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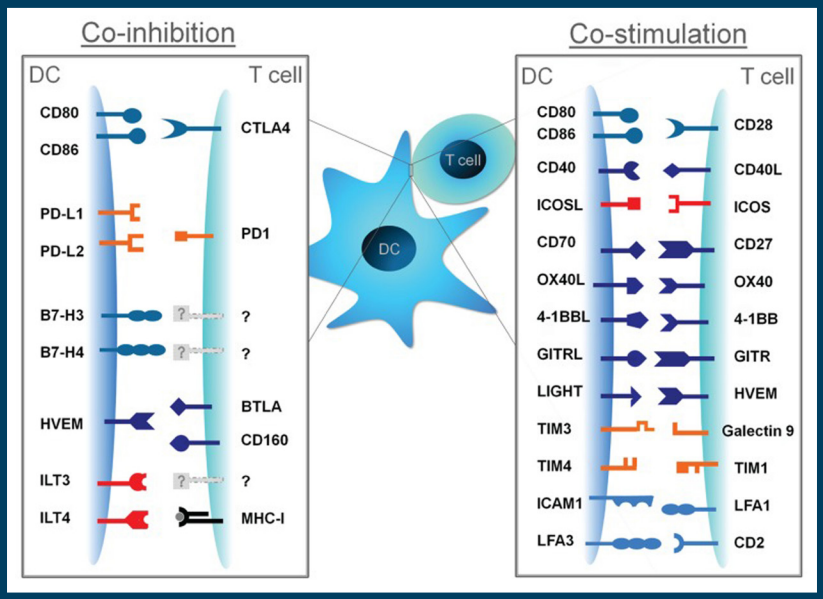

THE NATURE OF ACTIVATORY AND TOLEROGENIC DENDRITIC CELL-DERIVED SIGNAL 2

Topic Editors
Francesca Granucci, Ivan Zanoni
and Manfred B. Lutz

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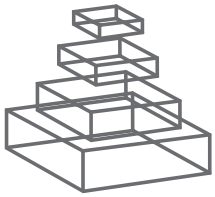

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THE NATURE OF ACTIVATORY AND TOLEROGENIC DENDRITIC CELL-DERIVED SIGNAL 2

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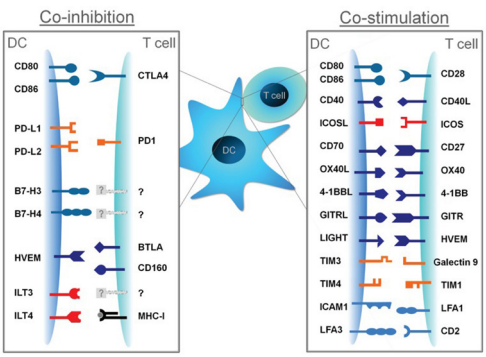

Co-stimulatory and co-inhibitory molecules and their cognate ligands. DC-derived signal II can promote T cell activation when conveyed by co-stimulatory molecules, or can attenuate T cell responses when conveyed by co-inhibitory molecules.

One of the most interesting issues in immunology is how the innate and adaptive branches of the immune system cooperate in vertebrate organisms to respond and destroy invading microorganisms without destroying self-tissues. More than 20 years ago, Charles Janeway proposed the innate immune recognition theory [1]. He hypothesized the existence of innate receptors (Pattern recognition receptors, PRRs) that, by recognizing molecular structures associated to pathogens (PAMPs) and being expressed by antigen presenting cells (APCs) and epithelial cells, could alert the immune system to the presence of a pathogen, making it possible to mount an immediate inflammatory response. Moreover, by transducing the alert signal in professional APCs and inducing the expression of costimulatory molecules, these receptors could control the activation of lymphocytes bearing clonal antigen-specific receptors, thereby promoting appropriate adaptive immune responses. Since adaptive immunity can be activated also following sterile inflammatory conditions, it was subsequently proposed by Polly Matzinger that the innate immune system could be also activated by endogenous danger signals, generically called danger associated molecular patterns (DAMPs) [2]. The first prediction has been amply confirmed by the discovery of Toll-like receptors [3; 4; 5] and cytoplasmic PRRs such as RIG-like receptors [6]. Other PRR families such as the NOD-like receptors and C-type lectins exert immunogenic or tolerogenic signals [7; 8; 9] and may recognize not strictly

pathogens but also endogenous danger signals that may lead to inflammasome activation [10; 11] .

Dendritic cells (DCs) have been identified as the cells of the innate immune system that, by sensing PAMPs or DAMPs transduce signals to the nucleus. This leads to a transcriptional reprogramming of DCs with the consequent expression of three signals, namely signal 1 (MHC+peptide), signal 2 (surface costimulatory molecules) and signal 3 (cytokines) necessary for the priming of antigen-specific naïve T cell responses (signal 1 and 2) and T cell polarization (signal 3). The reason why DCs are superior with respect to other professional APCs in naïve T cell activation has not been unequivocally defined but in vivo may mainly result from their migration capacity to secondary lymphoid organs. It has not been established whether DCs can provide a special "signal 2" or simply very high levels, compared with other APCs, of commonly expressed signals 1 and 2, so that a naïve T cell could reach the threshold of activation.

A second aspect of DC biology needs also to be taken into account. Concerning the question of how self-tissues are not destroyed following the initiation of adaptive immune responses, different mechanisms of central and peripheral auto-reactive T cell tolerization have been proposed [12]. In particular, it has been defined that high affinity T cells are deleted in the thymus, while low affinity auto-reactive T cells or T cells specific for tissue-sequestered antigens that do not have access to the thymus are controlled in the periphery. In a simplified vision of how peripheral T cell tolerance could be induced and maintained, it was thought that, in resting conditions, immature DCs, expressing low levels of signal 1 and low or no levels of signal 2, were able to induce T cell unresponsiveness. Nevertheless, it is now clear that a fundamental contribution to the peripheral tolerance is due to the conversion of naïve T cells into peripheral regulatory T cells (pTreg cells) and it is also clear that DCs need to receive a specific conditioning to become able to induce pTreg cell differentiation. Even more intriguing is that also DCs activated through PRRs, with particular Toll like receptor (TLR) agonists, are capable of generating pTreg cell conversion if these agonists induce the production of the appropriate cytokines. Thus, what is emerging is that immature DCs are not able to induce tolerance by default but need to receive specific signals in order to acquire the ability to transfer to T cells a tolerogenic, rather than an activatory, signal 2.

Given these premises, this Research Topic covers the following topics:
1. The responses induced specifically in DCs by PAMPs and DAMPs and the consequences of these responses.
2. The DAMP and PAMP receptors expressed by different DC subsets and the consequences in the activation of adaptive immune responses.
3. How DCs induce and maintain peripheral T cell tolerance and the stimuli that confer tolerogenicity.

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The nature of activatory and tolerogenic dendritic cell-derived signal 2

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Dendritic cells (DCs) were first described by Steinman and Cohn in 1973 (1) as cells provided of efficient antigen-presenting capacity. Steinmann received the Nobel Prize in 2011 for having revealed the pivotal role of DCs in linking innate and adaptive immunity and initiating antigen-specific immunity (2). The work conducted by Steinman was also instrumental in determining the role of DCs not only in activating adaptive immunity but also in controlling adaptive anti-self reactions by inducing and maintaining self-tolerance both at central and peripheral level (3).

Dendritic cells are highly heterogeneous. Two major classes of DCs have been described: classical or conventional and plasmacytoid DCs (pDCs). Classical DCs are cells of myeloid origin capable of efficiently capturing microbial antigens, and, after homing to lymph nodes and antigen processing, present these antigens to prime naive T cells. Diversely, pDCs are poorly phagocytic but recognize viruses very well. Upon encounter of microbial stimuli, they also differentiate into efficient antigen-presenting cells and express high levels of type I interferons (IFNs) (4). Based on tissue localization, transcription factor, and surface markers expression, classical DCs have been subdivided in a continuously growing number of subtypes (5, 6). Subsets expressing CD4/CD11b or CD8α/CD103 resides in secondary lymphoid organs; under healthy conditions lymph nodes contain additional subsets of partially matured steady state migratory DCs that transport self-antigens into lymph nodes for tolerance induction (7–10).

The DC capacity of inducing both immunity and tolerance may seem a contradictory aspect of DC biology, nevertheless the acquisition of these two different properties may depend on stimuli DC are exposed to or may be a specific feature of different DC subsets.

Dendritic cells sense the presence of exogenous microbial signals through germline-encoded pattern-recognition receptors (PRRs), which recognize molecular patterns expressed by various microorganisms or endogenous danger signals. The consequences of the activation of these receptors on DCs have been implicated in the acquisition of the immunogenic functions characterized by the increase of antigen presentation and costimulation as well as the release of proinflammatory cytokines. The attainment of the tolerogenic function by DCs seems instead to be more linked to the exposure to endogenous factors sensed in peripheral tissues under steady state conditions.

In the present Research topic, contributing articles describe a number of aspects determining the tolerogenic or immunogenic functions of DCs. In particular, the themes that will be discussed concern the role of DCs in controlling the threshold of activation of T cells; the role of the diverse costimulatory molecules, either secreted by DC subsets or expressed on the cell surface, in determining the DC immunogenic or tolerogenic functions; the role of endogenous or exogenous stimuli in influencing the DC functional state; some specific roles of DCs in preventing particular organ autoimmunity and, finally, possible therapeutic potentials of immunogenic or tolerogenic DCs.

Understanding the mechanisms that regulate the functional properties of DCs will allow exploiting these cells in new effective therapeutic strategies, including cancer immunotherapy and control of autoimmunity, to improve intervention outcomes. Understanding DC biology and their responses to activating stimuli will also help the identification of novel adjuvants to be used in new vaccine formulations.

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The dendritic cell response to classic, emerging, and homeostatic danger signals. Implications for autoimmunity

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Dendritic cells (DCs) initiate and control immune responses, participate in the maintenance of immunological tolerance and are pivotal players in the pathogenesis of autoimmunity. In patients with autoimmune disease and in experimental animal models of autoimmunity, DCs show abnormalities in both numbers and activation state, expressing immunogenic levels of costimulatory molecules and pro-inflammatory cytokines. Exogenous and endogenous danger signals activate DCs to stimulate the immune response. Classic endogenous danger signals are released, activated, or secreted by host cells and tissues experiencing stress, damage, and non-physiologic cell death; and are therefore referred to as damage-associated molecular patterns (DAMPs). Some DAMPs are released from cells, where they are normally sequestered, during necrosis (e.g., heat shock proteins, uric acid, ATP, HMGB1, mitochondria-derived molecules). Others are actively secreted, like Type I Interferons. Here we discuss important DAMPs in the context of autoimmunity. For some, there is a clear pathogenic link (e.g., nucleic acids and lupus). For others, there is less evidence. Additionally, we explore emerging danger signals. These include inorganic materials and man-made technologies (e.g., nanomaterials) developed as novel therapeutic approaches. Some nanomaterials can activate DCs and may trigger unintended inflammatory responses. Finally, we will review "homeostatic danger signals," danger signals that do not derive directly from pathogens or dying cells but are associated with perturbations of tissue/cell homeostasis and may signal pathological stress. These signals, like acidosis, hypoxia, and changes in osmolarity, also play a role in inflammation and autoimmunity.

Keywords: dendritic cells, autoimmunity, DAMPs, mitochondria, nanomaterial, acidosis, hypoxia, osmolarity

INTRODUCTION

Dendritic cells (DCs) are specialized antigen-presenting cells (APCs) that help maintain peripheral tolerance and orchestrate the adaptive immune response by presenting both endogenous and exogenous antigens and producing immune modulatory factors, such as costimulatory/inhibitory molecules and cytokines (Banchereau et al., 2000; Hammer and Ma, 2013). DCs reside in virtually all the tissues of our body in a predominantly antigen-capturing state and maintain immunologic tolerance by routinely migrating to the draining lymph nodes and presenting self-antigens to lymphocytes in a tolerogenic manner (Steinman et al., 2003; Reis e Sousa, 2006). Upon stimulation, DCs undergo a process of maturation/activation, which allows them to present in an immunogenic fashion the antigens captured in the periphery and initiate immune responses (Cella et al., 1997; Blander and Medzhitov, 2004). Danger signals are molecules released during infections (Janeway, 1989; Medzhitov and Janeway, 1997b) and/or tissue damage and cellular stress (Matzinger, 1994, 1998, 2002; Gallucci and Matzinger, 2001; Land and Messmer, 2012). Danger signals activate DCs and stimulate both the innate and adaptive immune response (**Box 1**).

The recognition of tissue and cell damage – which may involve several types of innate immune cells beside DCs and cascades such as the complement – has been proposed to initiate multiple processes (Bianchi, 2007), including: (a) the development of inflammation, (b) the induction of innate effector functions leading to pathogen clearance, (c) the stimulation of adaptive immunity, which generates immunological memory for future encounters with the same antigen, and (d) the stimulation of tissue repair processes, necessary to reconstitute tissue integrity compromised by the infection or, in some cases, by the immune response itself (Stoecklein et al., 2012) (**Figure 1**).

Dendritic cells collaborate with other cells of the innate immune system to initiate what is often called "sterile inflammation and immunity;" that is, the activation of the innate and adaptive immune response in the absence of pathogens (Rock and Kono, 2008). Mechanisms of sterile inflammation are key to our understanding of clinical situations such as transplant rejection, tumor immunity, chronic inflammatory diseases, trauma-induced systemic inflammatory response syndrome (SIRS), and other inflammatory conditions like ischemia-reperfusion injury and atherosclerosis – in which immune responses develop when no overt infection is present. Autoimmune diseases, in which the adaptive immune system mounts an immune response against self-antigens, are also an important class of pathologies in which

Box 1 | Terms and definitions.

In order to help our readers, here we define the terms we use throughout this review:

Pathogen-associated molecular pattern: molecular structures common to bacteria, viruses, or other microorganism, like LPS, flagellin, and peptidoglycan, that are able to activate DCs. These molecules are recognized by pattern recognition receptors (PRRs) expressed on both immune and non-immune cells. Since PAMPs are also expressed by the normal flora of mammalian mucosas, they are also called Microbial Associate Molecular Patterns (MAMPs).

Damage-associated molecular pattern: endogenous molecular structures that are normally contained within the cell interior and hidden from the immune system, and are liberated upon tissue damage. Examples include ATP, HSPs, and HMGB1. These molecules are recognized by a number of receptors, including PRRs, and are capable of inducing inflammation and immune responses in the absence of infection.

Danger signal: a danger signal may be any substance or event that is able to activate DCs and therefore initiate immune responses. For the purposes of this review, we have split the danger signals into three categories shown below. They all have in common an association with cellular stress and the ability to activate DCs.

Classic danger signals: we use the term "classic" to refer to danger signals that are not emerging or homeostatic (defined below). The classic danger signals are molecular species either associated with pathogens (PAMPs) or directly derived from tissue injury damage-associated molecular patterns (DAMPs) as described above, or secreted by activated immune cells as amplifiers of the immune activation.

Emerging danger signals: we use the term "emerging" to turn attention to new fields of research and technology that may pose new challenges to the immune system. Specifically, emerging danger signals include inorganic materials and man-made technologies (e.g., nanomaterials) often used as novel therapeutic strategies. These materials have the potential to activate DCs directly or indirectly by inducing tissue damage and release of DAMPs.

Homeostatic danger signals: with the term "homeostatic" we aim to highlight a less appreciated category of endogenous danger signals. Homeostatic danger signals generally represent perturbations in tissue steady state often as a result of inflammation. These perturbations include (but are not limited to) hypoxia, changes in acidity, or osmolarity, and metabolic stress.

the role of infectious triggers for immunity is still controversial (Mills, 2011; Stranger and De Jager, 2012). The aim of this review is to analyze the danger signals that modulate DC functions and explore how those danger signals may contribute to the pathogenesis of autoimmunity.

DENDRITIC CELLS IN AUTOIMMUNITY

The DC lineage comprises a complex population of several subsets – with recent advances demonstrating intricacies of gene transcription (Miller et al., 2012) and tissue localization (Gerner et al., 2012) that define each population. Indeed, DC subsets vary greatly in terms of lineage markers, cytokine production, and ability to stimulate different immune responses, largely depending on the tissue in which they reside or emigrate from [i.e., skin (Ginhoux et al., 2012) or gut (Rescigno, 2010)]. For simplicity, DCs are categorized as conventional DCs, strong APCs, or plasmacytoid DCs, the major producers of Type I Interferons (IFNs). DCs are either resident within the secondary lymphoid organs like lymph nodes and spleen, or migratory, coming into the lymph nodes from the peripheral tissues (Hammer and Ma, 2013).

The importance of DCs in the pathogenesis of autoimmune diseases is supported by the effects of their manipulation in experimental animal models. Indeed, the constitutive genetic depletion of DCs induces spontaneous development of autoimmunity characterized by autoantibodies against nuclear and tissue-specific antigens, multi-organ lymphocyte infiltration, and severe tissue damage, especially in the intestine (Ohnmacht et al., 2009). Therefore, DCs may be necessary for the establishment and maintenance of immunological self-tolerance (Reis e Sousa, 2006). Paradoxically, DCs also seem to be powerful inducers of autoimmunity. Mice in which DCs accumulate as a result of a DC specific defect in apoptosis also develop chronic lymphocyte activation and lupus-like systemic autoimmunity (Chen et al., 2006). Consequently, uncontrolled DC functions are sufficient to prime for autoimmune reactions. In many experimental models of autoimmunity, DCs accumulate in the secondary lymphoid organs and in the tissues targeted by the autoimmune process (Rosmalen et al., 2000; Kalled et al., 2001; Adachi et al., 2002; Colonna et al., 2006), although the causative mechanism for this accumulation remains unclear. Further support comes from studies demonstrating that the uptake of autoantibody-opsonized apoptotic cells increases DC presentation of self Ags in an inflammatory context (Frisoni et al., 2005). Moreover, *in vivo* priming with immunogenic (highly activated), self-antigen-loaded DCs induces, or accelerates autoimmunity (Bondanza et al., 2003; Eriksson et al., 2003), while the administration of tolerogenic DCs reduces disease and has been proposed as a potential therapeutic strategy in many models of autoimmune disease, such as type I diabetes (Feili-Hariri et al., 2002) and experimental autoimmune encephalomyelitis (EAE) (Menges et al., 2002; Toscano et al., 2010).

Dendritic cells may play a pathogenic role in autoimmunity by presenting self-antigens to T cells in an immunogenic fashion and by collaborating in the activation of autoreactive B cells. To do so, DCs have to be activated and express immunogenic costimulatory molecules and pro-inflammatory cytokines. Indeed, much evidence shows abnormally activated DC phenotypes in patients with different autoimmune diseases, as well as in murine models of autoimmunity (reviewed in Amodio and Gregori, 2012). In

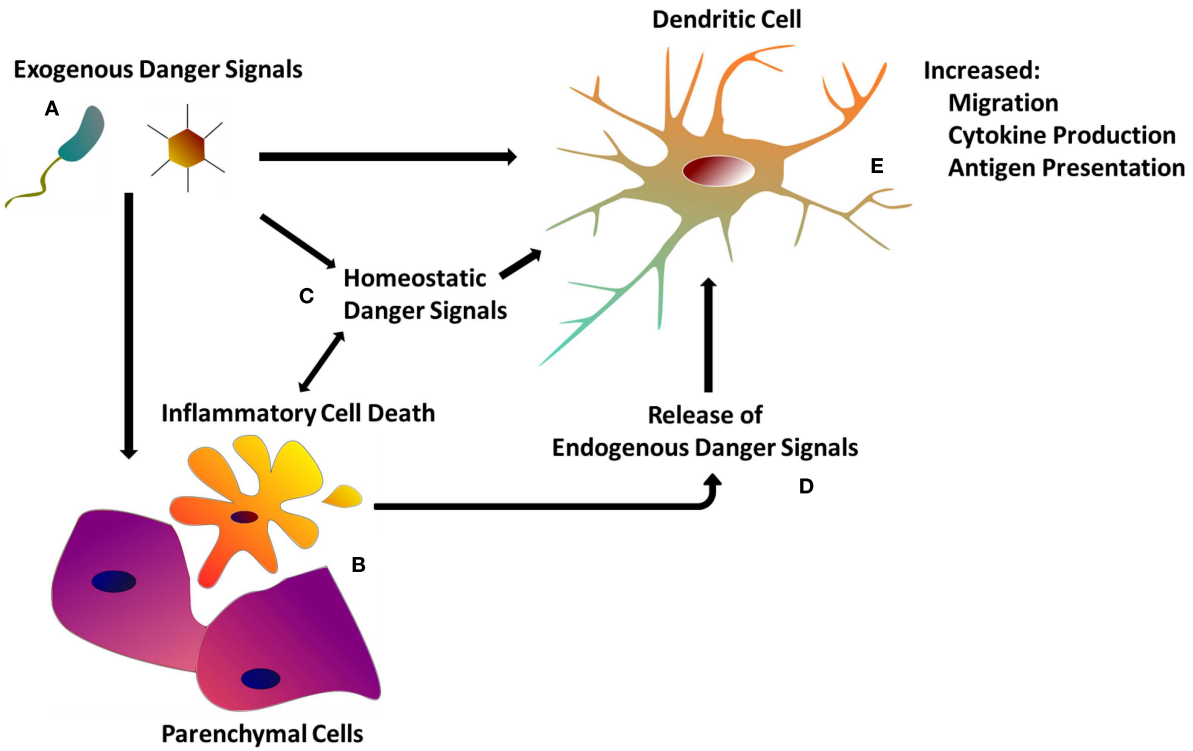

FIGURE 1 | The dendritic cell response to danger. **(A)** Exogenous danger signals include pathogen-associated molecular patterns, as well as exogenous particulate matter. Exogenous danger signals activate DCs directly via pattern recognition receptors, or indirectly though tissue damage **(B)** and homeostatic perturbations **(C)**. **(B)** Inflammatory cell death as a result of tissue injury or programed necrosis causes the release of endogenous danger signals **(D)** (see **Table 1**) which also activate DCs (see **Table 1**). **(C)** Homeostatic perturbations such as those found in inflamed tissue (e.g., decreased pH, hypoxia) may also act as endogenous danger signals, influencing DC immune functions. **(E)** DCs integrate both exogenous and endogenous danger signals in order to orchestrate the appropriate immune response.

some autoimmune strains of mice, DCs show abnormalities even when generated in culture, far removed from the autoimmune microenvironment (i.e., DCs from young mice, before the onset of the disease) (Sriram et al., 2012). These results suggest a genetic defect intrinsic to DCs, leading to their excessive activation, possibly through an uncontrolled production of danger signals (Elkon and Stone, 2011). In other cases, abnormalities were present only in DCs *in vivo* or isolated *ex vivo* from diseased mice, therefore pointing to a direct association with the autoimmune process (Colonna et al., 2006; Melli et al., 2009) and suggesting that in some situations DC abnormalities are a consequence rather than the cause of the autoimmune environment. In either case, determining the role of danger signals in the activation of DCs is key to better understanding the pathogenesis of autoimmune disease. Knowledge of danger signals will also facilitate the identification of novel therapeutic approaches aimed at the prevention of autoreactive lymphocyte activation. For example, neutralization of stimuli that induce abnormal DC activation may be a far more physiologic strategy than relying on general suppression of the lymphocyte response, as is common with most current therapeutic protocols.

