coordinate gene expression in nascent Teffs (Hogan *et al.*, 2010). While activated T cells that acquire effector functions express IL-2, IL-4, IL-17, T-bet, Edg3, and CD69 among others (Fontenot *et al.*, 2005), differentiating Tregs present a different transcriptional signature (Feuerer *et al.*, 2010; Fontenot *et al.*, 2005; Hill *et al.*, 2007; Sadlon *et al.*, 2010) driven by NFAT, Foxp3, and runt-related transcription factor 1 (Runx-1 or myeloid leukemia factor, AML1; Hermann-Kleiter and Baier, 2010, Hu *et al.*, 2007; Sakaguchi *et al.*, 2008). Indeed, the Treg transcriptome is enriched with gene products implicated in their suppressive function like IL-10, CD103, Killer cell lectin-like receptor subfamily G member 1 (Klrg1), neuropilin 1 (Nrp1), GITR, ICOS (CD278), fibrinogen-like protein 2 (Fgl2), probable G-protein coupled receptor 83 (Gpr83), and CTLA-4. However, it is still unclear, how exactly iDCs or tDCs skew the TCR signaling cascade in Tns to accomplish the subsequent selection of Treg-associated transcription factors. Furthermore, as discussed above, some mature and semimature DC expressing high levels of costimulatory molecules can also induce suppressive function on T cells (Reis and Sousa, 2006). Thus, the magnitude of antigen presentation/costimulation or activating cytokines alone cannot fully explain the function of all tDCs subsets.

4.2. Tolerogenic factors produced by tDC

The presence of IL-10 has been identified in numerous settings of tolerance (Tables 4.1–4.3). Indeed, secretion of IL-10 by tDCs is necessary for tolerance in a variety of models of Treg differentiation (Akbari *et al.*, 2001; McGuirk *et al.*, 2002; Wakkach *et al.*, 2003). IL-10 can initiate a powerful anti-inflammatory positive feedback loop because it can both modify and be produced by leukocytes and structural cells within tissues (e.g., IECs, AECs, and LSECs). Thus, when tDCs are induced by IL-10 in peripheral tissues, they acquire the ability to secrete IL-10 themselves and migrate to lymphoid organs, where tDC-derived IL-10 then contributes to Treg differentiation and proliferation. Having been instructed by tDCs, the activated Tregs enter the blood stream and home to the peripheral organ, where antigen recognition triggers their production of even more

IL-10 (Scott-Browne *et al.*, 2007; Shafiani *et al.*, 2010; Sharma *et al.*, 2009; Zhang *et al.*, 2009b). In the presence of this cytokine proliferation, cytokine production and migratory capacities of effector T cells are impaired (Moore *et al.*, 2001). Mechanistically, the Akdis and Blaser groups have shown that ligation of IL10R overrides costimulatory signaling via activation of SHP-1, which dephosphorylates the cytoplasmic tails of CD28, ICOS, and CD2, thus inhibiting the recruitment of phosphatidylinositol-3-kinase (PI3K; Akdis and Blaser, 2001; Akdis *et al.*, 2000, 2001; Taylor *et al.*, 2007, 2009b). Additionally, IL-10 signaling is also required for the stabilization of the suppressive phenotype of Tregs in the face of strong inflammatory signals (Murai *et al.*, 2009).

TGFβ is unique among cytokines in that it can induce Foxp3 expression and aTreg differentiation in the absence of DCs (Chen *et al.*, 2003). However, it is not clear whether and to what extent the tolerogenic capacity of tDCs relies on TGFβ production. Exploring this question is complicated by the fact that TGFβ effects are highly pleiotropic, and genetic mutants present complex phenotypes with multiple immune disorders and poor survival (Rubtsov and Rudensky, 2007). A strong argument for the importance of TGFβ production by tDCs has come from animals with a DC-restricted deletion of the TGFβ-activating integrin, $\alpha_v\beta_8$. These mutant mice develop autoimmunity similar to animals in which DCs are chronically depleted or TGFβR signaling is dysfunctional in T cells, suggesting that DCs are important to ensure the bioavailability of active TGFβ (Birnberg *et al.*, 2008; Gorelik and Flavell, 2000; Kim *et al.*, 2006; Ohnmacht *et al.*, 2009; Travis *et al.*, 2007). Antigen presentation by DCs in the presence of TGFβ results in the differentiation of Foxp3+ aTregs (Yamazaki *et al.*, 2008), which present a transcriptional signature that is similar to, but distinct from that of nTregs (Chen and Konkel, 2010; Feuerer *et al.*, 2010; Rubtsov and Rudensky, 2007). A recent study has shown that activation of Foxo3a and Foxo1 by TGFβ signaling precedes Foxp3 expression in aTregs (Harada *et al.*, 2010). However, we are only beginning to understand how Treg differentiation is controlled upstream of Foxp3.