THE EXTENDED FAMILY OF DANGER SIGNALS

The term "danger signal" was originally proposed by Polly Matzinger to indicate endogenous molecules released by stressed or necrotic cells, which are able to activate DCs (Matzinger, 1994, 1998; Gallucci et al., 1999; Gallucci and Matzinger, 2001). Five years earlier, Janeway (1989) had theorized that the innate immune system becomes activated by conserved molecular species expressed by evolutionarily distant microorganisms. These features were called pathogen-associated molecular patterns (PAMPs) and were proposed to trigger PRRs present on host cells (Medzhitov and Janeway, 1997a,b). With his innovative theory, Janeway (1992) updated the classic self-non-self-discrimination model of immunity by theorizing that the immune system can distinguish between self and infectious non-self, i.e., the pathogen (Medzhitov et al., 1997; Poltorak et al., 1998). As our understanding of the biochemical basis of the PRRs (e.g., TLRs, NLRs, RIG-I) has improved, it has become clear that host factors, derived from damaged tissues and cells, signal through the same receptors, serving as "danger signals" to stimulate immunity and therefore allow the immune system to discriminate what is dangerous or damaged from what is not, as previously theorized by Matzinger (1994, 2002) (**Figure 1**). These endogenous danger signals are a subset of what Seong and Matzinger (2004) named "DAMPs," analogous to the nomenclature of Janeway. Presently the term "danger signal" has a broad meaning and includes very different families of molecules that activate DCs: either exogenous molecules, such as the PAMPs;

or endogenous molecules released, activated, or secreted by host cells and tissues undergoing stress, damage, and non-physiological cell death, namely DAMPs (Matzinger, 1998, 2002) (**Figure 1**; **Table 1**). We support this inclusive nomenclature as it conveys the idea that both pathogens and trauma/stress are inducers of tissue and cell damage, which results in a pathologic status that is required for the activation of the innate immune system.

In the next section, we will review the DAMPs that activate DCs in autoimmunity, and we will then focus on danger signals that are less in the spotlight for immunologists, although already hypothesized in the original version of the Danger Model (Matzinger, 1994): the emerging and the homeostatic danger signals. Emerging danger signals are molecules that are newly developed by modern technology and that can activate DCs either by inducing cellular stress/damage or by attracting and carrying endogenous danger signals. Finally, we will review "homeostatic danger signals," danger signals that do not derive directly from pathogens or dying cells but rather are associated with perturbations of tissue/cell homeostasis and may be considered signs of stress and non-physiological conditions. We will not review PAMPs since they have been subjects of many excellent reviews.

Table 1 | Endogenous danger signals.

Signal	Source	Receptor/sensor(s)
Nucleic Acids	Dead/dying cells	TLR-7, -8, -9
Retroviral DNA	Cytoplasmic accumulation of endogenous retroviral DNA	STING
ATP, ADP, adenosine	Cell interior Dead/dying cells Mitochondria Activated platelets	P2X, P2Y A1, A2A, A2B, A3
Uric Acid	Nucleic acid breakdown Dead/dying cells	TLRs CD14 Inflammasome
HSPs	Cell interior Dead/dying cells Mitochondria	TLRs CD91
HMGB1	Cell Nucleus Necrotic cells Secreted by activated innate immune cells	TLRs RAGE CD24-Siglec10-TIM-3 CXCR4
Type I IFNs	Virally infected cells pDCs and mDCs	IFN receptors
Degradation products of the ECM	Extracellular matrix	TLR4 CD44 (hyaluronan)
MtDNA	Dead/dying cells Mitochondria	TLR9
N-formyl peptides	Dead/dying cells Mitochondria	High affinity formal peptide receptors
Acidity	Perturbation in homeostasis Inflammation Secreted by tumor cells	Acid-sensing ion channels Acid-sensing G-proteins
Osmolarity	Perturbation in homeostasis	mTOR
Hypoxia	Perturbation in homeostasis	Mitochondria

ENDOGENOUS DANGER SIGNALS

Classic endogenous danger signals are molecules that activate DCs (**Table 1**; **Figure 1**; **Box 2**). As DCs are central to antigen presentation and the initiation of the immune response, it was first shown that DCs respond to endogenous danger signals released from dying cells (Gallucci et al., 1999; Sauter et al., 2000), explaining the adjuvant effects of dying cells when co-injected with antigen *in vivo* (Gallucci et al., 1999; Shi et al., 2000, 2003).

Endogenous danger signals can be classified as "primary" when secreted by stressed cells or released by dying cells, or as "secondary" when produced by activated immune cells (Gallucci and Matzinger, 2001) (**Box 2**). Some cytokines act as both primary and secondary danger signals, when they are secreted by damaged/dying cells (**Box 2**). Many primary endogenous danger signals are released from the interior of the cell when the cell loses plasma membrane integrity upon necrosis (Rock and Kono, 2008; Sims et al., 2010). In contrast, the process of apoptosis keeps these pro-inflammatory signals contained and can actively up-regulate anti-inflammatory mediators (Behrens et al., 2007).

We will next describe some of the most studied DAMPs in autoimmunity. Covering all possible endogenous danger signals is not the purpose of this review: we will discuss a few signals in detail with specific reference to their role in autoimmune diseases (**Table 1**).

NUCLEIC ACIDS

Nucleic acids play a complex role in the pathogenesis of the autoimmune disease systemic lupus erythematosus (SLE). Nucleic acids are both the primary self-antigens and potent DAMPs, activating DCs through TLR7 and TLR9 and inducing the production of Type I IFNs (Barrat et al., 2005; Marshak-Rothstein and Rifkin, 2007). Nucleic acids often activate DCs either as part of an immune complex with autoantibodies (Leadbetter et al., 2002) or in complex with chaperones like heat shock proteins (HSP) and HMGB1. Oligonucleotides that inhibit the response to TLR7 and TLR9 ameliorate disease in lupus-prone mice (Barrat et al., 2007). Similarly the genetic deficiency of TLR7, or TLR7 and TLR9 combined prevent disease, while the single deficiency of TLR9 renders the disease worse, suggesting a double-edge effect of TLR9 (Santiago-Raber et al., 2009). In our hands, DCs from lupus-prone mice express constitutively high levels of Type I IFNs and IFN responsive genes and this IFN Signature was diminished by treatment with oligonucleotides inhibitory for TLR7 and TLR9, supporting a role for the response of DCs to nucleic acids in the excessive production of Type I IFNs in lupus (Sriram et al., 2012). The importance of TLR7

Box 2 | Primary vs. secondary endogenous danger signals.

Endogenous danger signals can be divided in two categories depending on the trigger and on the cellular source.

Primary endogenous danger signals: endogenous molecules that are normally contained within the cell interior or present in an inactive form, hidden from the immune system, and mostly performing non-immune functions. They are released upon tissue damage and are able to activate DCs by triggering a number of receptors, including PRRs. They are the first initiators of sterile immunity. Examples are listed in **Table 1**.

Secondary endogenous danger signals: endogenous molecules that are actively secreted by immune cells upon activation to mediate innate and adaptive immune activation in an autocrine and paracrine manner. They fuel and direct both sterile and non-sterile immunity. Secondary endogenous danger signals include lymphocyte-derived activators of DCs such as CD40-L (Ridge et al., 1998) and granulysin (Zitvogel and Kroemer, 2010); neutrophil-derived alarmins (Yang et al., 2009) and pro-inflammatory cytokines such as TNF-α, Type I IFNs, and HMGB1.

is further highlighted by the discovery that the Yaa translocation of a piece of chromosome X, containing 16 genes including TLR7, to the Y chromosome induces in male mice a severe lupus-like disease by creating a duplication of TLR7 (Pisitkun et al., 2006). Necrotic cells are a source of immunogenic nucleic acids (Eloranta et al., 2009) as well as neutrophils undergoing NETosis (Garcia-Romo et al., 2011).

ENDOGENOUS VIRAL ELEMENTS

A subset of PAMPs that can also be considered a form of special endogenous danger signals are endogenous viral elements (EVEs). EVEs are sections of viral DNA that have integrated into the mammalian genome during evolution (Stoye, 2012). The best characterized EVEs come from retroviruses, but may also derive from other viruses (Katzourakis and Gifford, 2010). Recent evidence demonstrates that defects in controlling retroviral elements are linked to autoimmunity. Indeed, mice deficient in the cytoplasmic three prime repair exonuclease 1 (Trex1) (formerly DNase III) express elevated IFN alpha (IFNα) levels in many organs and develop inflammatory myocarditis in the absence of viral infection (Morita et al., 2004). *Trex1*-deficient (*Trex1*−/−) murine embryonic fibroblasts accumulate endogenous retroviral DNA in the cytosol which induces excessive Type I IFN production via STING (Yang et al., 2007; Stetson et al., 2008; Gall et al., 2012). Loss-of-function mutations in *Trex1* are present in patients with Aicardi–Goutières syndrome as well as other autoimmune diseases such as familial chilblain lupus (Lee-Kirsch et al., 2007a; Gunther et al., 2009) and SLE (Lee-Kirsch et al., 2007b; Hur et al., 2008). This evidence suggests that cytoplasmic DNA derived from endogenous retroviruses is an endogenous danger signal that, if not scavenged by Trex1, can trigger an intracellular nucleic acid-sensing machinery (Pichlmair and Reis e Sousa, 2007; Thompson et al., 2011), leading to excessive production of the pro-autoimmunogenic Type I IFNs (Elkon and Stone, 2011). Although DCs are activated by Type I IFNs (Gallucci et al., 1999), and might therefore become hyperactivated, and thus initiate the autoimmune process, phenotypic, or specific functional abnormalities of DCs in Trex1−/− mice or in patients with Aicardi–Goutières syndrome have not yet been reported.

ATP

Other forms of nucleic acids acting as danger signals are the intracellular single nucleotides ATP and UTP. Extracellular ATP concentration is typically kept very low (10 nM) by ectonucleotidases (Robson et al., 2006). Under pathologic conditions, however, local concentrations of ATP can be increased – when liberated from damaged tissue or when released by an active process such as platelet or phagocyte activation (Zeiser et al., 2011). These adenosine derivatives activate DCs (Marriott et al., 1999) by stimulating the high affinity purinergic receptors P2Z/$P2X_7$ (Mutini et al., 1999). ATP and UTP activate the inflammasome in collaboration with TLRs (Mariathasan et al., 2004) and mediate at least some of the sterile inflammation induced by necrotic cells (Iyer et al., 2009; Aymeric et al., 2012). $P2X_7$-activated DCs have been shown to be crucial for the induction of tumor immunity (Ghiringhelli et al., 2009), the lack of $P2X_7$ signaling decreases the severity of EAE (Sharp et al., 2008), and oxidized ATP (oxATP), an inhibitor of the ATP receptor P2rx7, ameliorated autoimmune type I diabetes and autoimmune encephalitis in mice (Lang et al., 2010). Apoptotic cells can also release small amounts of ATP through the pannexin family of plasma membrane channels. At this low concentration, ATP binds low affinity purinergic receptors, including $P2Y_2$ and $P2X_7$ on phagocytes and recruits them to accelerate non-inflammatory clearance of apoptotic cells (Elliott et al., 2009). In a model of allergic lung inflammation, $P2Y_2$ is required for myeloid DC and eosinophil migration, although this receptor does not seem to be involved in DC maturation (Muller et al., 2010). Therefore, the ATP molecule may represent an important DAMP, conserved from prokaryotes (Atarashi et al., 2008) to plants (Heil et al., 2012) to mammals (Aymeric et al., 2012), acting as an activation signal at high doses when released by damaged cells, while at lower doses it may act as a "find-me" signal that helps housekeeping clearance of apoptotic cells and prevents immune activation (Elliott et al., 2009).

URIC ACID

Uric acid was one of the first DAMPs to be discovered and is partly responsible for the adjuvant properties of dying cells (Shi et al., 2003). Uric acid is a product of purine metabolism and is normally soluble inside cells. When released in the extracellular space uric acid precipitates and forms insoluble crystals of monosodium urate (MSU) capable of activating DCs *in vitro* and of acting as an adjuvant *in vivo* (Shi et al., 2003; Rock et al., 2010). Extracellular uric acid is highly inflammatory when it accumulates in joints to cause gout (Rock et al., 2013) or when it is released in large amounts during anti-cancer therapy causing tumor lysis syndrome

(Muslimani et al., 2011). The inflammatory nature of uric acid, as well as of other crystals like silica and alum, is in part due to their ability to activate the inflammasome (Martinon et al., 2006) with the extracellular delivery of endogenous ATP, the stimulation of IL-1β, and other pro-inflammatory cytokines, and the induction of reactive oxygen species (ROS) (Schorn et al., 2010; Riteau et al., 2012). These crystals may activate the inflammasome by causing direct cell damage to phagocytes, including DCs, inducing phagolysosome rupture (Hornung et al., 2008). Indeed, membrane fragments of these organelles can activate several responses, including polyubiquitination, autophagy, and pyroptosis and may play a role in host defense against intracellular parasites (Hilbi, 2009). Excessive activation of the inflammasome has been strongly linked to a group of hereditary autoinflammatory diseases called cryopyrin associated periodic syndromes (Lamkanfi and Dixit, 2012) but it may also play a role in more classic autoimmune diseases such as multiple sclerosis (MS) as mice deficient for components of the inflammasome (Nlrp3 and ASC) are protected from the development of EAE (Gris et al., 2010; Shaw et al., 2010).

HEAT SHOCK PROTEINS

Heat shock proteins are the most abundant proteins in cells, responsible for maintaining the correct folding of nascent and misfolded proteins, and of proteins that must remain inactive for long periods. HSPs are up-regulated in response to tissue stressors such as elevated temperature, osmotic shock, and cytotoxic agents (Srivastava, 2002). During cellular necrosis, HSPs are released into the extracellular space where they activate the innate immune response by triggering TLRs (Vabulas et al., 2002). As HSPs are protein chaperones, they also actively participate in DC antigen processing and presentation to initiate adaptive immunity (Srivastava, 2002). Srivastava's group showed that necrotic cells, but not apoptotic cells, release many HSPs such as HSP70, HSP90, calreticulin, and GP96 (Basu et al., 2000), which may activate immunity through the common receptor CD91 (Binder et al., 2000). In contrast, other HSPs, like the human heat shock protein 60, induce DC maturation and promote a Th1 phenotype via TLR4 (Flohe' et al., 2003). Like HMGB1 and autoantibodies, HSPs may contribute to the development of autoimmunity not only as endogenous adjuvants but also as chaperones that physically deliver autoantigens to APCs (Biswas et al., 2006). This is the mechanism by which HSP70 promotes autoimmunity in a mouse model of diabetes (Liu et al., 2003; Millar et al., 2003). Furthermore, autoantibodies against HSPs are found in SLE patients and allelic variants of the *Hsp70* genes are significantly associated with SLE (Furnrohr et al., 2010) and with several other autoimmune diseases such as MS (Ramachandran and Bell, 1995), Crohn's disease (Debler et al., 2003), Grave's disease (Ratanachaiyavong et al., 1991), and insulin-dependent diabetes mellitus (Pociot et al., 1993).

HIGH MOBILITY GROUP BOX PROTEIN 1

High mobility group box protein 1 (HMGB1) is a well-established endogenous danger signal (Lotze and Tracey, 2005), released by necrotic cells that have lost membrane integrity (Scaffidi et al., 2002) as well as secreted by phagocytic cells as a late mediator of inflammation (Gardella et al., 2002). HMGB1 normally has a nuclear localization and was originally described as a DNA binding protein (Javaherian et al., 1978; Bianchi et al., 1989), but a variety of other cellular functions have been attributed to HMGB1 largely depending on different post-translational modifications such as oxidation and hyperacetylation (Harris et al., 2012). Intracellular HMGB1 regulates gene transcription and autophagy (Tang et al., 2012). Extracellular HMGB1 acts as a classic DAMP when released by necrotic cells, or as a secondary danger signal when secreted by macrophages and DCs in response to LPS and TNF-α (Yang et al., 2005; Bianchi, 2007). Autocrine HMGB1 mediates DC activation and the ability to induce Th1 polarization (Dumitriu et al., 2005). The secretion of HMGB1 by monocytic cells is mediated by hyperacetylation of HMGB1, which localizes it to the cytoplasm where, upon a secondary signal, it is liberated into the extracellular compartment (Bonaldi et al., 2003).

HMGB1 is significantly increased in the serum of patients with brain and myocardial ischemia (Goldstein et al., 2006) and in septic patients in which it mediates the late phase of septic shock (Wang et al., 1999). HMGB1 is recognized by many receptors, like the receptor for advanced glycation end products (RAGE) (Kokkola et al., 2005) as well as by TLR2 and TLR4 (Park et al., 2004), CD24-Siglec10, and the recently reported TIM-3 (Chiba et al., 2012). When bound to the chemokine CXCL12, HMGB1 acts as a chemokine to recruit leukocytes to the site of inflammation by binding CXCR4 (Schiraldi et al., 2012). The shift from functioning as a chemokine to acting as a danger signal depends on its redox state: intracellular HMGB1 has three cysteines that are in a reduced state (all-thiol state); when it is secreted or released, the all-thiol HMGB1 can bind to CXCL12 and chemoattract unless two cysteines are oxidized, forming a disulfide bond and promoting HMGB1 danger signal functions (Yang et al., 2013). During apoptosis HMGB1 is kept intracellular, and uptake of apoptotic cells induces release of oxidized HMGB1 so that its pro-inflammatory activities are neutralized. Possible functions of this fully oxidized state are still under investigation (Yang et al., 2013).

Evidence suggests that HMGB1 participates in the pathogenesis of many autoimmune diseases like Rheumatoid Arthritis (RA), SLE, EAE, and diabetes (Zhang et al., 2009; Harris et al., 2012). For example, RA patients have increased levels of HMGB1 in the serum and synovial fluid that decreases upon therapy-induced amelioration of joint inflammation (Zetterstrom et al., 2008). Similarly, HMGB1 blockade ameliorates arthritis in animals, while its administration in the joints induces arthritis (Kokkola et al., 2003). In SLE patients, serum HMGB1 levels, as well as anti-HMGB1 autoantibody titers, positively correlate with disease activity (Jiang and Pisetsky, 2008; Abdulahad et al., 2011). HMGB1-containing nucleosomes from apoptotic cells have been shown to induce secretion of pro-inflammatory cytokines and expression of costimulatory molecules in macrophages and DCs and the administration of HMGB1-nucleosome complexes in mice induces lupus-like autoantibodies (Urbonaviciute et al., 2008; Urbonaviciute and Voll, 2011). In MS patients, extracellular HMGB1 is increased in the cerebrospinal fluid (CSF) and microglia and macrophages expressing cytosolic HMGB1 are increased in MS active lesions (Andersson et al., 2008). In mice, HMGB1 drives neuroinflammation in EAE and the inhibition of HMGB1 signaling with a neutralizing antibody ameliorates disease (Robinson et al., 2013). Clinical and experimental evidence also suggest a role for HMGB1 in the

pathogenesis of type I diabetes. In diabetes-prone NOD mice, HMGB1 blockade significantly inhibited insulitis progression and delayed diabetes onset, decreased the number, and maturation of DCs in the pancreatic lymph nodes, and increased the number of regulatory T cells (Han et al., 2008). Once diabetes has developed, hyperglycemia can induce HMGB1 secretion (Yao and Brownlee, 2010; Dandona et al., 2013) and HMGB1 may contribute to the nephropathy and the vascular complications of diabetes by fueling inflammation and promoting tissue damage, especially in the kidney (Lin et al., 2012). Higher levels of serum HMGB1 were associated in type 1 diabetes patients with a higher risk of mortality, cardiovascular disease (Nin et al., 2012a), and kidney damage (Nin et al., 2012b).

In summary, HMGB1 is a powerful danger signal that plays a complex role in the pathogenesis of autoimmune diseases. Blockade of HMGB1 has thus far proven to be beneficial in ameliorating a number of types of experimental autoimmunity, making its therapeutic potential very promising. Further investigation is still needed to dissect the role of HMGB1 as an activator of DCs in terms of its multiple functions as a cytokine, chemokine, transcription regulator, and possible functions yet to be discovered.

TYPE I INTERFERONS

Type I IFNs activate DCs *in vitro* and act as adjuvant *in vivo* (Gallucci et al., 1999). They are secreted by virally infected cells (Taniguchi and Takaoka, 2002) as a primary danger signal to alert both neighboring tissue cells and local immune cells to the presence of a viral infection. They are also secreted by immune cells as an amplifier of innate immunity (Liu, 2005). Type I IFNs (IFNs α and β) mediate the activation of DCs to promote adaptive immune responses such as cross-priming and isotype switching of responding murine B cells toward IgG2 (Theofilopoulos et al., 2005). Type I IFNs are particularly important in autoimmunity and especially in SLE in which an IFN Signature is present – an abnormally high expression of Type I IFN responsive genes in immune cells and tissues (Baechler et al., 2003; Bennett et al., 2003; Crow et al., 2003; Elkon and Stone, 2011). The hyper-activation of Type I IFNs may play a role in the early and most acute phases of disease because the IFN Signature is common in pediatric patients (Bennett et al., 2003) and in adults with central nervous system involvement and nephritis (Baechler et al., 2003). Supporting this notion, we have recently discovered that myeloid DCs and pDCs from lupus-prone Sle1,2,3 mice express an IFN Signature before the onset of autoimmunity. Even when generated in culture from bone marrow precursors, depleted of mature T and B cells, macrophages, and DCs, and thus, severed from the *in vivo* pro-autoimmune environment, DCs from Sle123 mice expressed an IFN Signature (Sriram et al., 2012). These results indicate that DCs are an independent cellular source of the pathogenic danger signals implicated in lupus (Elkon and Stone, 2011; Sriram et al., 2012). Since polymorphisms in genes that are part of the signaling pathway of Type I IFNs, such as IRF5, IRF7, and STAT4, are associated with a higher risk of developing SLE (Harley et al., 2009), the over-expression of Type I IFNs in these patients may be the result of the combination of genetic predisposition to produce high levels of Type I IFNs and exposure to danger signals that stimulate IFN production (Elkon and Stone, 2011; Niewold, 2011).

DEGRADATION PRODUCTS OF THE EXTRACELLULAR MATRIX

During tissue damage, not only are cells stressed and dying but components of the extracellular matrix (ECM) are disrupted leading to the production of low molecular weight degradation products such as hyaluronic acid (HA) and heparan sulfate, which have been shown to activate DCs and initiate inflammation (Termeer et al., 2002; Shirali and Goldstein, 2008; Brennan et al., 2012). Moreover, other components of the ECM such as fibrinogen, fibronectin extra-domain A (EDA), biglycan, and tenascin-C are up-regulated in response to tissue injury, and they all act as danger signals involved in the pathogenesis of RA (Goh and Midwood, 2011). Many of these molecules have been found increased in RA tissues; administration of danger signals, such as fibronectin EDA or tenascin-C, induces joint inflammation *in vivo*, while mice deficient in tenascin-C show a rapid resolution of inflammation (Goh and Midwood, 2011), indicating an important role for ECM molecules as initiator/amplifiers of the autoimmune process in RA.

EMERGING EXOGENOUS DANGER SIGNALS

The classic exogenous danger signals originate from pathogens (PAMPs), but exogenous particles not derived from microbes, such as silica, crystals, and nanoparticles can also induce DC activation and inflammatory disease. Indeed, it has long been known that inhalation of fine particulate material can cause inflammatory and fibrotic lung disease (i.e., pneumoconiosis). The inflammatory nature of non-living particulate matter, like crystals and nanoparticles, may stem in part from their hydrophobic character (Seong and Matzinger, 2004). Moreover, nanoparticles are preferentially taken up by phagocytes (Dobrovolskaia et al., 2008), including DCs, in which they cause destabilization and rupture of the phagolysosome (Hornung et al., 2008), leading to inflammasome activation (Cassel et al., 2008). Therefore, extracellular inorganic material may directly damage DCs, leading to inflammasome activation and DC maturation. This is the proposed mechanism of action for alum, a widely used adjuvant in human vaccines (Eisenbarth et al., 2008). Because of their immunogenic potential, it is important to consider the emerging use of nanomaterials to target DCs for novel therapeutics and their possible autoimmunogenic side effects.

NANOMATERIALS

The term nanoparticle may apply to almost any material with individual units at the nano scale. Nanomaterials can be generated from organic (e.g., liposomes, organic polymers) or inorganic materials (e.g., gold, silver) and engineered to display a molecule of interest or contain a therapeutic payload. Using nanoparticles and synthetic material systems, it may be possible to modulate the immune response through direct manipulation of DCs (Elamanchili et al., 2007).

The usefulness of pro-inflammatory nanoparticles has been demonstrated in a number of fields (Fadeel, 2012; Fadeel et al., 2012). For example, nanoparticle-based co-delivery of antigens and danger signals directly to DCs may be a promising strategy to direct anti-tumor immune responses (Kim and Mooney, 2011), as

well as useful in next-generation vaccine adjuvants (Demento et al., 2009). However, because materials designed as next-generation therapies are sometimes capable of inducing DC-mediated inflammation similarly to PAMPs or DAMPs (Koike et al., 2008; Li et al., 2010), nanomaterials may raise concerns for safety (Dobrovolskaia and McNeil, 2007; Stern and McNeil, 2008). For example, nanoparticle debris from prosthetic alloys can activate the inflammasome (Caicedo et al., 2009), nanoparticles are known to modulate various forms of inflammatory cell death (Andón and Fadeel, 2013), and some nanomaterials are strong activators of complement (Salvador-Morales et al., 2006).

Additionally, it is now clear that nanoparticles do not stay "naked" when introduced *in vivo* – they become coated by biomolecules (e.g., proteins) to form what is termed a bio-corona (Fadeel, 2012). The composition and biophysical properties of the bio-corona is likely to have major implications for safety and effectiveness of future therapies (Aggarwal et al., 2009). The protein bio-corona first develops as a "soft" coat which eventually becomes a compact "hard" shell, becoming difficult to remove (Mohamed et al., 2012). Proteins within the hard coat are therefore aggregated and may become unfolded or modified (for example, citrullinated), possibly resulting in DCs recognizing altered self-antigen as danger signals, although evidence for this is still lacking (Fadeel, 2012).

The study of nanoparticle characteristics is also likely to uncover as yet unknown characteristics of danger signals. With the ability to control the characteristics and the delivery of the "danger signal," we may better understand the immune response to those factors. For example, size of the nanoparticle seems to determine which DC populations interact with an injected particle: namely, larger particles require peripheral DCs for transport into the draining lymph nodes, whereas smaller particles act on lymph node resident cells directly (Manolova et al., 2008). Large particles also tend to be more inflammatory, with smaller particles often eliciting no reaction at all (Fadeel, 2012), possibly because larger sized particles more potently destabilize the phagolysosome.

Although most studies focus on the pro-inflammatory nature of particulate matter, some nanomaterials may suppress immune function (Mitchell et al., 2009; Zolnik et al., 2010) or can be engineered to evade the innate immune system (Moghimi, 2002). Indeed, gold has anti-inflammatory properties that have been exploited for many years in treatment for RA and gold nanoparticles are being engineered as novel treatments for a number of inflammatory diseases (Ulbrich and Lamprecht, 2010). Recently, Yeste et al. used gold nanoparticles to generate tolerogenic DCs in a murine model of EAE by delivering a tolerogenic compound in combination with oligodendrocyte antigen to DCs. These DCs in turn induced the generation of regulatory T cells which mitigated disease progression (Yeste et al., 2012).

Although gold may have some anti-inflammatory properties and may be useful as a delivery system, it also has many toxic side effects, earning itself a black box warning and precluding it as a practical therapy. Indeed, heavy metals can cause autoimmune-like syndromes (Schiraldi and Monestier, 2009) and therefore should be implemented cautiously as therapeutics. However, it is important to note that nanoparticles are being developed which are both biocompatible and biodegradable. For example, liposomes have been used as a delivery system to successfully treat experimental murine arthritis (Metselaar et al., 2003; Sethi et al., 2013), and biodegradable nanoparticles have been used to localize and target T cells (Fahmy et al., 2007). Nanoparticles coated with glycans have been used to target antigen to DCs (García-Vallejo et al., 2013), but the therapeutic use of DC-targeted nanoparticles in spontaneous autoimmunity remains largely unexplored (Ulbrich and Lamprecht, 2010). Nanomaterials therefore represent a promising strategy for next-generation therapy in the autoimmune diseases and an important area for future investigation to insure their safety and efficacy.

HOMEOSTATIC ENDOGENOUS DANGER SIGNALS

So far, we have briefly described the DAMPs investigated under the classic paradigm – cell stress/death from tissue damage, possibly induced by trauma, infections or foreign materials, provides endogenous danger signals, which propagate the immune response (**Figure 1**). But a number of investigations suggest the existence of endogenous danger signals that do not derive from damaged or dying cells. Rather, they are signals that alert immune cells, and possibly non-immune cells, about perturbations in the steady state of the cellular microenvironment, and we refer to these as "homeostatic" danger signals (**Figure 2**). Indeed, the immune system is closely tied to metabolic homeostasis (reviewed in Odegaard and Chawla, 2013; Pearce and Pearce, 2013). These homeostatic danger signals may include conditions often characteristic of chronically inflamed tissues such as localized acidosis, changes in osmolarity, decreased oxygen tension (hypoxia), oxidative stress with ROS, and other metabolic disturbances – all possible causes of cellular stress as hypothesized in the original Danger Model (Matzinger, 1994). Homeostatic changes also result from bacterial growth and acute tissue damage, further supporting the notion that cells may sense homeostatic changes as danger. Some of these homeostatic perturbations directly activate DCs, because DCs have specific sensors for these perturbations, while others lead to expression of more classic DAMPs: an example of the latter is the up-regulation of HSP like HSP70 by DCs in response to hyperthermia (40 °C), resulting in up-regulation of costimulatory molecules, pro-inflammatory cytokines, and T cell stimulation (Knippertz et al., 2011).

ACIDITY

Physiologic pH is normally held within relatively narrow range (pH 7.35–7.45). During localized inflammation (e.g., infection, arthritic joint) or tissue ischemia (e.g., lupus nephritis, MS lesions) extracellular pH can reach pH values as low as 5.5 (Treuhaft and McCarty, 1971; Nedergaard et al., 1991; Simmen and Blaser, 1993).

A significant amount of research on cell functions in acidic conditions comes from the cancer field. Tumor cells tend to use glycolysis for energy generation even when there is adequate oxygen supply – the Warburg effect (Cardone et al., 2005). The inefficiency of glycolytic metabolism, the active secretion of acid, and the poor perfusion in solid tumors is thought to contribute to an acidic microenvironment. Acidic microenvironments increase the invasiveness and metastatic potential of some cancers as a result of remodeling of the ECM (Martinez-Zaguilan et al., 1996; Rofstad et al., 2006) and activation of inflammatory processes that have been shown to promote tumor survival (Grivennikov, 2013).

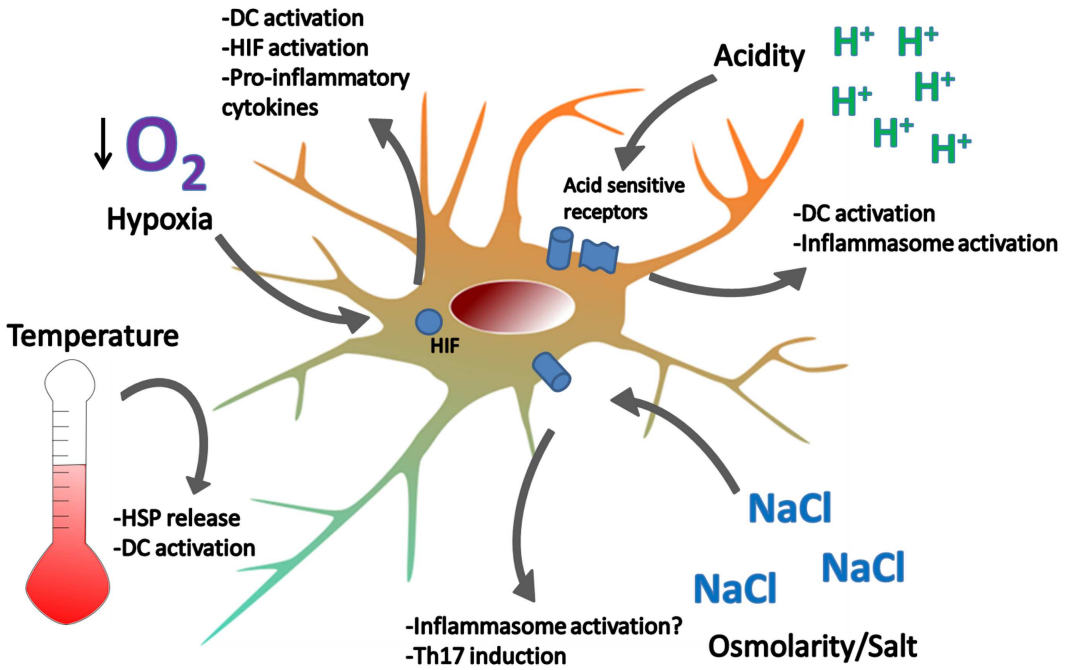

FIGURE 2 | Examples of homeostatic danger signals. Localized perturbations in homeostasis are common in infection, ischemia-reperfusion injury, and inflammation associated with autoimmune disease. Extreme temperatures can cause DC activation and cell death. Heat induces the release of HSPs which can activate DC. Hypoxia is sensed by HIF and leads to DC activation, NF-kB signaling, and cytokine production. Acidity is sensed by acid-sensing ion channels and acid-sensing G-proteins, leading to DC activation. Hypotonicity activates the inflammasome in macrophages and high salt concentrations promote Th17 responses. How DCs respond to changes in osmolarity remains unclear.

Several studies have indicated that acidity might act as a danger signal. For example, extracellular acidification can promote activation of the inflammasome and secretion of IL-1β by monocytes (Jancic et al., 2011) and seems to activate neutrophils through the activation of PI3K-Akt and ERK pathways (Trevani et al., 1999; Martinez et al., 2006). Additionally, non-immune cells may also produce inflammatory cytokines in response to extracellular acidification (Shime et al., 2008; Ichimonji et al., 2010) or may alter cell death programs (Jouan-Lanhouet et al., 2012).

Acidity in the extracellular microenvironment was initially proposed to inhibit DC differentiation (Gottfried et al., 2006), but some work suggests that DCs are instead activated by acidosis (Martinez et al., 2006). Other evidence has shown that acidic environments might improve antigen uptake and antigen presentation in DCs, although the mechanisms are still unclear (Vermeulen et al., 2004). Recently, acid-induced activation of DCs was found to be in part due to acid-sensing ion channels (Tong et al., 2011), but our understanding of DC responses to protons remains limited (Lardner, 2001). Low pH may also be sensed by proton-sensing G protein-coupled receptors (Seuwen et al., 2006), as seen in responses from synovial cells (Christensen et al., 2005; Tomura et al., 2005). Acidity is a common feature of inflammation found in many autoimmune diseases (e.g. within the arthritic joint, nephritic kidney, ischemic lesions of MS). However, to our knowledge, it is still unclear whether pathways triggered by acidity are involved in DC immune functions in the context of autoimmune diseases.

OSMOLARITY AND SALT

Cell volume expansion, as a result of extracellular hypotonicity, may also act as a danger signal. In response to hypotonic osmotic challenge lymphocytes first swell, then undergo a process termed regulatory volume decrease (RVD) (Garber and Cahalan, 1997; Cahalan et al., 2001). A reciprocal process occurs in hypertonic environments called regulatory volume increase (RVI). RVD is largely the result of the activity of potassium and chloride channels, with efflux of KCl giving way to water efflux as a result of decreased intracellular osmolarity. In some cases KCl efflux also leads to activation of other ion channels and cellular depolarization, thus having major implications in fundamental cellular processes like metabolism and cell death. Indeed, a similar process is seen during apoptosis. Apoptotic cell volume decrease seems to be due to the activation of the same potassium and chloride channels (Maeno et al., 2000, 2006). Cellular responses resulting from homeostatic signals are complicated by the observation that ion channels form complexes with integrins and may signal together (Arcangeli and Becchetti, 2006).

Earlier studies demonstrated that hypotonicity influence immune cell function, for example in neutrophil shedding of L-selectin (Kaba and Knauf, 2001). Mammalian target of rapamycin (mTOR), in conjunction with mitochondrial metabolism, may also be an important sensor for osmotic stress, as well as other homeostatic signals (Desai et al., 2002; Kwak et al., 2012). DCs are able to pinocytose, uptake fluids from the extracellular compartment as a way to sample for antigens and danger signals. This

process is very efficient in immature/resting DCs which can uptake fluids up to one thousand fold their volume (Sallusto et al., 1995). This large flow of fluids requires a tight control of the DC volume, and therefore DCs express aquaporins for this purpose (de Baey and Lanzavecchia, 2000). Inhibition of aquaporins leads to sudden increase in cell volume, which may ultimately result in DC cell death. The idea that hypotonicity may be regarded as a danger signal has recently gained credence with the finding that hypotonic solutions activate the NLRP3 inflammasome in macrophages via the efflux of K^+ and Cl^- ions and RVD (Compan et al., 2012). The role of alterations of cell volume in DC physiopathology remains largely unclear.

In contrast to hypotonicity, high salt concentrations do not seem to activate inflammasome activation, as hypertonic solutions are not able to induce caspase-1-dependent release of cytokines IL-18 and IL-1β (Compan et al., 2012). However, high salt concentrations may promote development of other pro-inflammatory conditions such as those associated with T helper 17 cells (Th17). Osmotic shock due to high salt was known to induce the production of IL-8 from human peripheral blood mononuclear cells (Shapiro and Dinarello, 1995), but recently two groups have demonstrated that high salt concentrations may promote pathogenic Th17 responses (Kleinewietfeld et al., 2013; Wu et al., 2013) and a high salt diet exacerbates EAE (Wu et al., 2013). These studies suggest that elevated salt concentrations, similar to levels found in tissues of animals on high salt diets, may exacerbate autoimmune reactions. It is still unclear how high salt concentrations affect DCs *in vivo* in the context of autoimmunity.

HYPOXIA

Decreased oxygen tension is another characteristic disturbance of inflamed or damaged tissue (Nizet and Johnson, 2009). Innate immune cells are thought to be better than adaptive immune cells at surviving in such low oxygen conditions (Sica et al., 2011), and a number of signaling pathways are relatively well known (Gloire et al., 2006; Semenza, 2009). In normoxic conditions, the α subunits of hypoxia-inducible factor (HIF), HIF-1α, and HIF-2α, are constantly produced and promptly degraded. Under hypoxic conditions, HIF-α accumulates, forms a dimer with the constitutively expressed HIF-1β subunit within the nucleus, and regulates transcription via hypoxia-responsive elements (HREs). Important gene targets are involved in adaptive response to hypoxia and include glycolytic enzymes, erythropoietin, and vascular endothelial growth factor.

A number of lines of evidence suggest that hypoxia acts as a homeostatic danger signal. Low oxygen levels leads to the generation of ROS, affecting both metabolism and mediators of inflammation. Furthermore, it is well established that HIF-1α signaling is closely tied to NF-kB signaling. In neutrophils, survival in hypoxic microenvironments, such as those found in infected tissue, is mediated by HIF-1α-dependent NF-kB activation (Walmsley et al., 2005). In a number of cell types, including DCs, hypoxia-induced HIF-1α prompts expression of TLR2 and TLR6 (Kuhlicke et al., 2007). Additionally, macrophages with a conditional deficiency in HIF-1α are compromised in their ability to clear bacteria (Cramer et al., 2003; Peyssonnaux et al., 2005), and TLR ligands like LPS may induce the HIF-NF-kB axis even under normoxic conditions (Spirig et al., 2010). Indeed, hypoxia-independent HIF induction may occur via other mechanisms, including in response to bacterial siderophores (Hartmann et al., 2008). Finally, hypoxia is also known to modulate cytokine production via HIF expression, inducing production of pro-inflammatory cytokines (Jeong et al., 2003; Nizet and Johnson, 2009) and enhancing the production of IFN-β in response to viral infection (Hwang et al., 2006).

Studies of DC responses to hypoxia are at times conflicting. It is unclear if hypoxia in itself activates DCs (Jantsch et al., 2008; Wang et al., 2010), and hypoxia may enhance (Jantsch et al., 2008) or inhibit (Mancino et al., 2008) LPS-induced costimulatory molecule expression. However, hypoxia does seem to augment DC production of pro-inflammatory cytokines, particularly in response to LPS (Jantsch et al., 2008; Mancino et al., 2008; Blengio et al., 2013). Similar activation has been demonstrated in human DCs associated with the inflamed joints of patients with juvenile idiopathic arthritis (Bosco et al., 2011).

HIF-1α expression is associated with a number of inflammatory and autoimmune diseases, including within the synovium of RA patients (Hollander et al., 2001) and in the muscle from patients with dermatomyositis and systemic sclerosis (Konttinen et al., 2004). HIF-1α is important for the development of B cells, with HIF-1α deficiency in chimeric mice leading to autoimmunity (Kojima et al., 2002). To our knowledge, the role of HIF signaling in DCs in the context of autoimmunity remains relatively unexplored.

MITOCHONDRIA

As a number of excellent reviews on mitochondria-derived danger signals have recently been published (Arnoult et al., 2011; Krysko et al., 2011; Galluzzi et al., 2012; Rath and Haller, 2012), we can only briefly touch on this important subject. Mitochondria are key in both the recognition and propagation of danger signals and we discuss them last because, as we describe in this section, mitochondria belong to more than one category of danger signal. As ancient symbionts, mitochondria harbor a number of molecules that have bacterial origin and therefore can be considered PAMPs. At the same time, these molecules are released from the cell interior upon cell stress/death and therefore they act as DAMPs and propagate the inflammatory response. Mitochondrial-derived danger signals include mitochondrial DNA (mtDNA), which contains CpG motifs capable of stimulating TLR9, *N*-formyl peptides which mimic bacterial peptides, and ATP as described above (Arnoult et al., 2011). The double nature of mitochondria as PAMPs and DAMPs brings the models of Janeway and Matzinger together (Seong and Matzinger, 2004), showing how the immune system co-evolved with his "enemy within" (Zhang et al., 2010). Moreover, as mitochondria are the cell power-house, they are key players in programs of cell death and in controlling homeostatic perturbations (Desai et al., 2002; Galluzzi et al., 2012).

Mitochondrial-derived danger signals are associated with the inflammatory response upon tissue damage and have been implicated in the inflammatory response to trauma. Severe traumatic injury may result in SIRS, a life threatening condition in which extensive tissue injury leads to systemic inflammation and shock reminiscent of sepsis (Stoecklein et al., 2012). Indeed, trauma

patients who have sustained major tissue injury have elevated levels of circulating mtDNA which contributes to the resultant sterile shock (Zhang et al., 2010).

Mitochondrial-derived CpGs are also an important danger signal in autoimmune disease. For example, injecting mtDNA into the joints of mice induces myeloid cell-mediated arthritis, but injection of nuclear DNA does not (Collins et al., 2004). The authors of this study also found mtDNA in the synovial fluid of patients with RA, but not from health controls (Collins et al., 2004). Recently, a mitochondrial-derived protein related to HMGB1, called mitochondrial transcription factor A, has been found to synergize with CpG to induce the production of Type I IFNs by pDCs (Julian et al., 2012).

Furthermore, as mitochondria are central to metabolism and cellular energy production, they represent a key sensor of homeostatic perturbations (Desai et al., 2002) and are known to regulate multiple cell death programs – linking homeostatic signals to cell death and release of danger signals (Galluzzi et al., 2012). Importantly, mitochondrial control of cell death includes the execution of both apoptosis and regulated necrosis, the latter a form of inflammatory cell death now known to propagate the immune response to pathogens (Vandenabeele et al., 2010). Regulated necrosis leads to the liberation of many danger signals, including those we have outlined in this review.

Perturbations in homeostasis or mitochondrial function also lead to the generation of ROS, which are an important danger signals in their own right. ROS are normal byproducts of respiration, but may also function as signaling molecules and antimicrobial agents under controlled conditions (Valko et al., 2007); as well as a danger signals under pathologic conditions (Galluzzi et al., 2012). Cells defend themselves against toxic ROS buildup using a multitude of antioxidants and enzymes including superoxide dismutase and catalase (Dröge, 2002). Superoxide dismutase converts oxygen radicals to hydrogen peroxide and oxygen; and catalase converts hydrogen peroxide to water and oxygen. However, when oxidative stress overwhelms these antioxidant mechanisms the result is activation of cell death programs (Galluzzi et al., 2012). In the context of autoimmune diseases like SLE, increased ROS may contribute to pathogenesis via modification of self-antigens, thereby increasing their auto-reactivity, and by promoting cell death resulting in the release of DAMPs (Perl et al., 2004; Oates and Gilkeson, 2006).

In addition to being a source and sensor of danger signals, mitochondrial dysfunction may directly influence the course of autoimmune and autoinflammatory diseases. This seems to be due to the participation of mitochondria in regulating the inflammasome and cell death. Indeed, inflammasome activation in the context of monogenic autoinflammatory diseases has been suggested to cause mitochondrial dysfunction (Escames et al., 2011). Mitochondrial dysfunction and subsequent cell death, in response to oxidative stress, may have a predominant role in the propagation of MS (Witte et al., 2010). Mitochondria from T cells of SLE patients also seem to be deregulated – having an elevated baseline transmembrane potential, predisposing them to necrotic cell death (Gergely et al., 2002; Perl et al., 2012) and other metabolic disturbances (Wu et al., 2012). Continued investigation of mitochondria at the cross-roads between metabolism and inflammation will very likely be important in our understanding of autoimmunity.

CONCLUSION AND FUTURE PERSPECTIVES

The immune response is often described as a double-edge sword. Too little immunity leads to uncontrolled infections, while too much immunity results in inflammatory damage, autoinflammatory disease, and some forms of autoimmunity. The delicate balance needed to keep immunity within this narrow range is the result of complex biochemical interactions. A threshold likely exists whereby tonic factors suppress noise in the system and other factors amplify or regulate immunologic signals in order to have a quick and efficient immune response (Germain, 2012). Danger signals may be both initiators of the immune response, as well as amplifiers of the immune response as a result of tissue injury and cell death. Indeed, inflammatory cell death seems to be required for proper pathogen clearance (Cho et al., 2009; Upton et al., 2010). Another layer of complexity comes in the fact that some molecules do not always act as danger signals, but rather serve other functions (e.g., HMGB1 acts as a chemokine, HSP as a chaperone, mitochondria as a power-house) and may even inhibit the immune response or promote tissue repair (Castiglioni et al., 2011; Stocki and Dickinson, 2012).

Danger signals are therefore diverse in form and function. Indeed, there are a number of conceptual frameworks in which danger signals are characterized, all of which have been extremely useful (Janeway, 1989; Matzinger, 1994). Here we have outlined, to the best of our ability, both the classic, emerging and homeostatic danger signals that influence DC biology. Although we describe some non-classic because they do not directly derive from pathogens or dead/dying cells, they are in fact well established. Man-made technologies (nanomaterials) are simply a novel form of particulate matter, which has been known for many years to induce inflammatory disease. Furthermore, the notion that homeostatic perturbations (e.g., osmotic stress, oxidative stress) act as danger signals is supported by a large body of literature (See "Mitochondria"). These data suggest that the immune system, set to recognize tissue/cell damage, can use sensors for basic physical and chemical perturbations of the tissue microenvironment to detect early causes of damage, even before the release of DAMPs. While much is now know about DC responses to exogenous and endogenous danger signals, many questions remain unresolved. Indeed, we have discussed signals as if they act independently, but we have little information on how signals interact with each other to control the immune response. Future studies will determine how the sum of these functions influences the development of autoimmunity.

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Tailoring gut immune responses with lipoteichoic acid-deficient *Lactobacillus acidophilus*

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As highlighted by the development of intestinal autoinflammatory disorders when tolerance is lost, homeostatic interactions between gut microbiota, resident immune cells, and the gut epithelium are key in the maintenance of gastrointestinal health. Gut immune responses, whether stimulatory or regulatory, are dictated by the activated dendritic cells (DCs) that first interact with microorganisms and their gene products to then elicit T and B cell responses. Previously, we have demonstrated that treatment with genetically modified *Lactobacillus acidophilus* is sufficient to tilt the immune balance from proinflammatory to regulatory in experimental models of colitis and colon cancer. Given the significant role of DCs in efficiently orchestrating intestinal immune responses, characterization of the signals induced within these cells by the surface layer molecules, such as lipoteichoic acid (LTA), and proteins of *L. acidophilus* is critical for future treatment and prevention of gastrointestinal diseases. Here, we discuss the potential regulatory pathways involved in the downregulation of pathogenic inflammation in the gut, and explore questions regarding the immune responses to LTA-deficient *L. acidophilus* that require future studies.