Some DCs can synthesize RA, a metabolite of vitamin A that is generated by RALDH. Most intestinal DCs express at least one of the three isoforms of this enzyme, while most DCs in other lymphoid tissues express little or no RALDH (Iwata *et al.*, 2004). When T or B cells are activated in the presence of DC-derived RA, they are ''imprinted'' to express gut homing receptors (Iwata *et al.*, 2004; Mora *et al.*, 2006). In addition, exposure of activated CD4 T cells to RA promotes their differentiation into Foxp3+ aTregs (Belkaid and Oldenhove, 2008; Benson *et al.*, 2007; Hill *et al.*, 2008; Mora *et al.*, 2008; Mucida *et al.*, 2009; Nolting *et al.*, 2009; Siddiqui and Powrie, 2008; von Boehmer, 2007). RA binds the nuclear RA receptor α (RARα) and regulates the expression of Foxp3 and Smad3 in T cells (Nolting *et al.*, 2009; Takaki *et al.*, 2008), but whether RARα is necessary for differentiation of Tregs *in vivo* is unclear. It has been suggested that RA is particularly relevant in aTreg differentiation in mucosal environments because the continuous exposure to commensal antigens requires a fine balance between tolerance and immunity (Manicassamy and Pulendran, 2009). Recent observations suggest that some DCs in the skin also express RALDH and may produce RA for dermal Treg differentiation (Guilliams *et al.*, 2010). More experimentation will be necessary to evaluate the exact role of RA-producing DCs for tolerance versus immunity *in vivo*.

tDCs also express several membrane receptors that may instruct antigen-specific Tns during their activation. Among these are the immunoglobulin-like transcript (ILT) receptors, which are found on tDCs that stimulate Treg differentiation (Gregori *et al.*, 2009, 2010; Vlad *et al.*, 2010). The proximal signaling cascade for ILTs is not known and the impact of ILT recognition by T cells is also not well established. However, multiple groups have shown an important role for these molecules in cancer, transplantation, and autoimmunity by using animals deficient for the expression of ILTs, blocking antibodies, and recombinant ILT3 (Vlad *et al.*, 2009, 2010; Wu and Horuzsko, 2009). DCs also express programmed death-1 ligands (PD-Ls), PD-L1 and PD-L-2, which control T cell activation through engagement of PD-1 and CD80 (in case of PD-L1) (Keir *et al.*, 2007). PD-1 is a critical determinant of ''exhausted'' T cells that arise during chronic viral infections, and it also contributes to Treg differentiation (Francisco *et al.*, 2009; Keir *et al.*, 2007; Riley, 2009; Wang *et al.*, 2008). The effects of PD-1 signaling resemble those of the IL10R by limiting PI3K activation and shutting down costimulatory signaling through SHP-1. However, PD-1 is not thought to be expressed by Tns, but is only upregulated during activation, so its role (if any) in the initial phase of Treg education is uncertain.