Keywords: *Lactobacillus acidophilus*, lipoteichoic acid, S-layer proteins, gut inflammation, dendritic cells, immune regulation

INTRODUCTION

The gastrointestinal tract possesses a highly specialized immunologic system comprised of both innate and adaptive immune components. These defense systems act in concert to maintain a state of alertness or physiological inflammation in the gut that enables the recognition and clearance of invading pathogens while remaining tolerant to the commensal microbiome (Sansonetti, 2004). By virtue of their antigen processing and presenting abilities, dendritic cells (DCs) are at the forefront of intestinal immune responses (Chang et al., 2012). DCs in the lamina propria constantly sample an array of food and microbial antigens and present them to resident T cells. Under steady state conditions, intestinal DCs induce the development of Th1 and Th17 effector T cells; however, at the same time, a specialized subset of regulatory $CD103^+$ DCs promote the generation of induced regulatory T cells (iTregs; Siddiqui and Powrie, 2008) that prevent exacerbated Th1 and Th17 effector responses, and thus limit collateral tissue damage. Tregs express the transcription factor FoxP3 and suppress proinflammatory immune responses through the production of anti-inflammatory cytokines, including interleukin (IL)-10 and transforming growth factor-beta (TGF-β), and the surface expression of inhibitory molecules, such as cytotoxic T lymphocyte antigen 4 (CTLA-4) and lymphocyte activation gene-3 (LAG-3; Huang et al., 2004; Li et al., 2007; Rubtsov et al., 2008; Wing et al., 2008; Bos and Rudensky, 2012). Indeed, the transfer of total $CD4^+CD25^+$ Tregs efficiently mitigated established colitis in an experimental model of the disease (Mottet et al., 2003), and a deficiency of this cell population has been found in patients with ulcerative colitis (Takahashi et al., 2006). Although these studies highlight the role of thymic-derived or natural Tregs, subsequent studies have emphasized the importance of iTregs for disease resolution (Haribhai et al., 2009). Therefore, the induction of peripheral Tregs by regulatory DCs in the gut seems to be particularly crucial for microbial coexistence and colonic health. In support of this notion, colonic Tregs were found to express T cell receptor (TCR) repertoires that were distinct from those found on Tregs from other organs and were also specific for antigens encoded by commensal bacteria (Lathrop et al., 2011).

In addition to the aforementioned regulatory immune cells, and equally important for gut immune homeostasis is the composition of the gut microbiota (Round and Mazmanian, 2009; Consortium, 2012; Holmes et al., 2012). Recent elegant studies have contributed to our understanding of intestinal immune modulation and the promotion of regulatory responses by the microbiota. For instance, monocolonization of germ-free (GF) mice with the human commensal, *Bacteroides fragilis*, induced the development of IL-10-secreting colonic Tregs (Round and Mazmanian, 2010). Moreover, *Clostridium*-colonized GF mice demonstrated a marked increase in the number of $CD4^+$ Tregs in the colon (Atarashi et al., 2011). Interestingly, a significant percentage of the Tregs were not positive for Helios, a transcription factor expressed by natural Tregs (Thornton et al., 2010), indicating that these Tregs were locally derived through regulatory signaling cascades (Atarashi et al., 2011). In line with these reports, our work has shown that oral treatment with a novel strain of *Lactobacillus acidophilus* deficient in lipoteichoic acid (LTA) effectively ameliorated inflammation-induced colitis and colonic polyposis, and restored intestinal homeostasis in experimental models (Mohamadzadeh

et al., 2011; Khazaie et al., 2012). Nonetheless, despite current advances in the field, the specific signals delivered by microbes to innate immune cells, particularly DCs, to foster tolerance are not completely understood. To this end, this review focuses on the immunomodulating characteristics of specific cell surface components of *L. acidophilus* and discusses potential mechanisms whereby LTA-deficient *L. acidophilus* is able to promote the suppression of pathogenic intestinal autoinflammation.

Lactobacillus acidophilus AND ITS SURFACE LAYER COMPONENTS

Oral consumption of probiotics has been associated with multiple health benefits, including induction of mucus-secreting cells, maintenance of intestinal permeability, production of antimicrobial factors, colonization resistance, and immune cell activation or regulation (Gareau et al., 2010). Attesting to the importance of a well-balanced microflora, several systemic and intestinal disorders are associated with gut dysbiosis or alterations in the intestinal microbial composition (Nishikawa et al., 2009; De Palma et al., 2010; Giongo et al., 2011; Blumberg and Powrie, 2012; Jeffery et al., 2012). Among the beneficial bacteria used to maintain physiological intestinal balance, lactobacilli have been tested in clinical trials with favorable outcomes (Ouwehand et al., 2002). These benefits are, in part, due to induced changes in the immune system, as specific *Lactobacillus* species are known to stimulate DCs to produce stimulatory and regulatory cytokines that direct subsequent T cell responses (Christensen et al., 2002; Mohamadzadeh et al., 2005; Konstantinov et al., 2008). The immunomodulatory effects of lactobacilli are attributed to the interactions between bacterial cell surface components and pattern recognition receptors (PRRs) expressed on innate cells, such as Toll-like receptors (TLRs) and C-type lectins (CLRs; Konstantinov et al., 2008; Mohamadzadeh et al., 2008). Given the species-specific differential signaling of lactobacilli cell surface components, detailed examination of these proteins is imperative for the achievement of tailored immune responses. Dissecting the downstream consequences of host immune cell–microbial interactions is of particular importance in cases where preexisting inflammation or a propensity for inflammatory conditions might be exacerbated or promoted, respectively, by otherwise harmless bacterial constituents.

Lactobacillus acidophilus, one of the most widely consumed beneficial microbes (Sanders and Klaenhammer, 2001), is a Gram-positive bacterium that expresses the highly conserved LTA and other surface-exposed (S-layer) molecules, such as the proteins encoded by *slpA*, *slpB*, and *slpX*. S-layers have putative roles in cell adhesion, cell shape determination, as protective barriers, and as anchoring sites for accessory proteins, all of which may contribute to bacterial survival and host–microbial cell interactions within the gastrointestinal tract. Under laboratory growth conditions, the dominant S-layer protein found on *L. acidophilus* is SlpA (Boot et al., 1996), which is coexpressed with the lesser expressed protein SlpX (Goh et al., 2009). On the other hand, SlpB, due to a chromosomal inversion, is only coexpressed with SlpX in a small fraction of laboratory-grown *L. acidophilus* (Boot et al., 1996) or in some mutants devoid of SlpA (Boot et al., 1996; Konstantinov et al., 2008; Goh et al., 2009). While deletion of SlpA leads to decreased binding ability *in vitro* (Buck et al., 2005), the absence of SlpX did not result in morphological changes, reduced adherence to epithelial cells *in vitro*, or increased sensitivity to cellular stresses (Goh et al., 2009). Still, a *L. acidophilus* mutant lacking SlpX and SlpB is cleared faster *in vivo* than the wild-type strain (Zadeh et al., 2012), suggesting that SlpX and SlpB, albeit to a lesser extent, may also contribute to the gastrointestinal interactions of *L. acidophilus*. In terms of immunomodulatory effects, DCs stimulated *in vitro* with a SlpB-dominant strain ($SlpA^-$) produced higher levels of the proinflammatory cytokines IL-12 and tumor necrosis factors-alpha (TNF-α) than those challenged with the parental *L. acidophilus* strain ($SlpA^+$; Konstantinov et al., 2008), indicating a potential regulatory role for *L. acidophilus* SlpA that could very well account for our recent exciting observations (Mohamadzadeh et al., 2011; Khazaie et al., 2012). Additionally, the $SlpA^-$ mutant demonstrated reduced binding to DC-specific ICAM-3-grabbing non-integrin (DC-SIGN), a CLR expressed on DCs, and no differences in the ability to activate TLR2 (Konstantinov et al., 2008), implying that *L. acidophilus* SlpA does not signal to DCs via TLR2. Conversely, *L. helveticus*-derived SlpA, although very similar to *L. acidophilus* SlpA, was recently reported to downregulate inflammation-associated gene expression when tested *in vitro* using an epithelial cell line, but promoted proinflammatory effects in macrophages via TLR2, also *in vitro* (Taverniti et al., 2012). The authors ascribed these discrepancies to differences in the models employed; nonetheless, the *in vivo* role of *L. acidophilus* SlpA remains to be elucidated and is currently under extensive scrutiny in our laboratories to decipher its immunoregulatory effects using a range of experimental animal models.

In contrast, LTA is regarded as the Gram-positive counterpart of the potent and proinflammatory Gram-negative stimulus, lipopolysaccharide (LPS; Sriskandan and Cohen, 1999; Su et al., 2006). LTA is a zwitterionic glycolipid found in the cell wall of many Gram-positive bacterial strains, including *L. acidophilus*, which is believed to facilitate adhesion, colonization, and invasion of host cells (Reichmann and Gründling, 2011). In addition to the likely role of LTA in *Lactobacillus* adhesion to mucosal surfaces, this molecule promotes immune cellular activation via TLR2 signaling, which then activates downstream proinflammatory cytokine signaling cascades (Schwandner et al., 1999; Chiu et al., 2009; Chang et al., 2010; Saber et al., 2011). Notwithstanding, conflicting reports suggested that LTA from certain *Lactobacillus* species induces anti-inflammatory cytokine production (IL-10), and only results in the generation of proinflammatory mediators in preexisting inflammatory conditions [i.e., co-culture with interferon-gamma (IFN-γ); Kaji et al., 2010; Kang et al., 2011]. Taken together, these data contend that the functions of LTA might differ between bacterial species (beneficial lactobacilli versus pathogenic) as well as depend on the status of the local cytokine milieu (steady state versus proinflammatory). However, a caveat of these studies is that the work was performed *in vitro*, which prompts the following question: what is the physiological role of lactobacilli-derived LTA?

IMMUNE REGULATION INDUCED BY LTA-DEFICIENT *L. acidophilus*

To clarify the *in vivo* effects of *L. acidophilus*-LTA, we recently developed a *L. acidophilus* strain lacking the gene encoding

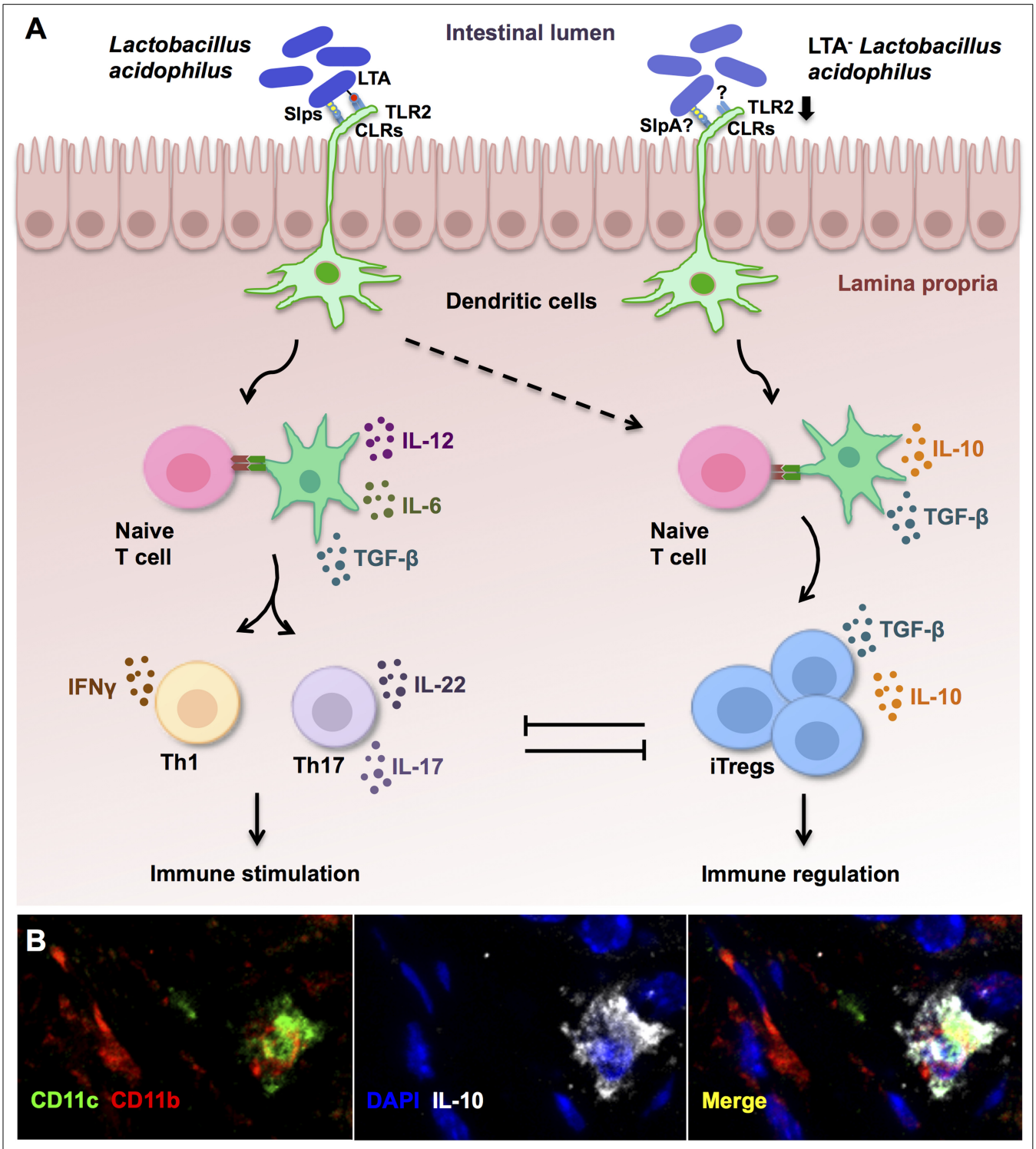

FIGURE 1 | Immune regulation established by lipoteichoic acid (LTA)-deficient *Lactobacillus acidophilus*. (A) In steady state conditions, molecules expressed on the cell surface of *L. acidophilus* activate dendritic cells (DCs) to promote effector Th1 and Th17 responses that are held in check by the accompanying generation of induced regulatory T cells (iTregs). However, in preexisting inflammation or susceptible individuals, immune activation by *L. acidophilus*-LTA exacerbates inflammatory responses and fails to promote immune regulation. Oral intake of mutant strains lacking LTA expression (LTA$^-$ *L. acidophilus*) predominantly results in suppression of exacerbated immune responses via the induction of regulatory IL-10-secreting DCs **(B)**, which then promote the conversion of naive T cells into iTregs. **(B)** Confocal microscopy analysis of DCs (CD11c$^+$, green; CD11b$^+$, red) that produce IL-10 (white) in the colons of healthy control mice after treatment with LTA-deficient *L. acidophilus*.

phosphoglycerol transferase, an enzyme required for the biosynthesis of LTA. As opposed to treatment with the wild-type strain, oral inoculation with LTA-deficient *L. acidophilus* not only prevented chemical and pathogenic T cell-induced colitis, but also quickly resolved established colitis, as measured by diminished percent weight loss, lower diarrhea and fecal occult blood scores, and reduced disease activity index (Mohamadzadeh et al., 2011). By the same token, LTA-deficient *L. acidophilus* dramatically reversed colonic preneoplasia in genetically predisposed animals (Khazaie et al., 2012). While protection from colitis in our studies correlated with an increase in IL-10-producing DCs and the number of iTregs (Mohamadzadeh et al., 2011; Khan et al., 2012), polyposis reversal coincided with an overall dampening of local and systemic immunity that was linked with restoration of Treg function and stability (Khazaie et al., 2012). Importantly, proinflammatory Tregs have also been identified in colorectal cancer (CRC) patients (Blatner et al., 2012), further supporting the clinical applicability of LTA-deficient *L. acidophilus* for the treatment of intestinal maladies given its potential ability to prevent the formation of proinflammatory $FoxP3^{+}ROR\gamma t^{+}$ Tregs.

Moreover, *in vitro* co-culture of DCs with LTA-deficient *L. acidophilus* led to a regulatory DC phenotype, as demonstrated by enhanced IL-10 secretion, low expression of costimulatory molecules, and concomitant decreases in IL-12 and TNF-α production. Alternatively, no beneficial effects could be induced in $IL\text{-}10^{-/-}$ mice *in vivo*, highlighting the important role of this anti-inflammatory cytokine in the control of pathogenic intestinal inflammation in our system, similar to previous findings by others (Asseman et al., 1999; Grangette et al., 2005; Rubtsov et al., 2008). Activation of mitogen-activated protein kinases (MAPK) signaling pathways differentially controls features of both innate and adaptive immune responses (Dong et al., 2002). Favored IL-10 production by regulatory DCs has previously been found to be dependent on extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) activation, while suppressed IL-12 secretion resulted from impaired p38 activation (Qian et al., 2006). Indeed, significant and sustained ERK1/2 activation was measured in the colonic tissues of mice orally treated with LTA-deficient *L. acidophilus*, whereas the wild-type strain promoted p38 phosphorylation (Saber et al., 2011). Furthermore, DC stimulation with LTA-deficient *L. acidophilus* resulted in only weak TLR2-dependent cytokine production and did not enhance the expression of this PRR; these data indicate that LTA is in fact the proinflammatory molecule most strongly associated with TLR2 activation by *L. acidophilus* in DCs, and that the *in vivo* regulatory response noted after LTA-deficient *L. acidophilus* treatment is a direct consequence of its absence. Collectively, the favorable effects of LTA-deficient *L. acidophilus* may be due to weak TLR2 activation and downstream signaling, together with the predominant activation of alternative DC-related PRRs, such as CLRs (Konstantinov et al., 2008), by different surface-associated molecules, including SlpA (summarized in **Figure 1A**).

CONCLUDING REMARKS AND FUTURE DIRECTIONS

Although the exact signaling pathways whereby LTA-deficient *L. acidophilus* promotes the generation of regulatory DCs and, consequently, iTregs, are currently under intensive investigation, data obtained thus far clearly demonstrate that IL-10-dependent pathways (**Figure 1**) underlie the protective effects of LTA-deficient *L. acidophilus*. In addition, work by others point to SlpA as a potential regulatory molecule in *L. acidophilus* (Konstantinov et al., 2008). Notably, as seen in the wild-type *L. acidophilus* strain, the presence of this S-layer protein alone is not sufficient to counterbalance the proinflammatory actions of LTA. Additional studies performed in our laboratories demonstrated that a mutant strain expressing LTA and SlpA, but not SlpX and SlpB, was unable to afford any protection against colitis (Zadeh et al., 2012). In fact, oral treatment with this $LTA^{+}SlpA^{+}$ *L. acidophilus* strain led to a higher number of TNF-α-producing colonic DCs, in addition to sustained IL-12 production by DCs in the colon, when compared to the LTA-sufficient parental strain (Zadeh et al., 2012). These findings may be interpreted to imply that the other S-layer proteins expressed by *L. acidophilus* NCFM also contribute to the regulation of LTA-induced inflammation; however, attempted deletion of SlpA in this strain resulted in slightly lower expression levels of the protein when compared to the parental strain, which then suggests that even small perturbations in the amount of SlpA expressed can exacerbate LTA-mediated inflammation. Consequently, ongoing studies aim to investigate the specific contribution of the S-layer components (i.e., SlpA) to conserve and support gut homeostasis by creating restricted mutant strains of *L. acidophilus* using molecular techniques previously described (Goh et al., 2009) and purifying our protein of interest, SlpA. Thus, the therapeutic value of both $SlpA^{+}SlpB^{-}SlpX^{-}LTA^{-}$ *L. acidophilus* and purified SlpA will be determined *in vivo*.

In other respects, it is likely that LTA-deficient *L. acidophilus* confers additional benefits to the host through mechanisms independent of the immunomodulatory effects mentioned above. For instance, intestinal epithelial cells not only create a protective barrier against invading pathogens, but also sense and interact with microbes through PRRs to influence subsequent innate immune responses (Wells et al., 2011). Accordingly, the status of the mucosal epithelium is central to gastrointestinal health and accumulating evidence indicates that aberrant epigenetic modification of colonic tissue contributes to CRC development (Lao and Grady, 2011). As these changes can arise in the presence or absence of pathogenic intestinal inflammation, we recently tested the effects of LTA-deficient *L. acidophilus* treatment on the epigenetic landscape of the intestinal mucosa and found that this bacterium induced the expression of CRC-associated, epigenetically controlled genes that are often downregulated in cancer-promoting pathogenic conditions (Lightfoot et al., 2012). These important results create a strong position to precisely define the bacterial gene products that may dampen detrimental gut inflammation and protect against inflammatory conditions, including inflammatory bowel disease and colon cancer, not only through immune cell modulation, but also via direct interactions with the gut epithelium.

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The nature of activatory and tolerogenic dendritic cell-derived signal II

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Dendritic cells (DCs) are central in maintaining the intricate balance between immunity and tolerance by orchestrating adaptive immune responses. Being the most potent antigen presenting cells, DCs are capable of educating naïve T cells into a wide variety of effector cells ranging from immunogenic $CD4^+$ T helper cells and cytotoxic $CD8^+$ T cells to tolerogenic regulatory T cells. This education is based on three fundamental signals. Signal I, which is mediated by antigen/major histocompatibility complexes binding to antigen-specific T cell receptors, guarantees antigen specificity. The co-stimulatory signal II, mediated by B7 family molecules, is crucial for the expansion of the antigen-specific T cells. The final step is T cell polarization by signal III, which is conveyed by DC-derived cytokines and determines the effector functions of the emerging T cell. Although co-stimulation is widely recognized to result from the engagement of T cell-derived CD28 with DC-expressed B7 molecules (CD80/CD86), other co-stimulatory pathways have been identified. These pathways can be divided into two groups based on their impact on primed T cells. Whereas pathways delivering activatory signals to T cells are termed co-stimulatory pathways, pathways delivering tolerogenic signals to T cells are termed co-inhibitory pathways. In this review, we discuss how the nature of DC-derived signal II determines the quality of ensuing T cell responses and eventually promoting either immunity or tolerance. A thorough understanding of this process is instrumental in determining the underlying mechanism of disorders demonstrating distorted immunity/tolerance balance, and would help innovating new therapeutic approaches for such disorders.

Keywords: activation, tolerance, co-stimulation, co-inhibition, dendritic cells, T cell priming

INTRODUCTION

The immune system is endowed with the unique capacity to protect against invading pathogens, yet not react to self. Among the different constituents of the immune system, dendritic cells (DCs) play a central role in drawing the thin line between immunity and tolerance. Discovered in 1973 (Steinman and Cohn, 1973), DCs are recognized as the most potent antigen presenting cells (APCs). Their ability to initiate and modulate various forms of T cell responses, earned them the position of being master orchestrators of adaptive immunity (Banchereau and Steinman, 1998). DCs are spread throughout the body, residing in different tissues as sentinels, monitoring their surrounding environment for any signs of danger. Equipped with pathogen recognition receptors (PRRs), DCs are capable of sensing pathogenic invasion (Medzhitov and Janeway Jr., 2002) and self-structures associated with cellular stress (Matzinger, 2002). Upon danger sensing, DCs will undergo functional changes, also known as maturation, crucial for the ensuing induction of T cell responses (Banchereau et al., 2000). A hallmark of DC maturation is the expression of the chemokine receptor CCR7 that allows mature DCs to migrate to draining lymphoid tissues where they activate naïve T cells in a process based on three signals. The first signal results from the ligation of T cell receptors (TCRs) to pathogen-derived peptide antigens that are presented by major histocompatibility complex (MHC) molecules of DCs, which are upregulated upon maturation. This principal stimulation signal is important to assure antigen specificity of the immune response. Although TCR triggering is crucial for naïve T cell activation, it is not sufficient by itself to initiate an efficacious immune response. The concept of a second co-stimulatory signal was first introduced by Lafferty and Woolnough (1977). They deduced from organ transplantation studies that alloantigens presented by transplanted tissues failed to elicit any immune responses unless accompanied by hematopoietic stimulator cells (Lafferty and Woolnough, 1977). This concept was corroborated by seminal observations by the group of Schwartz, implying that T cells activated solely by TCR engagement were rendered unresponsive and anergic (Jenkins and Schwartz, 1987). This was followed by the discovery of the main elements of co-stimulation: CD28 (Aruffo and Seed, 1987) and CD80 (Freeman et al., 1989), the latter being initially identified as a B cell activation marker and eventually recognized as the ligand of CD28 (Linsley et al., 1990). Subsequently, more pathways contributing to signal II were identified. Based on the nature of their signal, these molecules can be divided into co-stimulatory molecules that promote T cell proliferation, and co-inhibitory molecules that attenuate T cell responses. The nature of signal II is vital in determining the T cell response, which is further defined by a third polarizing signal. This third signal promotes the selective development of naïve T cells into one of the identified types of effector or tolerogenic T cells (De Jong et al., 2005). Although signal III is generally recognized to be mediated

by soluble DC-derived cytokines, there are indications that signal II may also contribute to T cell polarization. A final putative DC-derived signal is suggested to provide polarized T cells with homing directions to the site of infection or injury (Sigmundsdottir and Butcher, 2008). Thus, DCs control the delicate balance between immunity and tolerance through the signals they convey to T cells.

Although the combined effect of all DC-derived signals is important for full blown T cell responses, signal II is key for allowing these responses and licensing them to become either immunogenic or tolerogenic. Here, we shed light on the multifaceted signal II by reviewing current knowledge of to date identified co-stimulatory and co-inhibitory pathways (**Figure 1**), their mode of action, relation to disease, and any possible clinical applications based on utilizing these pathways.

CO-STIMULATORY MOLECULES

CD80/CD86/CD28 PATHWAY

Following the discovery of the CD80/CD28 interaction, B7-2 (CD86) was identified as a second ligand for CD28 (Azuma et al., 1993). The CD80/CD86/CD28 pathway was suggested to deliver the strongest co-stimulatory pathway as CD28-deficient cells failed to proliferate in the presence of APCs (Green et al., 1994). The consequences of CD28 engagement by its ligands comprise stimulation of T cell proliferation, dramatic upregulation of IL-2 (Linsley et al., 1991a), promotion of T cell survival by enhancing Bcl-XL expression (Boise et al., 1995), and enhanced glycolytic flux to meet energetic requirements associated with a sustained response (Frauwirth et al., 2002). Those effects were shown to be dependent on activating the signaling cascades of phosphoinositide-3 kinase (PI3K), protein kinase B (PKB, also known as Akt), and nuclear factor kappaB (NF-κB; Song et al., 2008).

Several reports pointed out a possible role for CD28 signaling in T cell polarization. Murine T cells were shown to produce enhanced levels of IL-4 and IL-5, characteristic for T helper (Th) 2, upon strong CD28 stimulation (Rulifson et al., 1997). Strong CD28 signaling was also demonstrated to inhibit Th17 responses (Purvis et al., 2010). Although it is generally accepted that memory T cells, unlike naives, are less dependent on co-stimulation via CD28, it was shown that this co-stimulatory pathway is important in controlling T cell recall responses (Ndejembi et al., 2006).

In addition to its key role in initiating and sustaining efficient T cell responses, the CD28 pathway is also involved in controlling immune tolerance. Co-stimulation of developing thymocytes by CD28 was shown to induce the expression of Foxp3 and promote the differentiation of regulatory T cells (Tregs; Tai et al., 2005). Furthermore, T cell activation in the absence of CD28 co-stimulation leads to a state of anergy characterized by dramatically reduced production of IL-2 and other effector cytokines upon subsequent TCR triggering (Schwartz, 1997). There is ample evidence that DCs utilize this mechanism to maintain tolerance to self. At steady state conditions, immature DCs present self-derived antigens accompanied by low levels of CD80/CD86 and therefore fail to supply specific T cells with adequate signal II, leading eventually to the deletion, anergy, or regulation of auto-reactive T cells that escaped thymic selection (Steinman and Nussenzweig, 2002). Thus, the CD80/CD86/CD28 pathway is as involved in promoting tolerance as in mediating immunity.

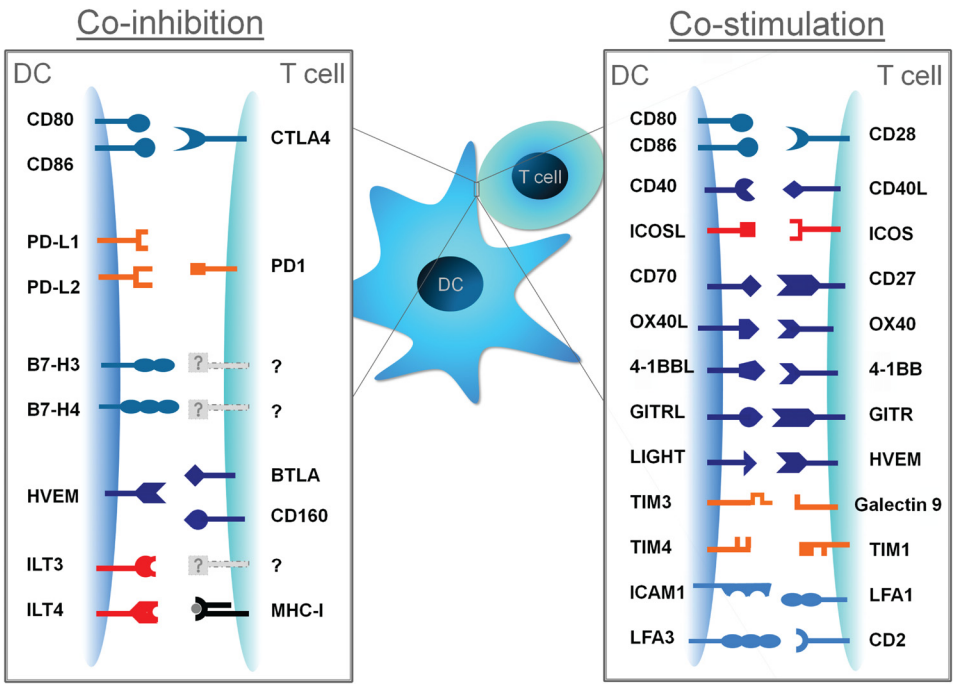

FIGURE 1 | Co-stimulatory and co-inhibitory molecules and their cognate ligands. DC-derived signal II can promote T cell activation when conveyed by co-stimulatory molecules, or can attenuate T cell responses when conveyed by co-inhibitory molecules.

Since many immunogenic tumors lack expression of CD80 and CD86, it was postulated that tumor-infiltrating T cells would receive chronic TCR stimulation without co-stimulation leading to T cell anergy. This hypothesis was tested by inducing the expression of CD80/CD86 molecules on tumor cells prior to injection into mice. Forced expression of CD80/CD86 in tumor cells resulted into $CD8^+$ T cell-dependent tumor rejection (Townsend and Allison, 1993). However, this method had barely any effect on pre-established tumors (Fallarino et al., 1997), implying that other pathways promoting immune tolerance toward established tumors are involved.

CD40/CD40L PATHWAY

CD40 was the first co-stimulatory molecules to be identified from the tumor necrosis factor (TNF) receptor (TNFR) family. First discovered as B cell receptor, CD40 is also expressed by DCs, macrophages, epithelial cells, and even activated T cells. Its ligand (CD40L or CD154), a member of the TNF family, is expressed not only by activated T cells, but also by natural killer (NK) cells and plasmacytoid DCs (pDCs; Quezada et al., 2004). In addition to promoting humoral immunity by activating B cells, the CD40/CD40L pair is pivotal for cellular immunity as it mediates a dialog between T cells and DCs. Indeed, CD40 engagement on DCs was shown to activate NF-κB pathway (Quezada et al., 2004) and consequently inducing DC maturation (Caux et al., 1994) and enhancing DC longevity (Miga et al., 2001). Initially, CD40-induced maturation of DCs was suggested to be sufficient in licensing $CD8^+$ cytotoxic responses (Schoenberger et al., 1998). However, further investigation in the CD40 pathway revealed that additional signals are necessary for optimal DC activation. CD40 cross-linking alone is not enough to induce IL-12 production, necessary for cytotoxic and Th1 responses, but DC pre-activation by microbial products followed by CD40 ligation dramatically increased IL-12 production (Schulz et al., 2000). This finding indicates that combined triggering of CD40 and PRRs, like Toll-like receptors (TLRs), is critical for DC licensing. The CD40-induced IL-12 also implies a central role for CD40/CD40L pathway in T cell differentiation, by favoring Th1 polarization. Blocking CD40/CD40L interactions lead to abrogated Th1 responses with reciprocal upregulation of Th2 cytokines (Hancock et al., 1998).

The adjuvant effect of CD40 ligation, reflected by DC activation, prompted the application of agonistic anti-CD40 antibodies for cancer therapy. Injecting agonistic anti-CD40 antibodies evoked cytotoxic T cell responses and eradicated the tumor in a mouse model of lymphoma (French et al., 1999). Furthermore, application of fully humanized anti-CD40 agonistic antibody resulted in objective partial responses in 14% of advanced solid tumor patients (Vonderheide et al., 2007). A similar approach was based on the administration of soluble CD40L, which was less efficient as it lead to partial responses in 6% of treated tumor patients (Vonderheide et al., 2001). More clinical trials applying CD40 ligation, singularly or in conjunction with other therapeutic modalities, were carried out and showed promising results (Khong et al., 2012).

Due to its activatory nature, the CD40/CD40L is decisive in regulating tolerance. It was shown that DCs derived from CD40-deficient mice conferred tolerance by priming IL-10 secreting Tregs (Martin et al., 2003). This effect on tolerance prompted investigating the possibility of exploiting CD40 blocking to enhance allograft survival. Although applying anti-CD40L antibodies as a monotherapy was able to block many effector mechanisms, it failed to induce sufficient allograft tolerance (Jones et al., 2000). However, combinations with other immunosuppressive therapies such as cytotoxic T lymphocyte (CTL)-associated antigen-4-immunoglobulin (CTLA-4-Ig; Larsen et al., 1996) and rapamycin (Li et al., 1998) were shown to result in long-term graft survival. Collectively, CD40/CD40L pathway, in conjunction with other pathways, is vital for initiating active immunity and regulating tolerance.

ICOSL/ICOS PATHWAY

The inducible T cell co-stimulator (ICOS) was identified as the third member of the CD28/CTLA-4 family of co-stimulatory molecules (Hutloff et al., 1999). ICOS expression by T cells requires prior TCR activation and CD28 co-stimulation (McAdam et al., 2000). The ligand (ICOSL) is expressed by DCs (Wang et al., 2000), B cells, and a variety of non-hematopoietic tissues (Ling et al., 2000). ICOSL/ICOS pathway exerts its co-stimulatory effects on already activated T cells by supporting proliferation and cytokine production (Hutloff et al., 1999). Additionally, ICOS is proposed to play an important role in T cell polarization. Initially, ICOSL/ICOS was suggested to support Th2 responses. Blocking ICOSL/ICOS interactions was shown to block Th2-lead airway responses without influencing Th1-mediated inflammation (Coyle et al., 2000). Similarly, another study showed that the majority of T cells expressing ICOS *in vivo* co-produced Th2-type cytokines (Lohning et al., 2003). In contrast, disrupting ICOSL/ICOS pathway was found to inhibit Th1-mediated disorders like allograft rejection (Guo et al., 2002) and experimental allergic encephalomyelitis (Rottman et al., 2001). ICOS was shown to be involved driving Th17 responses (Park et al., 2005), further complicating the role of ICOSL/ICOS in T cell polarization. An attempt to resolve this controversy was by showing that engaging ICOS on activated T cells amplified the effector responses of these cells regardless of their polarized state (Wassink et al., 2004).

Benefiting of the activatory effect of ICOSL/ICOS pathway in the context of cancer therapy was evaluated. Induced ICOSL expression on tumor cells was demonstrated to promote tumor regression by inducing CD8 cytotoxicity (Liu et al., 2001). Nevertheless, this strategy was ineffective in case of weakly immunogenic tumors (Ara et al., 2003). Surprisingly, it was recently revealed that tumor cell-expressed ICOSL augments Treg activation and expansion within the tumor local environment (Martin-Orozco et al., 2010). This suggests that triggering ICOSL/ICOS pathway may not be the most optimal option for cancer treatment. On the contrary, blocking its ICOSL/ICOS-mediated suppression may be beneficial in cancer therapy.

The tolerogenic effect of ICOSL/ICOS pathway is not restricted to tumors, as there are indications of its involvement in maintaining immune tolerance. ICOS-deficient mice displayed reduced numbers of natural Tregs (nTregs), which may be owed to a decrease in survival and/or proliferation of these cells (Burmeister et al., 2008). Another indication of ICOS involvement in tolerance

is the finding that ICOS triggering on T cells dramatically increased the production of the anti-inflammatory cytokine IL-10 (Hutloff et al., 1999). Consistently, high ICOS expression by T cells was selectively associated with the anti-inflammatory IL-10 (Lohning et al., 2003). These findings argue for targeting ICOSL/ICOS pathway to induce tolerance for therapeutic purposes. However, it is very important to clearly dissect the conditions under which this pathway induces activation or tolerance.

CD70/CD27 PATHWAY

CD70 is another member of the TNF family of co-stimulatory molecules. Its ligand CD27 was identified first as a novel T cell differentiation antigen (van Lier et al., 1987). The contribution of CD27 to immunity was later recognized to be dependent on its binding partner CD70, which is expressed under the control of antigen receptors and TLRs in lymphocytes and DCs, respectively (Tesselaar et al., 2003). Similar to CD40, engaging CD27 induced the activation of NF-κB pathway (Akiba et al., 1998). The first indication of the co-stimulatory properties of the CD70/CD27 pathway was provided by triggering CD27, which augmented CD3-induced T cell proliferation (van Lier et al., 1987). This effect was later explained by promoting survival of newly stimulated T cells, in contrast to CD28 that prompts cell cycle entry and induces proliferation (Hendriks et al., 2003). This survival effect relies completely on IL-2 receptor signaling and the autocrine production of IL-2 (Peperzak et al., 2010).

The contribution of CD70/CD27 pathway to T cell polarization is debatable. $CD8^+$ T cells from CD27 knockout mice maintained the capacity of differentiation into CTLs and interferon-gamma (IFN-γ) production, implying that CD27 is not involved in the development of cytotoxic CD8 responses (Hendriks et al., 2000). On the other hand, transgenic expression of CD70 on steady state immature DCs was found to break $CD8^+$ tolerance and permit the differentiation of effector $CD4^+$ and $CD8^+$ cells from naïve precursors (Keller et al., 2008). Moreover, the murine $CD8\alpha^+$ DC subset was revealed to favor the differentiation of Th1 cells in a CD70-dependent and IL-12-independent mechanism (Soares et al., 2007). This is further supported by showing that human Langerhans cells (LCs), an epidermal subset of DCs, are capable of inducing $CD8^+$ anti-viral responses in a CD70-dependent manner (van der Aar et al., 2011). A recent study also demonstrated that CD70/CD27 pathway impedes the differentiation of Th17 effector cells and attenuates accompanying autoimmunity in a mouse model of multiple sclerosis (Coquet et al., 2012). These findings imply that CD70 involvement in T cell polarization may depend on the type of DCs expressing CD70 and the type of stimuli to which these DCs are exposed.

The activatory effect of CD70/CD27 pathway can be exploited for anti-tumor therapy. Induced expression of both CD70 and CD40L by tumor cells was shown to impede tumor growth and initiate anti-tumor immunity (Couderc et al., 1998). Furthermore, the application of CD70, encoded in a vaccinia virus, was shown to confer protection against introduced tumors (Lorenz et al., 1999). Evidence for possible clinical benefit from mobilizing the CD70/CD27 pathway was provided by a recent clinical trial utilizing DCs expressing CD70, CD40L, and constitutively active TLR4 (TriMix-DC) in the treatment of metastatic melanoma patients. These TriMix-DCs were able to initiate a broad anti-tumor T cell response, resulting in prolonged progression-free survival (Van Nuffel et al., 2012). This paves the way for a novel strategy in cancer immunotherapy based on mobilizing the CD70/CD27 pathway.

Several reports have implicated CD70/CD27 pathway in autoimmunity. Elevated expression of CD70 by pathogenic T cells was observed in rheumatoid arthritis (Lee et al., 2007) and lupus erythematosus patients (Han et al., 2005). Moreover, blocking CD70/CD27 pathway seems to help ameliorating inflammation in mouse models of arthritis (Oflazoglu et al., 2009) and colitis (Manocha et al., 2009). However, the study reporting Th17 inhibiting effects of CD70 signaling (Coquet et al., 2012) may argue against the blockade of CD70/CD27 pathway, especially since Th17 effector cells are involved in various auto-inflammatory diseases.

OX-40L/OX-40 PATHWAY

OX-40L and OX-40 belong to the TNF family and TNFR family, respectively. OX-40, also known as CD134, was first described on activated $CD4^+$ T cells (Paterson et al., 1987). The expression of OX-40 is in fact restricted to recently antigen-activated T cells and not naïve or memory T cells, implying that it is specialized in delivering co-stimulation to activated T cells (Sugamura et al., 2004). The ligand, OX-40L (CD252), is expressed on DCs and macrophages, especially after TLR or CD40 ligation (Ohshima et al., 1997). Additionally, responding T cells express OX-40L themselves (Soroosh et al., 2006). Engagement of OX-40 on T cells promotes long-term survival by inducing the expression of the anti-apoptotic molecules Bcl-2 and Bcl-xL (Rogers et al., 2001). This study suggests that the differential expression kinetics of OX-40 and CD28, the latter being constitutively expressed by T cells, bares functional specialization. Whereas CD28 is essential for the initial priming of naïve T cells into effector T cells, OX-40 is crucial for the expansion (later proliferation) and survival of these effector cells.

Several studies have pointed out a central role for OX-40 in regulating the balance between Th1 and Th2 responses. Co-stimulating T cells through OX-40 was shown to induce IL-4 expression and inhibited IFN-γ production (Flynn et al., 1998). Furthermore, DC treatment with thymic stromal lymphopoietin (TSLP), known for its Th2 skewing properties, leads to the expression of OX-40L and the subsequent priming of Th2 cells (Ito et al., 2005). OX-40-favored Th2 response was proposed to be mediated by an initial induction of nuclear factor of activated T cells (NFAT) c1 in an IL-4 receptor-independent manner, followed by an IL-4 receptor-dependent effect on GATA-3 (So et al., 2006). However, it was shown later that DC-derived OX-40L maintained both Th2 and Th1 responses, owed to OX-40-enhanced survival of effector T cells regardless of their polarization (Jenkins et al., 2007). Thus, it seems that the role of OX-40/OX-40L in the differentiation of Th2 cells is restricted to promoting the survival of already established Th2 cells that differentiated under the effect of other DC-derived factors.

OX-40/OX-40L is also involved in controlling immune tolerance. The first evidence of this role is the expression of significant amounts of OX-40 on naturally occurring $Foxp3^+$ Tregs. OX40 signaling appears to be dispensable for the development of nTregs,

since this population exists in OX-40-deficient mice. However, OX-40 signaling is important for the survival of nTregs as OX-40-deficient mice displayed lower counts of this population of Tregs (Takeda et al., 2004). The effect of OX-40 triggering on the functions of nTregs remains controversial. Whereas one study showed that OX-40 signaling in $CD4^+$ T cells render them resistant to suppression by nTregs (Takeda et al., 2004), another study reported abrogated suppression following OX-40 triggering on nTregs (Valzasina et al., 2005). Another mechanism by which OX-40L/OX-40 is assumed to contribute to tolerance regulation is by influencing the development of induced Tregs (iTregs). Under conditions promoting iTreg differentiation, OX-40 engagement on T cells was shown to inhibit Foxp3 expression by these T cells (So and Croft, 2007). Nevertheless, the surrounding environment during iTreg differentiation seems to determine the outcome of OX-40 signaling, which was reported to promote the expansion of iTregs if IL-4 and IFN-γ were absent from the milieu (Ruby et al., 2009). In conclusion, OX-40L/OX-40 appears to be central in maintaining the survival of T cells in general, but its influence on T cell functions requires further elucidation.

4-1BBL/4-1BB PATHWAY

4-1BB (CD137) is yet another member of the TNFR family. Its expression is induced on T cells following TCR activation (Pollok et al., 1993). The ligand, 4-1BBL of the TNF family, is expressed on activated APCs (Vinay and Kwon, 1998). Engagement of T cell 4-1BB was reported to induce IL-2 production independently of CD28, when accompanied by strong TCR signaling (Saoulli et al., 1998). Furthermore, 4-1BB interaction with its ligand was demonstrated to provide a co-stimulatory signal particularly to $CD8^+$ T cells, enhancing proliferation, cytotoxicity (Shuford et al., 1997), and survival (Lee et al., 2002). Similar to other TNFR family members, 4-1BB enhanced survival is dependent on NF-κB activation, which in turn induces the two pro-survival molecules: Bcl-xL and Bfl-1 (Lee et al., 2002). When compared to co-stimulation with CD80/CD86, 4-1BBL appears to be more effective in driving $CD8^+$ memory T cells into a fully differentiated effector state (Bukczynski et al., 2004). Furthermore, 4-1BB ligation was also shown to augment Th1 cytokines and suppress Th2 cytokines, implying a possible role for 4-1BB in T cell polarization (Kim et al., 1998). Collectively, these properties raised the interest in 4-1BBL/4-1BB pathway as potential therapeutic target especially in cancer therapy. Several studies demonstrated a beneficial effect of activating 4-1BB in inducing anti-tumor immunity and tumor regression thereafter (Driessens et al., 2009). Nevertheless, great caution should be taken before transferring these observations into clinical applications especially after reporting possible tolerogenic effects following 4-1BB triggering. Engaging 4-1BB by agonist antibodies was reported to ameliorate the severity of autoimmunity in murine models of experimental autoimmune encephalomyelitis (EAE) (Sun et al., 2002) and systemic lupus erythematosus (Foell et al., 2003), and to inhibit rejections of intestinal allografts in mice (Wang et al., 2003). These findings imply a link between 4-1BBL/4-1BB pathway and tolerance. Indeed, 4-1BB co-stimulation was shown to synergize with IL-2 in promoting nTreg expansion (Elpek et al., 2007). In an experimental model of rheumatoid arthritis, treatment with 4-1BB agonist antibodies inhibited disease progression, which was attributed to the induction of indoleamine 2,3-dioxygenase (IDO; Seo et al., 2004). Altogether, 4-1BBL/4-1BB pathway contributes to immunity and tolerance, allowing multiple therapeutic applications through this pathway.

GITRL/GITR PATHWAY

Glucocorticoid-induced TNFR related gene (GITR) was first discovered as a dexamethasone-induced molecule in murine T cell hybridomas (Nocentini et al., 1997). The expression of the human ortholog was subsequently identified in human lymphocytes and shown to be independent of glucocorticoid treatment. Similar to the TNFR family members OX-40 and 4-1BB, GITR is only expressed on recently activated T cells, implying a role in promoting effector functions rather than involvement in initial priming of naïve T cells (Gurney et al., 1999). The GITR ligand (GITRL) is expressed by APCs and is upregulated upon activation (Tone et al., 2003). GITRL/GITR pathway provides co-stimulation to naïve T lymphocytes demonstrated by enhanced proliferation and effector functions in the setting of suboptimal TCR stimulation (Ronchetti et al., 2004). Additionally, GITR triggering promoted naïve T cell survival through the activation of NF-κB and mitogen-activated protein kinase (MAPK) pathways, though it was not sufficient to inhibit activation-induced cell death initiated by TCR signaling (Esparza and Arch, 2005). GITRL/GITR pathway does not seem to have an impact on T cell polarization. Although applying an agonist antibody against GITR initially enhanced Th2 responses in a mouse model of helminth infection, this effect was short lived and GITR-independent (van der Werf et al., 2011).