4.3. DCs and metabolism

Immune responses precipitate dramatic changes in the metabolic state of many cells. Changes in intra- and extracellular metabolites are becoming increasingly recognized as integral part of the ''information content'' of tissues in which immune responses are induced. For example, differentiation of inflammatory cells and the induction of T cell memory *in vivo* can be modified by the dietary abundance of amino acid and fatty acid metabolism (Pearce, 2010; Pearce *et al.*, 2009; Sundrud *et al.*, 2009). DCs also modulate T cell differentiation by modifying metabolic parameters surrounding T cells. DCs can release IDO and heme oxygenase-1 (HO-1) to control the abundance of environmental tryptophan and carbon monoxide (CO), respectively. In the presence of extracellular IDO, T cells proliferation is compromised and aTregs differentiation is enhanced, although the precise molecular basis for this effect is unclear (Belladonna *et al.*, 2009; Curti *et al.*, 2009; Katz *et al.*, 2008; Löb and Königsrainer, 2009; Mellor and Munn, 2004). IDO expression by DCs is induced by IFNγ and TGFβ suggesting that this enzyme may represent a feedback mechanism by which DCs modulate their own immunogenicity during inflammation (Jurgens *et al.*, 2009; Orabona *et al.*, 2006). HO-1 degrades heme, thereby producing CO, which inhibits DC immunogenicity (Rémy *et al.*, 2009). Indeed, HO-1 has a potent anti-inflammatory effect that may be mediated through Treg activity (Chora *et al.*, 2007; Yamashita *et al.*, 2006), but the mechanisms are still incompletely understood.

The serine/threonine kinase mTOR plays a pivotal role in DC immunogenicity and the control Treg differentiation. Activation of TLR signaling stimulates mTOR and promotes sDC function (Cao *et al.*, 2008; Schmitz *et al.*, 2008), whereas blockade of mTOR activity by hypoxia, amino acid starvation, or rapamycin enhances Tregs (Ben-Shoshan *et al.*, 2008; Cobbold *et al.*, 2009; Haxhinasto *et al.*, 2008; Sauer *et al.*, 2008; Thomson *et al.*, 2009). mTOR is involved in the regulation of numerous essential cellular processes, such as cell cycle progression, protein synthesis, lipid metabolism, and mitochondrial biogenesis (Delgoffe and Powell, 2009; Laplante and Sabatini, 2009; Thomson *et al.*, 2009). Treatment of DCs with the mTOR inhibitor rapamycin interferes with antigen processing and presentation, partly by regulating autophagy and production of MHC complexes, and also alters the response to cytokines, chemokines, growth factors, and TLRs agonists (Thomson *et al.*, 2009). It has been reported that rapamycin-treated DCs do not directly induce aTreg differentiation (Turnquist *et al.*, 2007); however, DC exposure to a combination of rapamycin and TGFβ effectively potentiates the capacity of DCs to induce aTreg differentiation (our unpublished results). It will be important to assess whether and how maturation and differentiation signal alter the metabolic state (e.g., oxidative vs. glycolytic) of iDCs that give rise to either sDCs or tDCs, and how such metabolic changes may be linked to the phenotypic and functional characteristics of these versatile cells.

5. CONCLUDING REMARKS

It is becoming increasingly clear that both mature and immature DC subsets can support immunological tolerance through Tregs and other mechanisms. A variety of environmental cues that may arise naturally or by pharmacological or experimental intervention can coerce iDCs to acquire a stable tolerogenic disposition that is preserved, even in the face of concomitant maturation signals. These tDCs can induce or enhance the suppressive function of existing Tregs and convert activated Tns into aTregs. At present, we have only rudimentary knowledge of the rules that govern tolerogenic versus immunogenic functions of DCs, and the signals that tDCs use to transmit their suppressive message to T cells are also still incompletely understood. A better understanding of these issues may offer new opportunities for the treatment of autoimmunity, allograft rejection, allergy, asthma, and various forms of hypersensitivity. Therapeutic applications of tDCs, either by cellular therapy or by targeting of endogenous DCs with novel drugs, could accomplish effects that elude traditional strategies for immune suppression. Specifically, while systemic immunosuppressants exert broadly paralyzing effects on immune cells, tDCs can induce tolerance to the specific antigens that elicit pathologic immune responses in a patient without compromising the immune defense against pathogens or tumors. While the prospect of clinical translation is exciting and seems almost within reach, substantial gaps in our knowledge remain to be filled before we will be able to exploit the full potential of tDC-based therapy.

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