A role for GITRL/GITR pathway in immune tolerance was initially demonstrated by the constitutive expression of GITR on Tregs (Shimizu et al., 2002). Factually, Tregs isolated based on the expression of GITR could prevent the development of colitis induced in an adoptive transfer model (Uraushihara et al., 2003). However, engaging Treg-expressed GITR, by agonist antibodies, was shown to abrogate their suppressive capacity (Shimizu et al., 2002). In the beginning, this effect was interpreted by mere activation of Tregs upon GITR stimulation, but this explanation was underscored by the fact that Treg preincubation with anti-GITR did not cause the subsequent loss of suppression (Shimizu et al., 2002). Eventually, it was revealed that triggering GITR on effector T cells rendered them resistant to suppression by Treg (Stephens et al., 2004), providing a plausible explanation for the anti-tolerogenic effects of GITR stimulation. This postulates a model where APC-expressed GITRL would bind GITR on recently stimulated T cells allowing them to resist suppression. Simultaneously, GITR ligation on Tregs would allow their expansion and their subsequent domination at later stages of the immune response (Stephens et al., 2004).

Based on the activatory nature of GITRL/GITR pathway and its characteristic inhibition of tolerance, employing this pathway in cancer therapy was evaluated. The administration of an agonistic antibody against GITR has been shown to augment CD8 anti-tumor immunity (Cohen et al., 2006). In addition to mobilizing anti-tumor responses, triggering GITR was also shown to attenuate Treg-mediated suppression within the tumor (Ko et al., 2005), making GITRL/GITR a promising target for cancer therapy.

LIGHT/HVEM PATHWAY

The TNFR family member herpes virus entry mediator (HVEM) was initially discovered as a receptor for herpes simplex virus (Montgomery et al., 1996). It is expressed on resting T cells, monocytes, and immature DCs. HVEM has multiple binding partners: LIGHT and lymphotoxin-α (LT-α) from the TNF superfamily; and CD160 and B and T lymphocyte attenuator (BTLA) from the Ig superfamily. HVEM interaction with these ligands creates a complex network of pathways, which collectively regulates adaptive immune responses (Ware and Sedy, 2011). In this section we will only focus on the co-stimulatory pathway resulting from LIGHT/HVEM interactions. LIGHT is expressed by immature DCs (Tamada et al., 2000a) and is induced upon activation on T cells, in contrast to HVEM (Morel et al., 2000). LIGHT/HVEM interaction was revealed to be required for DC-mediated allogenic T cell responses. Indeed, activating T cell HVEM enhanced T cell proliferation at suboptimal TCR stimulation conditions (Tamada et al., 2000a). Disrupted LIGHT/HVEM interaction was shown to result in inhibited T cell proliferation, further supporting the importance of this pathway in co-stimulation (La et al., 2002). Similar to other TNFR family members, HVEM mediates its effects by activating NF-κB pathway (Harrop et al., 1998). Interestingly, LIGHT/HVEM pathway can also contribute to T cell activation indirectly by inducing DC maturation, reminiscent of the role of CD40 in inducing DC maturation (Morel et al., 2001). LIGHT/HVEM pathway is also suggested to contribute to T cell polarization. T cells co-stimulated through HVEM displayed enhanced production of Th1 cytokines (Tamada et al., 2000b). Accordingly, LIGHT-deficient mice showed reduced IFN-γ levels, prolonging allograft survival in these mice (Ye et al., 2002). Due to the complexity of the signaling network of HVEM and LIGHT, reported findings should be interpreted as these observations may involve other pathways.

TIM FAMILY

In addition to the CD28/B7 and TNFR/TNF co-stimulatory families, the recently identified TIM (T cell Ig domain and mucin domain) family is a new contributor to signal II. This family of genes was initially identified while searching for Th1-specific markers (Monney et al., 2002). In humans, three TIM family members: TIM1, TIM3, and TIM4 have been identified thus far. Mice posses an additional member: TIM2 (Kuchroo et al., 2008). In this section we will only focus on TIM3 and TIM4, which were reported to be expressed by DCs.

TIM3 was first discovered as a specific marker for Th1 cells (Monney et al., 2002), and was shown to induce the death of these cells by binding to its ligand galectin-9 (Zhu et al., 2005). TIM3 expression was also detected on DCs, and its ligation by galectin-9 induced the production of the inflammatory cytokine TNF-α. The absence of TIM3 signaling was shown to result in impaired TLR responsiveness, implying a synergistic relation between TIM3 and TLR signaling pathways (Anderson et al., 2007). Although TIM3 triggering on T cells and DCs leads to ERK (extracellular signal-regulated kinases) phosphorylation and IκBα degradation, different tyrosine phosphorylation patterns in T cells and DCs were detected, providing a plausible explanation for the differential effects of TIM3 between different cell types (Anderson et al., 2007). Thus far, interactions between DC-expressed TIM3 and T cell-expressed galectin-9 have not been investigated. However, previous findings prompt a model where DC-expressed TIM3 promotes inflammation and the differentiation of TIM3-expressing Th1 cells. IFN-γ-induced galectin-9 would interact with TIM3 from other T cells, inducing cell death and thereby self-limiting the immune response. Additionally, TIM3 is suggested to contribute to tolerance. A crucial role for TIM3 in clearing apoptotic cells by phagocytosis was recently revealed. Blocking this function resulted in inhibited cross-presentation of self-antigens and the development of auto-antibodies (Nakayama et al., 2009). In a completely different mechanism, TIM3 expressed by tumor-infiltrating DCs was shown to interact with the alarmin HMGB1, disturbing the recruitment of tumor cell-derived nucleic acids into DC endosomes, attenuating immune responses to these tumors (Chiba et al., 2012).

In contrast to the other members of the TIM family, TIM4 is exclusively expressed by APCs and not by T cells (Meyers et al., 2005). Through binding to TIM1 on T cells, TIM4 was shown to provide T cells with a co-stimulatory signal promoting T cell expansion, cytokine production, and survival. These effects were mediated by induced phosphorylation of the signaling molecules LAT (linker of activated T cells), Akt, and ERK1/2 in stimulated T cells (Rodriguez-Manzanet et al., 2008). Notably, the strength of TIM4 signal is decisive in determining the stimulatory effect, as weak TIM4 signaling inhibits T cell proliferation instead of potentiating it (Meyers et al., 2005). Similarly, TIM4 was shown to inhibit the proliferation of naïve T cells, which lack the expression of TIM1 (Mizui et al., 2008). These data imply that TIM4 has at least two binding partners: an activating ligand (TIM1) and an inhibitory one to be identified. Through these ligands, TIM4 exerts bimodal regulation of immune responses. Analogous to TIM3, the role of TIM4 in regulating immunity is also evident through mediating the engulfment of apoptotic cells. *In vivo* blocking of TIM4 resulted in the development of auto-antibodies (Miyanishi et al., 2007).

ADHESION MOLECULES PROVIDING CO-STIMULATORY SIGNALS

Leukocyte adhesion and detachment from other cells is tightly regulated by adhesion molecules. A specific set of these molecules is involved in regulating DC/T cell interactions. This set includes the following molecules: intercellular adhesion molecule 1 (ICAM-1) and lymphocyte function-associated antigen-3 (LFA-3), expressed by DCs, and their respective ligands LFA-1 and CD2, expressed by T cells. The seminal discovery of the involvement of LFA-1 in mediating T cell functions prompted a hypothesis that LFA-1 would act by enhancing adhesion and thereby increasing the range of avidities that can promote antigen recognition (Springer et al., 1982). Subsequently, ICAM-1 was identified as the ligand of LFA-1 (Rothlein et al., 1986). LFA-1 ligation by ICAM-1 was shown to induce proliferation of TCR-stimulated T cells in an IL-2-dependent mechanism, proposing that ICAM-1/LFA-1 interaction as a co-stimulatory pathway (Van Seventer et al., 1990). In addition to co-stimulation, ICAM-1/LFA-1 interaction stabilizes the immunological junction (Bleijs et al., 2001) and the ICAM-1/LFA-1 pathway appears to contribute to T

cell differentiation as repeated T cell stimulation with ICAM-1 promoted IFN-γ production by these cells (Semnani et al., 1994).

Moreover, blocking ICAM-1/LFA-1 interactions during T cell stimulation drastically increased Th2 cytokines (Salomon and Bluestone, 1998). More recently, ICAM-1/LFA-1 interaction during $CD8^+$ T cell priming was demonstrated to be essential for the establishment of effective T cell memory (Scholer et al., 2008). The effects of the ICAM-1/LFA-1 pathway are believed to result from influencing multiple cellular signaling cascades. LFA-1 was found to interact with the transcriptional co-activator JAB1, implying an influence on c-Jun-driven transcription (Bianchi et al., 2000).

In parallel, T cell CD2 interaction with its ligand LFA-3 was recognized for contributing to T cell activation by strengthening the adhesion between T cells and APCs and thereby enforcing TCR contact with its ligands (Davis and van der Merwe, 1996). Moreover, CD2 signaling was also shown to restore responsiveness in anergized human T cells (Boussiotis et al., 1994). CD2 blocking *in vivo* was revealed to induce T cell unresponsiveness, further supporting the notion that LFA-3/CD2 pathway contributes to immune activation (Xu et al., 2004). Conversely, specific mobilization of LFA-3/CD2 interactions was demonstrated to induce, single handedly, non-proliferating Tregs secreting high amounts of IL-10 (Wakkach et al., 2001). In light of these contradictions, further characterization of the role of LFA-3/CD2 co-stimulatory pathway is required.

CO-INHIBITORY MOLECULES

CD80/CD86/CTLA-4 PATHWAY

Cytotoxic T lymphocyte-associated antigen-4 (CD152) is a CD28 homolog that was discovered in 1987 (Brunet et al., 1987). The closely related structures of these two molecules suggest overlapping functional qualities. Indeed, CTLA-4 binds to CD80 and CD86, though at greater affinities. However, CTLA-4 was the first described co-stimulatory molecule with inhibitory effects in a stark contrast to the activatory properties of CD28 (Linsley et al., 1991b). The effects of CTLA-4 include inhibition of proliferation, cell cycle progression, and IL-2 synthesis (Walunas et al., 1996). Additionally, CTLA-4 seems to have an influence on T cell polarization. T cells lacking CTLA-4 expression were shown to adopt a Th2 phenotype (Bour-Jordan et al., 2003). Furthermore, neutralizing CTLA-4 signaling in T cells was recently shown to enhance IL-17 production and promote the differentiation of Th17 cells (Ying et al., 2010).

The prominent role of CTLA-4 in tolerance is clearly demonstrated by CTLA-4-deficient mice, which succumb at 3–4 weeks of age to massive lymphoproliferative disease (Tivol et al., 1995). Furthermore, the suppressive functions of naturally occurring Tregs, which constitutively express CTLA-4, were dependent on CTLA-4 signaling (Read et al., 2000), corroborating its role in tolerance. CTLA-4 contribution to tolerance is postulated to arise from controlling T cell responses in an intrinsic or extrinsic manner (Rudd et al., 2009). First, CTLA-4 antagonizes the CD28 stimulatory signaling by competing with CD28 on binding to CD80/CD86. Interestingly, CTLA-4 expression on cells is induced in a CD28-dependent mechanism (Alegre et al., 1996), implying that CTLA-4 serves as an internal checkpoint that prohibits excessive stimulation by CD28. Extrinsic inhibitory effects of CTLA-4 are suggested to be exerted through different mechanisms. CTLA-4 molecules expressed by Tregs were shown to engage CD80/CD86, expressed by DCs, promoting the activity of IDO. The modified catabolic properties of DCs lead to localized deprivation of tryptophan and thereby reduced T stimulatory capacity of these DCs (Fallarino et al., 2003). Another suggested mechanism for the extrinsic effects of CTLA-4 was demonstrated by the capacity of CTLA-4 to capture CD86, expressed by APCs, internalize it for ensuing degradation in a process called *trans*-endocytosis (Qureshi et al., 2011). Tregs were also observed to suppress T cells by establishing a direct interaction through CTLA-4, which binds to CD80 and CD86 expressed by those T cells (Taylor et al., 2004). Finally, unstimulated T cells were revealed to produce a soluble form of CTLA-4, which may possibly convey the inhibitory effects to other cells (Magistrelli et al., 1999). Collectively, CTLA-4 is unequivocally vital for tolerance.

Due to its role in maintaining tolerance, blocking CTLA-4 interaction with CD80 and CD86 was postulated to promote anti-tumor immunity. Indeed, *in vivo* administration of blocking antibodies against CTLA-4 resulted into effective anti-tumor immunity and tumor rejection (Leach et al., 1996). Nevertheless, CTLA-4 blockade efficacy in tumor therapy was correlated with the stage and immunogenicity of the tumor. At early stages small tumors were sensitive to the effects of CTLA-4 blockade (Shrikant et al., 1999), whereas advanced tumors were resistant due to the strongly tumor-induced T cell tolerance (Sotomayor et al., 1999). In an attempt to circumvent this hurdle, anti-CTLA-4 blocking antibodies were tested in combination with other therapeutic modalities. Combined anti-CTLA-4 application and Treg depletion resulted in maximal tumor rejection, which was dependent on the expansion of tumor-specific $CD8^+$ T cells (Sutmuller et al., 2001). Those promising experimental observations lead to the development of two fully human anti-CTLA-4 antibodies: ipilimumab (Bristol-Myers Squibb, New York, NY, USA) and tremelimumab (Pfizer, New York, NY, USA). Early clinical trials in metastatic melanoma and ovarian carcinoma patients demonstrated that blocking CTLA-4 resulted in extensive tumor necrosis with lymphocyte and granulocyte infiltrates in a large number of patients (Hodi et al., 2003). Further large scale clinical trials have shown irrefutable evidence of the efficacy of anti-CTLA-4 antibodies, leading eventually to FDA approval of these antibodies (Kirkwood et al., 2012). Despite its novelty, this therapeutic strategy is challenged by autoimmune complications resulting from the administration of anti-CTLA-4 antibodies (Sanderson et al., 2005).

The tolerogenic effects arising from CTLA-4 engagement with CD80/CD86 can also be utilized for inducing tolerance toward transplanted tissues. This notion has been supported by observations in animal experimental models. Administration of recombinant CTLA-4-Ig fusion protein after renal or cardiac transplantation enhanced allograft acceptance and reduced inflammatory responses (Azuma et al., 1996). This led to the development of humanized CTLA-4-Ig (Belatacept). Kidney transplantation patients receiving Belatacept showed reduced allograft rejection and maintained better renal functions, compared to patients receiving cyclosporine. These findings resulted in gaining FDA approval for using Belatacept for the prevention of acute rejection post-renal transplant (Vincenti et al., 2011).

PD-L1/PD-L2/PD-1 PATHWAY

Programed cell death-1 (PD-1) is another member of the CD28 family that is expressed by activated T and B cells (Agata et al., 1996). Two ligands were identified to interact with PD-1: PD-L1 (Dong et al., 1999) and PD-L2 (Latchman et al., 2001). Those ligands are characterized by differential expression patterns. PD-L1 is constitutively expressed and further enhanced on activated lymphocytes, including Tregs and DCs. It is also expressed by a wide variety of non-hematopoietic cell types including the vascular endothelial cells, neurons and pancreatic islet cells. In contrast, PD-L2 expression is restricted to DCs and macrophages under certain conditions (Greenwald et al., 2005). Interestingly, PD-L2 displays three times higher binding affinity to PD-1 in comparison to PD-L1, which on the other hand was also identified to bind to CD80 (Butte et al., 2007). The varying binding and expression properties of PD-L1 and PD-L2 suggest distinct functions in regulating T cell responses. Along with its ligands PD-1, is recognized for its vital role in regulating adaptive immune responses (Sharpe et al., 2007). Indeed, triggering of PD-1 by one of its ligands during TCR signaling can block T cell proliferation, cytokine production and cytolytic activity, and impair T cell survival (Riley, 2009). The intracellular domain of PD-1 contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) as well as an immunoreceptor tyrosine-based switch motif (ITSM), which are phosphorylated upon ligand engagement. Subsequently protein phosphatases, such as Src homology phosphatase-1 (SHP-1) and SHP-2, are recruited to TSM where they are activated and inhibit proximal TCR signaling events by dephosphorylating key intermediates in the TCR signaling cascade (Chemnitz et al., 2004). Similar to CTLA-4, triggering PD-1 limits glucose metabolism and Akt activation, albeit through different mechanisms (Chemnitz et al., 2004). Consistently, a recent study also demonstrated that PD-1 exerted its inhibitory effects by affecting Akt and Ras pathways and thereby inhibiting cell cycle progression and T cell proliferation (Patsoukis et al., 2012).

The first indication of the importance of PD-1 in immune tolerance came from PD-1-deficient mice, which developed strain-specific autoimmunity. The absence of PD-1 caused the development of cardiomyopathy secondary to the production of auto-antibodies against cardiac troponin in BALB/c mice (Nishimura et al., 1999), while C57BL/6 developed a lupus-like autoimmune disease (Nishimura et al., 2001). In humans, polymorphisms in the PD-1 gene were also associated with susceptibility to several autoimmune diseases including systemic lupus erythematosus (Prokunina et al., 2002), type I diabetes (Nielsen et al., 2003), and multiple sclerosis (Kroner et al., 2005). These observations were supported by functional studies demonstrating the contribution of the PD-L1/PD-L2/PD-1 pathway to central tolerance. In the thymus, interactions between PD-1, expressed by $CD4^{-}CD8^{-}$ thymocytes, and PD-L1 broadly expressed in the thymic cortex, were deemed crucial in regulating positive selection (Nishimura et al., 2000). PD-1 was also shown to participate in thymic negative selection (Blank et al., 2003). Gene expression profiling studies of central tolerance in non-obese diabetic (NOD) mice also implicated PD-1 and PD-L1 in central tolerance (Zucchelli et al., 2005). PD-L1/PD-L2/ PD-1 pathway also contributes to peripheral tolerance through multiple mechanisms. Self-reactive $CD8^{+}$ T cells lacking PD-1 display increased responsiveness to self-antigens presented by resting DCs, suggesting that DC-expressed PD-L1 and PD-L2 may control T cell activation (Probst et al., 2005). PD-L1/PD-L2/PD-1 pathway can also regulate reactivation, expansion, and functions of effector T cells (Keir et al., 2006). Additionally, PD-1 triggering of TCR-stimulated, transforming growth factor-beta (TGF-β)-treated T cells profoundly enhanced the *de novo* generation of $Foxp3^{+}$ Tregs from $CD4^{+}$ naïve precursors. Further engagement of PD-L1 on the iTregs sustained Foxp3 expression and enhanced the suppressive capacity of these cells (Francisco et al., 2009). Consistently, PD-L1 was shown to mediate the effects of the immune suppressant vitamin D (VitD). DCs treated with VitD were shown to induce IL-10 producing Tregs in a PD-L1-dependent mechanism (Unger et al., 2009). Interactions between PD-1 and PD-L1 are also proposed to maintain tolerance by modifying DC–T cell contact. PD-1 ligation was shown to inhibit the TCR-induced stop signals, disrupting the stable DC–T cell contact and subsequently allowing tolerized T cells to move freely and prohibiting clustering around antigen-bearing DCs (Fife et al., 2009). Another plausible mechanism for PD-L1/PD-L2/PD-1 pathway-induced tolerance is that PD-L1 expressed by Tregs would engage PD-1 expressed by DCs and modulate DC function and thereby impeding immune responses (Francisco et al., 2010).

The inhibitory effects of PD-L1/PD-L2/PD-1 pathway can be hijacked by tumors to evade anti-tumor immune responses. PD-L1 expression has been confirmed on many tumors including glioblastoma and melanoma as well as cancers of the head and neck, lung, ovary, colon, stomach, kidney, and breast. High expression PD-L1 levels by tumor cells, tumor-infiltrating lymphocytes, or both associated with aggressive tumor behavior, poor prognosis, and elevated risk of mortality (Zang and Allison, 2007). Moreover, DCs generated from peripheral blood of ovarian cancer patients displayed high levels of PD-L1, prompting impaired T cells responses, which were restored by blocking PD-L1/PD-1 interactions (Curiel et al., 2003). *In vivo*, forced PD-L1 expression by squamous cancer cells rendered them resistant to T cell-mediated immunity. This resistance, however, was broken upon treatment with anti-PD-L1 blocking antibodies (Strome et al., 2003). A recent study also revealed that platinum based chemotherapeutics enhanced anti-tumor T cell responses by disrupting PD-L2/PD-1 interactions through reducing PD-L2 levels on both DCs and tumor cells (Lesterhuis et al., 2011). These experimental observations prompted the development of humanized anti-PD-1 and anti-PD-L1 antibodies for clinical application. Early stage clinical trials with these antibodies demonstrated clinical activity, which was characterized by durability accompanied with minimal side effects (Zitvogel and Kroemer, 2012).

There is also evidence that viral infections can make use of PD-L1/PD-L2/PD-1 pathway. Animal models of chronic viral infections had elevated PD-1 expression on exhausted viral antigen-specific T cells. The activity of these T cells was restored following PD-L1 blocking, suggesting a novel strategy for combating chronic viral infections (Barber et al., 2006).

In line with its inhibitory role, PD-L1/PD-L2/PD-1 pathway can be harnessed for the induction of tolerance when needed. Administration of recombinant PD-L1-Ig, with agonistic effect for

PD-1, prolonged the survival of cardiac allografts in mice (Ozkaynak et al., 2002). Furthermore, PD-L1 expression on murine liver allografts is central for spontaneous tolerance (Morita et al., 2010).

B7-H3 PATHWAY

B7-H3 belongs to the B7 family of co-stimulatory molecules. Similar to other Ig superfamily members, B7-H3 is a transmembrane molecule. It possesses a short cytoplasmic tail with no known signaling domain. B7-H3 is expressed on a wide a variety of tissues and tumor cell lines. However, its expression on leukocytes is only detectable following stimulation. B7-H3 expression can be induced on DCs and monocytes by inflammatory cytokines, whereas a combination of phorbol myristate acetate and ionomycin can induce it on T cells. B7-H3 was shown to bind a receptor expressed by activated T cells. This receptor is distinct from CD28, CTLA-4, ICOS, and PD-1 and yet to be identified (Chapoval et al., 2001). Triggering receptor expressed on myeloid cells (TREM)-like transcript 2 (TLT-2), constitutively expressed by $CD8^+$ T cells and activation-induced on $CD4^+$ T cells, was proposed to be the binding partner of B7-H3 (Hashiguchi et al., 2008). However, this was strongly refuted by another study providing evidence of non-existing interaction between B7-H3 and TLT-2 (Leitner et al., 2009). Initially, B7-H3 was suggested to be a positive co-stimulatory molecule that induces T cell proliferation, IFN-γ production and CTL generation in humans (Chapoval et al., 2001). Nevertheless, this was contradicted by another study demonstrating that B7-H3 is a potently inhibited T cell stimulation under different conditions and regardless of the stimulation status of the T cells in question (Leitner et al., 2009). This is corroborated by data from murine studies where applying an agonistic fusion protein, B7-H3-Ig, was shown to inhibit proliferation, IL-2 and IFN-γ production of TCR-stimulated T cells. This inhibitory effect was demonstrated by exacerbated airway inflammation in B7-H3-deficient mice compared to wild type counterparts (Suh et al., 2003). Moreover, blocking B7-H3 caused enhanced T cell proliferation *in vitro* and worsened EAE *in vivo*. This effect may be explained by the inhibitory influence of B7-H3 signaling over NF-κB, NFAT, and AP-1 that are involved in regulating T cell activation (Prasad et al., 2004). Notably, the effects of B7-H3 were overridden by CD28 co-stimulation, implying that B7-H3 functions optimally in the absence of co-stimulation (Suh et al., 2003). Of interest, tumors are suggested to hijack the B7-H3 to evade anti-tumor immune responses. This is demonstrated by increased disease severity when cancer cells upregulated B7-H3 expression (Hofmeyer et al., 2008). Collectively, further characterization of the B7-H3 pathway is required to resolve functional discrepancies, which may be explained by the existence of two receptors for B7-H3 with opposite functions, yet to be identified.

B7-H4 PATHWAY

B7-H4 is the last among the B7 family members that was identified. Unlike other B7 family members, which are type I membrane molecules, B7-H4 is characterized by a glycosylphosphatidylinositol (GPI) domain that links to the cell membrane (Prasad et al., 2003). In humans, B7-H4 mRNA was detected in a variety of tissues. However, immunohistochemical analysis did not reveal any B7-H4 protein expression by these tissues. Likewise, no B7-H4 expression could be detected on freshly isolated T cells, B cells, monocytes, and DCs, but it was induced after activating these cells *in vitro*. The ligand of B7-H4 has not been identified yet, but it is suggested to be expressed by stimulated T cells and to be distinct from other CD28 family members (Sica et al., 2003). B7-H4 is widely regarded as a co-inhibitory molecule. Indeed, treatment of TCR-stimulated T cells by a fusion B7-H4-Ig protein resulted in inhibited T cell proliferation and cytokine production, an effect that required B7-H4 cross-linking (Sica et al., 2003). The inhibitory effects of B7-H4 are proposed to arise from arrested cell cycle progression in T cells (Sica et al., 2003), and impaired induction of JunB, known for its role in inducing IL-2 production in activated T cells (Prasad et al., 2003). A recent study also showed that B7-H4 signaling inhibits phosphorylation of MAP kinases, ERK, p38, Jun N-terminal kinase (JNK), and Akt, usually elicited upon TCR triggering of T cells (Wang et al., 2012a).

In line with *in vitro* findings, mice suffering from graft versus host disease demonstrated prolonged survival upon the *in vivo* application of B7-H4-Ig (Sica et al., 2003). Expectedly, *in vivo* administration of an antagonizing antibody against B7-H4 blocked the inhibitory effect of B7-H4 pathway and led to accelerated disease development in a mouse model of EAE (Prasad et al., 2003). Furthermore, B7-H4-deficient mice showed better control of *Leishmania major* infection as Th1 responses were augmented in these mice (Suh et al., 2006). B7-H4 deficiency also enhanced neutrophils-mediated immunity, implying that B7-H4 may have a role in regulating innate immunity too (Zhu et al., 2009). In addition to its role as a co-inhibitory molecule, B7-H4 seems to mediate the effect of Tregs. It was shown that Tregs, but not conventional T cells, induce high levels of IL-10 production by APCs and consequently trigger B7-H4 expression that renders these APCs immunosuppressive (Kryczek et al., 2006a). The overall tolerogenic effect of B7-H4 can be exploited by tumors to evade immune responses. B7-H4 expression was reported for several tumors including lung cancer, ovarian cancer (Choi et al., 2003), gastric cancer (Jiang et al., 2010), and tumor-associated macrophages (Kryczek et al., 2006b). Blockade of B7-H4 on these macrophages was actually effective in reversing their suppressive effect and restored anti-tumor T cell immunity (Kryczek et al., 2006b). Additionally, manipulating B7-H4 pathway has potential in the field of transplantation. A recent study showed that B7-H4 expression was shown to prolong islet allograft survival in mice (Wang et al., 2012b). Thus, the B7-H4 pathway serves as an interesting therapeutic target in different diseases, though several aspects of this pathway remain elusive.

HVEM/BTLA/CD160 PATHWAY

As mentioned earlier, the molecules HVEM, BTLA, CD160, and LIGHT interact directly with each other forming a complex pathway network regulating adaptive immune responses. HVEM, expressed by immature DCs, can provide negative co-stimulatory signals through binding to its ligands BTLA and CD160 on T cells (Ware and Sedy, 2011). BTLA belongs to the Ig superfamily and is a structural homolog of CTLA-4 and PD-1. It is also a transmembrane glycoprotein that can be phosphorylated on tyrosines located in conserved cytoplasmic ITIM motif (Watanabe et al.,

2003). T cell expression of BTLA was shown to be very low on naïve cells. However, it is upregulated upon antigen-stimulation peaking at day 2 and declining around day 7 post-stimulation. This expression can be retrieved upon secondary stimulation of activated T cells. Interestingly, anergic T cells and Th1 cells demonstrated high BTLA expression unlike Th2 cells and Tregs that have low BTLA expression (Hurchla et al., 2005). The unique BTLA expression pattern and expression kinetics indicate that BTLA may interfere at certain stages of T cell activation with specificity to certain types of effector T cells.

Herpes virus entry mediator delivers its inhibitory signal to T cells by binding to BTLA, which induces the phosphorylation of its ITIM domain and the recruitment of SHP-2, leading to attenuated antigen-driven T cell activation (Sedy et al., 2005). In addition to inhibiting T cell responses, there is evidence that HVEM/BTLA pathway promotes T cell survival in a mechanism dependent on NF-κB activation (Cheung et al., 2009). Interestingly, BTLA was also shown to mediate Treg suppression by interacting with HVEM expressed by Tregs. This was supported by showing that Tregs from HVEM-deficient mice had lower suppressor activity and that wild type Tregs failed to suppress effector T cells from BTLA-deficient mice (Tao et al., 2008). The inhibitory effects of BTLA are also observed *in vivo*. In an EAE model, BTLA-deficient mice displayed increased severity and persistence of disease when compared with wild type controls (Watanabe et al., 2003). BTLA deficiency was also reported to exacerbate allergic airway inflammation (Deppong et al., 2006) and to cause the development of auto-antibodies leading to a hepatitis-like syndrome with advancing age (Oya et al., 2008). Moreover, a single-nucleotide polymorphism (SNP) in the ITIM region of BTLA was reported to associate with increased susceptibility to rheumatoid arthritis (Lin et al., 2006). Another study also revealed an association between another BTLA SNP and rheumatoid arthritis, but not with systemic lupus erythematosus or Sjogren's syndrome (Oki et al., 2011). Similar to B7-H3 and B7-H4, the inhibitory effects of BTLA can be exploited by tumors to evade immunity. Melanoma-specific $CD8^+$ T cells were shown to persistently express BTLA. Interrupted BTLA signaling, achieved by applying CpG oligonucleotide vaccine formulations, lead to functional recovery of melanoma-specific $CD8^+$ T cells (Derre et al., 2010).

Herpes virus entry mediator can also interact with CD160, a GPI anchored membrane molecule that is mainly expressed by $CD8^+$ T cells and activated $CD4^+$ T cells. Cross-linking CD160 with a specific antibody on stimulated T cells was shown to strongly inhibit T cell proliferation and cytokine production. Similarly, the inhibitory effect of CD160 was also elicited by binding to its ligand HVEM (Cai et al., 2008). Although both BTLA and CD160 bind to the cysteine-rich domain-1 (CRD-1) of HVEM with comparable affinity, CD160 dissociates from HVEM at a slower rate compared to BTLA. Moreover, mutagenesis study of HVEM revealed that CD160 has a distinct binding site on HVEM, albeit overlapping with BTLA (Kojima et al., 2011). Those differences between CD160 and BTLA, though subtle, suggest that these molecules do not have redundant functions. Further delineation of the elusive HVEM/CD160 pathway and its functional implications are required to unravel its specific role in regulating immune responses.

ILT3 AND ILT4/HLA-G PATHWAYS

The inhibitory receptor Ig-like transcript-3 (ILT3; Cella et al., 1997) and ILT4 (Colonna et al., 1998), both expressed by monocytes, macrophages, and DCs, belong to a family of Ig-like inhibitory receptors that are closely related to the killer cell inhibitory receptors. Both ILT3 and ILT4 were shown to transmit signal through a long cytoplasmic tail containing ITIM motifs, which inhibit cell activation by recruiting the protein phosphatase SHP-1 (Cella et al., 1997; Colonna et al., 1998). In the case of ILT3, the extracellular region consists of two Ig-like domains, which are speculated to contain the putative binding site of the yet to be identified ILT3 ligand (Cella et al., 1997). On the other hand, the binding partner of ILT4 was shown to be the MHC class I molecule human leukocyte antigen G (HLA-G; Colonna et al., 1998). In addition to triggering an inhibitory signal, ILT3 cross-linking was shown to lead to its internalization and delivery into an antigen presenting compartment, suggesting a role in antigen processing (Cella et al., 1997). DC expression of ILT3 and ILT4 was shown to be induced under the effect of $CD8^+CD28^-$ alloantigen-specific T suppressor cells (Chang et al., 2002). Immature monocyte-derived DCs (MoDCs) also upregulated ILT3 and ILT4 expression upon treatment with either IL-10 or/and IFN-α (Manavalan et al., 2003). VitD treatment only induced ILT3 expression in MoDCs (Manavalan et al., 2003) and primary human blood $BDCA1^+$ DCs (Chu et al., 2012). Expectedly, ILT3 expression, by both MoDCs and pDCs, was downregulated following activation (Ju et al., 2004).

Tolerogenic DCs over-expressing ILT3 or ILT4 demonstrated impaired NF-κB activation and consequently reduced transcription capacity of NF-κB-dependent co-stimulatory molecules (Chang et al., 2002). Those DCs were shown to be capable of transforming alloreactive effector T cells into antigen-specific Tregs (Manavalan et al., 2003). Similarly, triggering ILT4 by HLA-G tetramers was shown to impair maturation and T cell stimulatory capacity of human DCs (Liang and Horuzsko, 2003). Interestingly, ILT3 was shown to maintain its T cell inhibitory effect when it was expressed as soluble ILT3-Fc that lacks ILT3's cytoplasmic tail, indicating that ILT3 delivers its inhibitory signal by binding to its partner on activated T cells (Kim-Schulze et al., 2006). Recently it was shown that ILT3 capacity to convert T cells into suppressive cells is dependent on BCL6 signaling in these T cells (Chang et al., 2010). ILT3 is also proposed to be important for controlling inflammation, as silencing ILT3 expression in DCs enhances TLR responsiveness, which is reflected by enhanced secretion of inflammatory cytokines such as IL-1α, IL-1β, IL-6, and IFN-α. ILT3-silenced DCs could also attract more lymphocytes by secreting high levels of the chemokines CXCL10 and CXCL11 in response to TLR ligation. Eventually, impaired ILT3 expression in DCs rendered them more stimulatory for T cells, which also secreted higher levels of cytokines like IFN-γ and IL-17 (Chang et al., 2009). Another suggested mechanism by which both ILT3 and ILT4 contribute to tolerance is by possibly mediating the effects of IDO. DCs cultured in tryptophan-deprived local environment upregulated the expression of ILT3 and ILT4, favoring the development of $Foxp3^+$ Tregs (Brenk et al., 2009). Finally, ILT4 was shown to be central for the development of type I Tregs, induced by IL-10-treated DCs (Gregori et al., 2010).

The effects of ILT3 and ILT4/HLA-G pathways are also evidenced *in vivo*. Immune modulation exerted by ILT4/HLA-G interactions is believed to mediate maternal tolerance toward the semi allogenic fetus (Hunt et al., 2005). Moreover, *in vivo* treatment with VitD was shown to upregulate the expression of ILT3 on DCs in healing psoriatic lesions. Nevertheless, ILT3 was revealed to be dispensable for the induction of Tregs and completely overridden by the inhibitory effects of VitD (Penna et al., 2005). Consistently, maternal VitD intake during pregnancy was found to enhance ILT3 and ILT4 gene expression levels in cord blood, pointing out a plausible mechanism for early induction of immune tolerance (Rochat et al., 2010). Enhanced ILT3 and ILT4 levels were also observed at an early stage of venom-specific immunotherapy, implying a possible role in inducing tolerance toward allergic reactions (Bussmann et al., 2010). Owed to its inhibitory effects, ILT3 is suggested to be employed by tumors as a mean of evading anti-tumor immunity. Indeed, soluble ILT3 protein was found at high levels in the serum of patients with melanoma, and carcinomas of the colon, rectum, and pancreas produce. This soluble ILT3 was active in inducing suppressor $CD8^+$ T cells that block anti-tumor immunity, which was restored upon blocking or depleting ILT3 (Suciu-Foca et al., 2007). A similar mechanism is also utilized by viruses, as demonstrated by a point mutation in one of HIV Gag epitopes that increased binding to ILT4 and consequently programed myelomonocytic cells to become tolerogenic (Lichterfeld et al., 2007). The inhibitory effects of ILT3 can also be harnessed for allograft acceptance. Indeed, soluble recombinant ILT3-Fc was shown to suppress T cell-mediated rejection of allogenic islet transplants in mice (Vlad et al., 2008). In correlation to its inhibitory effect, blood monocytes during multiple sclerosis relapses demonstrated lower ILT3 expression, which was restored upon treatment with IFN-β, unraveling a plausible therapeutic target in the treatment of multiple sclerosis (Jensen et al., 2010). Similarly, a SNP in the ILT3 extracellular region was correlated with low surface expression and increased serum cytokine levels in lupus patients (Jensen et al., 2012).

CONCLUDING REMARKS AND FUTURE PROSPECTS

Since the identification of the CD80/CD86/CD28 classical co-stimulatory pathway, the concept of DC-derived signal II was dramatically expanded to accommodate the ever increasing number of newly discovered co-stimulatory and co-inhibitory pathways. An increasing body of reports reflects the complexity of these pathways and implies possible interactions to form a sophisticated network controlling adaptive immune responses. The existence of multiple co-stimulatory and co-inhibitory pathways postulates for overlapping functions. Nevertheless, this notion of redundancy should be considered carefully. The components of these pathways have distinct expression patterns and kinetics, which means that these pathways are not simultaneously operative. In addition, mobilizing these pathways can trigger distinct signaling cascades and thereby leading to variable outcomes.

Dendritic cell expression of co-stimulatory and co-inhibitory molecules is dictated by several factors. The specific type of DC is a major determinant of this expression. In humans, DCs are classified into groups based on origin, specific expression of certain surface markers, and functional properties. For example, human blood DCs are divided into two major subsets: pDCs and myeloid DCs (myDCs). The latter can be further divided into three subsets: $BDCA1^+$ DCs, $BDCA3^+$ DCs, and $CD16^+$ DCs. In parallel, skin DCs are also classified into epidermal LCs, dermal $CD1a^+$ DCs, and dermal $CD14^+$ DCs. Similar classification can be expected in other tissue-resident DCs. Most of the findings concerning co-stimulatory and co-inhibitory molecules in humans were based on experiments performed on the *in vitro* generated MoDCs, which serve as a great tool for delineating immunological functions and mechanisms. However, there are strong indications of differential expression of co-stimulatory and co-inhibitory molecules among different DC subsets. These variations can be partially related to the intrinsic qualities of every DC subset. For instance, pDCs and LCs lack the expression of TLR4, and consequently they are not able to upregulate CD80 and CD86, observed in other subsets in response to lipopolysaccharide (LPS).

Another central determinant of co-stimulatory and co-inhibitory molecules expression by DCs is the type of stimulus, to which DCs are exposed. As mentioned earlier, DCs respond to pathogen stimulation by upregulating CD80 and CD86. However, there are indications that certain co-stimulatory molecules are strictly expressed upon activation with a specific class of pathogens. A clear example is CD70 expression by LCs upon TLR3 triggering by double-stranded RNA derived from viruses, granting LCs advantage in eliciting strong anti-viral $CD8^+$ T cell responses. Although dermal DCs and MoDCs express TLR3, they do not upregulate CD70 in response to double-stranded RNA, implying a combined effect of the type of stimulus and the type of DC in inducing CD70 expression. Similarly, pDC stimulation with CpG B, a TLR9 ligand, induced the expression of CD70, which was not observed using another type of stimulation or in other DC subsets (Shaw et al., 2010). Another example demonstrating the effect of pathogenic stimulation is the upregulation of OX40L only upon exposure to the soluble egg antigen from the parasite *Schistosoma mansoni*. Furthermore, DC treatment with certain immune modulating agents can influence the expression of co-stimulatory and co-inhibitory molecules. VitD-treated DCs displayed induced expression of PD-L1 and ILT3, concurrent with inhibited expression of CD80 and CD86. On the other hand, DCs under the influence of IL-10 had normal expression levels of CD80 and CD86 but over-expressed ILT3 and ILT4. It is also evident that DCs are strongly influenced by cues derived from the local environment. The well-documented effect of VitD, the major component of local skin milieu, is a clear example. The influence of other known tissue-related environmental factors on co-stimulation requires further elucidation. Thus, optimal understanding of the role of DC-derived signal II requires determining the total repertoire of co-stimulatory and co-inhibitory molecules expressed by different DC subsets and under different conditions.

In addition to the differential DC expression of co-stimulatory and co-inhibitory molecules, the respective ligands of these molecules are also described to be expressed by T cells following different kinetics. Some of these ligands are constitutively expressed, like CD28, whereas others are restricted to recently TCR-activated T cells such as 4-1BB and GITR. Furthermore, some of these ligands were shown to be exclusively expressed by

certain types of effector T cells, like the Th1-specific expression of TIM3. Taken together, the different expression modalities of the co-stimulatory and co-inhibitory pathway constituents imply that these pathways are mobilized at certain stages of T cell priming and under specific conditions.

Despite the stimulatory or inhibitory nature of signal II, there are some indications pointing out a role in T cell polarization, typically undertaken by cytokine-based signal III. For instance, OX-40L/OX-40 and 4-1BBL/4-1BB pathways are proposed to promote the differentiation of Th2 and Th1 effector cells, respectively. Nevertheless, the observed polarizing effect was in many occasions revealed to be the mere outcome of promoted T cell survival rather than active polarization signaling mediated by these co-stimulatory or co-inhibitory molecules. Therefore, reported contributions of signal II to T cell differentiation should be interpreted carefully and further investigated.

The vast immunological consequences of signal II have transformed its pathways, both stimulatory and inhibitory, into therapeutic targets for the treatment of a wide variety of diseases. Mobilizing co-stimulatory pathways and blocking co-inhibitory interactions showed promising results in promoting anti-tumor immunity and it is proposed to be beneficial for the treatment of chronic viral responses. Assuming that mature DCs provide optimal positive co-stimulatory signals while priming anti-tumor T cells, blocking co-inhibitory pathways may augment the efficacy of these T cells. In that respect, concurrent targeting of multiple co-inhibitory pathways might be necessary. Neutralizing the key inhibitory check point CTLA-4 permits extensive primary T cell activation, but by itself is not sufficient for driving an anti-tumor immune response, especially in the case of advanced tumors. However, the additional circumvention of yet another co-inhibitory check point, which is dictated by the tumor itself, may solve this problem. Selecting the second inhibitory target would highly depend on the type of the treated tumor, as different types of tumors were revealed to preferentially express certain co-inhibitory receptors (PD-L1, PD-L2, B7-H3, etc.). The synergistic effects of such a combinatorial blocking strategy may not only mount efficient anti-tumor T cell responses, but also allow the persistence of such responses within the local tumor environment.

On the other hand, promoting tolerance by blocking activation and mobilizing co-inhibitory pathways is a promising strategy for raising allograft tolerance. Similarly, immune suppressant agents were also revealed to manipulate these pathways in a comparable manner to induce tolerance. Nevertheless, these therapeutic modalities should be applied with great care to avoid any possible adverse effects like inducing susceptibility to infection or autoimmune reactions. Targeting these therapies to a specific pathway or a specific cellular compartment, like a certain DC subset, may be an option to bypass any possible complications.

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Costimulatory molecules on immunogenic versus tolerogenic human dendritic cells

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Dendritic cells (DC) are sentinels of immunity, essential for homeostasis of T cell-dependent immune responses. Both functions of DC, initiation of antigen-specific T cell immunity and maintenance of tissue-specific tolerance originate from distinct stages of differentiation, immunogenic versus tolerogenic. Dependent on local micro milieu and inflammatory stimuli, tissue resident immature DC with functional plasticity differentiate into tolerogenic or immunogenic DC with stable phenotypes. They efficiently link innate and adaptive immunity and are ideally positioned to modify T cell-mediated immune responses. Since the T cell stimulatory properties of DC are significantly influenced by their expression of signal II ligands, it is critical to understand the impact of distinct costimulatory pathways on DC function. This review gives an overview of functional different human DC subsets with unique profiles of costimulatory molecules and outlines how different costimulatory pathways together with the immunosuppressive cytokine IL-10 bias immunogenic versus tolerogenic DC functions. Furthermore, we exemplarily describe protocols for the generation of two well-defined monocyte-derived DC subsets for their clinical use, immunogenic versus tolerogenic.

Keywords: dendritic cells, tolerance, immunity, IL-10, regulatory T cell, costimulation, inhibitory molecules

INTRODUCTION

DENDRITIC CELLS – SENTINELS OF IMMUNITY

Ralph Steinman started as a postdoc in the laboratory of Zanvil Cohn and James Hirsch at the Rockefeller University in the 1970s. The focus of his research was the identification and functional characterization of dendritic cells (DC) granted in 2011 with the Nobel Prize for medicine. Steinman identified this novel cell type in murine spleens and thereby opened a complete new field in immunology. The link between innate and adaptive immunity was revealed, concomitantly the origin of antigen-specific T cell-mediated immune responses (Steinman, 2012).

The family of DC is divided into two major subtypes with distinct functions: plasmacytoid and conventional DC. Plasmacytoid DC express receptors for recognition of viral antigens and produce high amounts of type I interferons after activation. Thus, the main function of this DC subtype is the initiation of anti-viral responses. Conventional DC are further divided into numerous subtypes residing in specific tissues in an immature state. They express a broad range of receptors for recognition of bacterial and viral components (Wu and Liu, 2007).

Dendritic cells turned out to be uniquely equipped for activation of naïve T cells and therefore are referred to as "professional" antigen-presenting cells. They are located in nearly all peripheral tissues. Here, immature DC differentiate from blood-derived progenitors under the influence of tissue-specific factors. Tissue residing DC form a close network, optimally positioned to sense invading pathogens. They excessively capture antigens by phagocytosis, macropinocytosis, or receptor-mediated endocytosis and further process these antigens into peptides. The peptides are loaded onto major histocompatibility complex (MHC) molecules and finally presented on DC surface. Due to their strong migratory capacity, antigen taken up by immature DC in the periphery is efficiently transported to T cell areas of local lymph nodes (Banchereau and Steinman, 1998) (**Figure 1**). Here, antigens are presented to T cells, which results in tolerance in absence of inflammation or immunity under inflammatory conditions. Therefore, the constant migration of immature DC to lymph nodes and the presentation of self-antigens are crucial parts of maintenance of peripheral tolerance. Under this aspect, it is not surprising that the vast majority of DC found in lymphoid organs under steady state conditions exhibit an immature phenotype (Wilson et al., 2003). These immature DC constitute of migratory immature DC from the periphery and tissue resident lymphoid DC (Shortman and Naik, 2007).

Recent reports showed that DC not only determine the type of T cell immunity, but also patterns of homing receptors expressed on T cells and thus their migratory behavior (Dudda and Martin, 2004; Sigmundsdottir and Butcher, 2008; Schwarz et al., 2011; Naik et al., 2012). Blood-derived DC mostly express both gut and skin homing markers and, thus, are able to migrate to both organs. These DC induce T cells with multi-homing properties. After immigration into particular tissues, DC within gut or skin do not further exhibit this ability and induce rather tissue-specific T cells. These functional changes of DC are a result of tissue-specific maturation processes (Johansson-Lindbom et al., 2003).

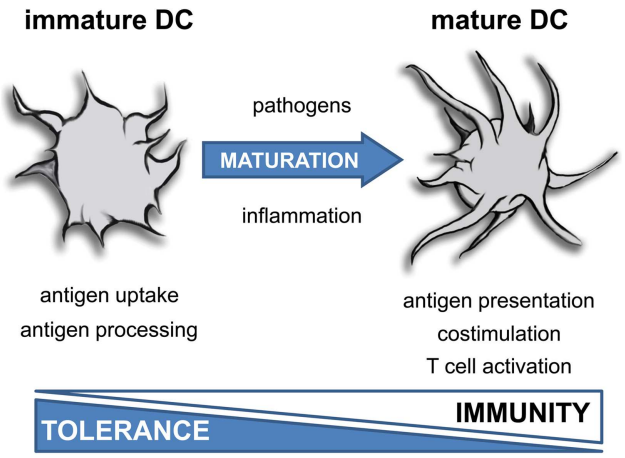

FIGURE 1 | Function of dendritic cells depends on maturation. Inflammatory mediators induce terminal differentiation of immature DC into fully matured immunogenic DC. This process is associated with a dramatic change in morphology, a reduced uptake of antigens and impaired antigen processing activity. Furthermore, mature DC exhibit a strong costimulatory and T cell activating capacity.

DENDRITIC CELLS AS POTENT INDUCERS OF IMMUNITY AND TOLERANCE

Dendritic cell function strictly depends on their current activation state. Under steady state and dependent on their localization, DC display an immature phenotype that correlates with low expression of costimulatory molecules and weak T cell stimulating properties (Banchereau and Steinman, 1998). Furthermore, functional properties of DC subsets are adapted to tissue functions. Particular tissues benefit from the unique capability of DC to either induce antigen-specific responses or tolerance. DC located in the mucosa of lung or gut are confronted with a continuous influx of foreign antigens. Mediated by tolerogenic mediators like IL-10 and TGF-β, the local micro milieu strongly prevents DC activation to avoid pathologic inflammation and DC in these environments rather promote tolerance than immunity (Akbari et al., 2001; Weiner, 2001). In contrast, lymphnodes and blood are protected against uncontrolled influx of antigens and the local environment lacks tolerogenic mediators. Immature DC located in lymph nodes and blood likewise maintain peripheral tolerance, but as a consequence of a different local milieu, these DC need less stimulation for maturation into immunostimulatory DC (Iwasaki and Kelsall, 1999).

Pathogens exhibit a broad range of molecular patterns that are recognized by specific receptors such as Toll-like receptors (TLR) expressed by DC. Direct recognition of invading pathogens activates immature DC and induces their differentiation. In addition to these pathogen-triggered signals, local inflammation influences the differentiation process of DC (Medzhitov, 2001).

As a result of maturation, DC undergo a dramatic change in their morphology and develop cellular extensions that enlarge cellular surface and improve the interaction with T cells (**Figure 2A**). DC also downregulate IL-10-receptor (IL-10R) expression rendering them insensitive to the immunosuppressive function of this cytokine (Steinbrink et al., 1999; Thurner et al., 1999). But the major events in DC maturation are probably the upregulation of MHC and costimulatory molecules on their surface (**Figure 2B**). The maturation process also drastically enhances their migratory capacity. Through upregulation of homing receptors like CCR7, migration to lymph nodes is accelerated. Those migratory DC follow gradients of chemokines such as CCL19 and CCL21 and enter T cell areas of secondary lymphoid organs (Dieu et al., 1998; Sallusto and Lanzavecchia, 2000). Importantly, activated DC cease any further uptake and procession of antigens. This ensures that antigens which are transported and presented by activated DC reflect the current situation at the site of inflammation. Assimilation of self-antigens on the way to lymph nodes and subsequent activation of self-reactive T cells are thereby prevented. Altogether, these events render mature DC potent inducers of T cell proliferation (**Figure 2C**) and T cell differentiation.

Activation of naïve T cells requires several distinct signals delivered by DC: signal I is mediated by MHC in complex with a peptide processed from captured antigens and is received by a specific T cell receptor. For entire T cell activation a costimulatory signal (signal II) is mandatory, as a T cell receptor signal in absence of costimulation renders respective T cells anergic (Corthay, 2006). In addition, a third signal in form of soluble factors such as IL-12, IL-15, IL-6, or TNF-α is also important for functional activation of naïve T cells. An integration of all signals designs the T cell differentiation process: inflammatory versus tolerogenic (Curtsinger et al., 1999). In strong contrast to naïve T cells, reactivation of effector or memory T cells is rather signal II independent, ensuring rapid execution of effector function at sites of inflammation independent of accessory cells (Byrne et al., 1988; Croft et al., 1994).

IMPACT OF SIGNAL II ON DC FUNCTION

Pattern of costimulation hence is a central feature distinguishing tolerogenic and immunogenic DC. But it is not solely absence or presence of costimulation that defines DC function. A complex network of transmembrane receptor/ligand pairs acts together with the T cell receptor and soluble factors to enhance T cell activation (**Figure 3**). Under these molecules, CD28, ICOS, and CD40L play a prominent role. At the same time, T cells also express inhibitory molecules such as CTLA-4 or PD-1, that down regulate T cell activation. Ultimately, it is the combination of several circumstances including the subtle interplay of signal II that produces an immunogenic or tolerogenic immune response. Here, we give a short overview of costimulatory molecules from the B7 family and TNF-receptor family that either support tolerogenic or immunogenic function.

COSTIMULATORY MOLECULES OF THE B7 FAMILY

CD80/CD86

CD80 (B7.1) and CD86 (B7.2) expression on DC probably constitutes the most important costimulatory pathway in T cell activation (Lenschow et al., 1996). Signaling through binding partner CD28 on T cells confers optimal mRNA stabilization and production of IL-2, a factor that promotes expansion and survival of primary T cells (Linsley et al., 1991). A variety of inflammatory or pathogen-derived mediators quickly up regulate expression

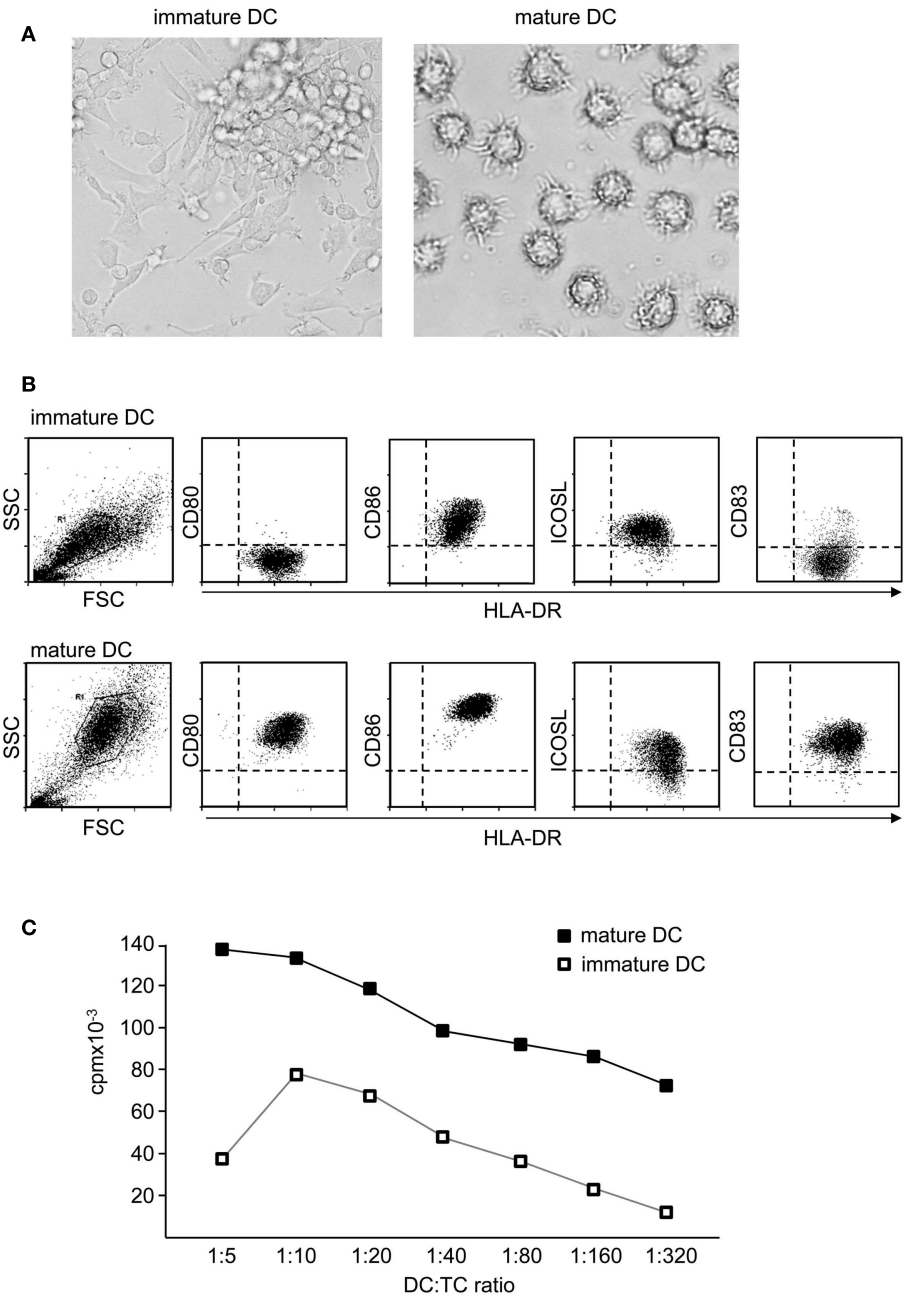

FIGURE 2 | Mature dendritic cells are potent activators of naïve T cells. **(A)** Functional properties of DC depend on their maturation state. In contrast to immature DC, terminally differentiated DC show a typical morphology with strong cellular extensions and **(B)** induce specific maturation markers like CD83 and costimulatory molecules like CD80 or CD86, whereas ICOSL is rather down regulated or unaltered. Also antigen presentation is enhanced, displayed by higher levels of MHC molecules. **(C)** In coculture with alloreactive T cells, immature DC induce only comparable weak T cell proliferation, whereas mature DC are potent activators of T cells.

of CD80 and CD86, therefore both molecules serve as very early costimulatory signals (**Figure 2B**). CD28-mediated costimulation also strongly interferes with tolerogenic properties of immature DC. A strong CD28 signal can inhibit differentiation into induced Treg by preventing stabilization of IL-10R on T cells (Tuettenberg et al., 2009). Interestingly, the same costimulatory molecules are also responsible for shutting down T cell activation. This is realized by a simple trick: T cell activation is accompanied by upregulation of CTLA-4 on T cell surface. CTLA-4 binds with higher affinity to CD80/CD86 than CD28 and thereby competes for interaction with both costimulators. CTLA-4-mediated signaling down regulates T cell responses and thus, provides a very simple negative

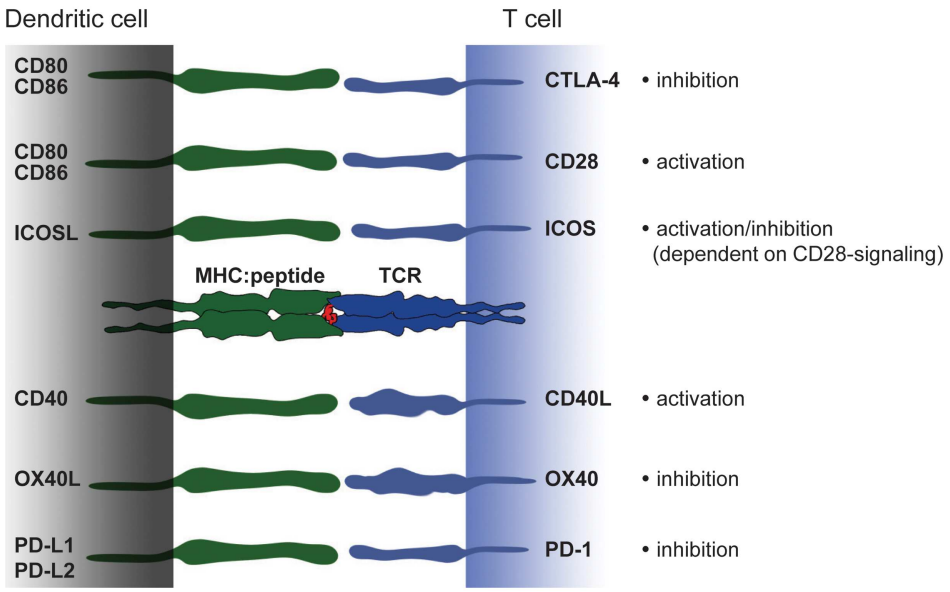

FIGURE 3 | Costimulatory molecules and their ligands – a brief overview. Modulation of T cell activation is mediated by an interplay of different costimulatory molecules expressed on DC that have either immunogenic or tolerogenic function. The picture shows an overview of members from the B7 and TNF-receptor family expressed on DC and their binding partners on T cells. In the last decade a number of new costimulatory molecules have been identified. However, in the context of monocyte-derived DC CD80 and CD86 constitute powerful members of the costimulatory family. Strong CD80/CD86-derived signals can overcome e.g., ICOSL-mediated signaling and thereby turning a rather tolerogenic signal into an immunogenic.

feedback loop carried out by the same ligand (Greene et al., 1996; Carreno et al., 2000).

Both, CD80 and CD86 are commonly used for describing fully matured DC. Often this is conveyed between species, a fact that has to be handled with care. *Human* immature DC constitutively express intermediate amounts of CD86 and lack CD80 (**Figure 2B**) (Jonuleit et al., 1997). Hence, for characterization of *human* DC maturation, CD80 is considerably more reliable, as it is exclusively induced on mature DC while CD86 is already present on immature DC and further up-regulated upon stimulation. In contrast, in the murine system CD86 is the main activation marker of bone-marrow derived DC, strongly up-regulated after maturation (Inaba et al., 1992, 1994) while CD80 expression is less pronounced on *murine* DC.

ICOS-Ligand

ICOS is expressed on $CD4^+$ T cells upon T cell receptor-mediated activation (Hutloff et al., 1999) and specifically interacts with ICOS-Ligand (ICOSL; B7-H2) on antigen-presenting cells (Yoshinaga et al., 1999). ICOS regulates general T cell features such as growth, proliferation and survival. In addition, depending on the inflammatory environment, ICOS/ICOSL interaction drives T cell polarization (Kopf et al., 2000).

Moreover, a central role for ICOS in mediating tolerance has been suggested in mouse and men (Rottman et al., 2001; Herman et al., 2004). In view of this aspect, it is interesting that immature *human* DC express high amounts of ICOSL on their surface (**Figure 2B**). This is an important fact, as immature DC thereby convey a strong ICOS-signal in context of weak CD28-stimulation which was shown to stabilize IL-10R-expression on stimulated T cells. Under these circumstances, low amounts of IL-10 produced by immature DC act on IL-10-sensitized T cells allowing immunosuppressive functions that prevent differentiation into inflammatory T effector cells (**Figure 4**). Altered T cell polarization results in low proliferative capacities and production of IL-10 instead of IFN-γ. Finally, these T cells differentiate after repetitive stimulation into induced Treg (Jonuleit et al., 2000b). This process is again driven by the balance of distinct engaging costimulatory signals: the induction of IL-10-producing Treg critically depends on ICOS/ICOSL interaction and is prevented by strong CD28 signaling (Witsch et al., 2002; Tuettenberg et al., 2009). Interestingly, activated human plasmacytoid DC express high levels of ICOSL and rather low CD28 ligands. Also this DC subset promoted differentiation of naïve T cells into IL-10-producing regulatory T cells in an ICOS-dependent fashion (Ito et al., 2007). This again illustrates the plasticity of a DC-derived immune response, as we showed recently that the same population of plasmacytoid DC is also able to elicit T cell proliferation in presence of regulatory T cells (Hubo and Jonuleit, 2012). Therefore it is important to note, that DC function cannot be attributed to the expression of single molecules but has always to be considered in the context of the local milieu.

Several groups reported a central role for ICOS-mediated costimulation in tolerance also in *mice*. Here, interaction of ICOS/ICOSL is required for Treg induction (Busse et al., 2012) or for maintenance of peripheral tolerance (Rottman et al., 2001; Herman et al., 2004). Taken together, ICOS/ICOSL interaction plays an important role in development of adaptive tolerance by DC rendering ICOS an interesting target for immunotherapy.

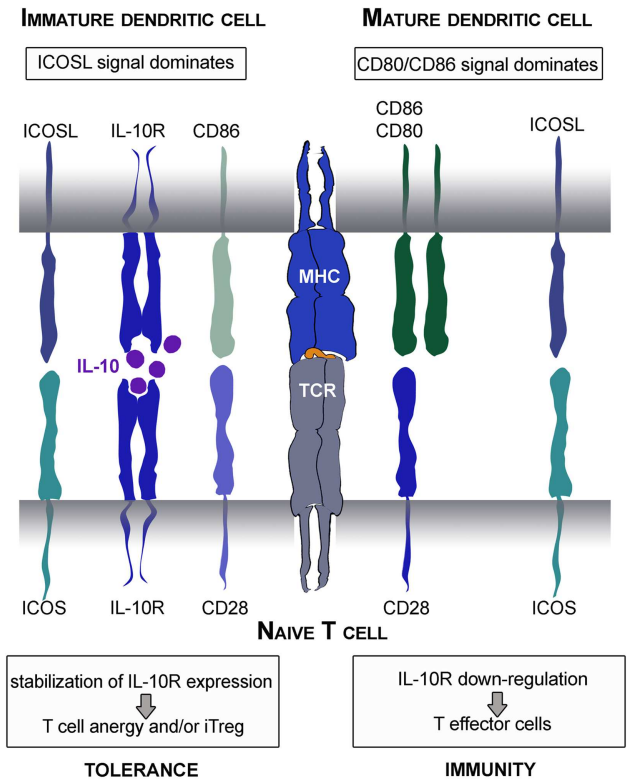

FIGURE 4 | Tolerogenic or immunogenic function of ICOS/ICOSL interaction depends on CD28 signaling. Immature DC express low amounts of CD28 ligands and thereby provide strong ICOS signals. This leads to stabilization of IL-10R on the surface of stimulated naïve T cells. Subsequently, the immunomodulatory cytokine IL-10 produced by DC mediates its function resulting in the differentiation into anergic Treg. In contrast, mature DC provide strong CD28 signals that overcome the tolerogenic ICOS function resulting in stabilization of IL-2 mRNA and synthesis and thereafter, the differentiation into inflammatory T effector cells.

PD-1 ligands

Programed cell death-1 (PD-1) has two ligands, PD-L1 and PD-L2. PD-L1 is constitutively expressed on resting DC as well as on other immune and non-immune cells (Yamazaki et al., 2002). After stimulation of immature DC with pathogen-derived factors like LPS or after CD40-mediated signaling, PD-L1 expression is further enhanced. Compared to PD-L1, PD-L2 expression is restricted to antigen-presenting cells like B cells, macrophages and DC (Zhong et al., 2007). Here, the molecule is up-regulated in response to anti-CD40, GM-CSF, IL-4, IFN-γ, and IL-12 (Loke and Allison, 2003). Interaction of T cells and DC via PD-L/PD-1-axis transfers inhibitory signals into T cells by inhibiting activation of PI3K. Subsequently, production of cytokines like IFN-γ is repressed, cell survival proteins are impaired and apoptosis is induced (Keir et al., 2008).

One mechanism of tolerogenic DC to shut down self-reactive T cells in the periphery is achieved through PD-1 signaling by induction of Treg. Just like ICOS/ICOSL-mediated induction of Treg, also the tolerogenic function of PD-1 underlies similar immune mechanisms: strong costimulation delivered by mature DC via CD28 overcomes PD-1-mediated inhibitory effects (Chemnitz et al., 2004). In summary, PD-1 signaling down regulates immune responses and so participates in peripheral tolerance (Nishimura et al., 2001; Krupnick et al., 2005).

In general, inhibitory effects in the immune system have a high potential to become pathologic, e.g., within a growing tumor. Cancer has generated several mechanisms to efficiently evade immune responses; among others overexpression of inhibitory molecules is critical. PD-1L was found to be expressed in high amounts on a multitude of solid tumors (Hamanishi et al., 2007; Nakanishi et al., 2007) thereby provoking a suppressive microenvironment that was suggested to explain the failure of anti-tumor immunotherapies. Also numerous autoimmune diseases such as type I diabetes, multiple sclerosis, systemic lupus erythematosus, and rheumatoid arthritis are linked with dysregulated PD-1 shown by analysis of single-nucleotide polymorphisms (Prokunina et al., 2002; Ferreiros-Vidal et al., 2004). Thus, targeting this costimulatory pathway might be beneficial to generate new therapies.

COSTIMULATORY MOLECULES OF THE TNF-RECEPTOR FAMILY

CD40

During interaction of DC and T cells, further receptor-ligand pairs are up-regulated and new possibilities for T cell modulation develop. These molecules include CD40L (CD154; member of the TNF superfamily) on activated T cells and CD40 expressed by activated DC and other antigen-presenting cells (Grewal and Flavell, 1998).

The CD40/CD40L pathway regulates cellular and humoral immunity and plays an important role in T cell priming and differentiation (MacDonald et al., 2002). Using blocking antibodies and knockout models, CD40/CD40L interaction was shown to be required for protective immunity (Reichmann et al., 2000; Habib et al., 2007). CD40 ligation on DC increases expression of costimulatory, adhesion and MHC molecules and promotes the production of T cell stimulatory cytokines such as IL-12 (Lapteva et al., 2007; Haenssle et al., 2008). Recombinant CD40L therefore is often used to induce DC maturation. However, CD40/CD40L interaction alone is insufficient for induction of the important effector molecule IL-12 in human DC. Additional IFN-γ, produced during DC-T cell crosstalk is required for IL-12 production. Since naïve T cells do not produce IFN-γ, their activation by mature DC does not result in IL-12 production by DC (Snijders et al., 1998). Nevertheless, some reports show that CD40-stimulated DC, despite their mature phenotype, induce T cell anergy (Wiethe et al., 2003) via IL-10 production and stabilization of IL-10R on T cells (Tuettenberg et al., 2010). This is not only true for conventional DC but also for plasmacytoid DC that produce large amounts of IL-10 after CD40L activation, resulting in induction of Treg (Gilliet and Liu, 2002).

In *mice* it was shown that also the level of CD40L expression influences the intensity of DC-T cell interaction and thereby modulates the outcome of an immune response. Low CD40L expression on T cells induces IL-10 production that impairs T cell expansion and antigen reactivity. Such anergized T cells were able to gain capabilities to suppress T cell activation. In contrast, strong interaction mediated by high levels of CD40L rather induced IL-12 production thus promotes immunity (Murugaiyan et al., 2007).

In summary, the T cell response resulting from CD40/CD40L interaction is complex and strictly depends on signal strength and presence of third signals such as IFN-γ that favor T cell-dependent immunity or tolerance.

OX40 ligand

OX40 ligand (OX40L) is ubiquitously expressed on a multitude of antigen-presenting cells. In addition, also non-immune cells like endothelia and smooth muscle cells show OX40L expression (Imura et al., 1996; Ohshima et al., 1997; Burgess et al., 2004). OX40L is induced on DC after CD40L stimulation or in response to inflammatory mediators like TNF-α (Ohshima et al., 1997; Migone et al., 2002; Fillatreau and Gray, 2003). OX40L signaling plays a central role in immunity and tolerance, controls T cell survival and homeostasis and at least supports the generation of long-lasting memory T cells. Interestingly, OX40L signaling preferentially promotes Th2 differentiation in the absence of IL-12 and independent of IL-4 (Flynn et al., 1998; Ito et al., 2005). However, together with IL-12, OX40L-mediated T cell stimulation rather induces Th1 polarization (De Smedt et al., 2002).

Besides its potent costimulatory potential regarding T cell activity, OX40/OX40L interactions are also capable to modulate peripheral tolerance in mice and men. Interestingly, Vu et al. reported that Treg stimulation via OX40 represses Foxp3 expression and thereby leads to reduced Treg function. In addition, it was shown that strong OX40-mediated signaling prevents TGF-β-promoted Foxp3 induction and further differentiation into induced Treg (Ito et al., 2006; Vu et al., 2007). Therefore, it is not remarkable that OX40/OX40L interference became a therapeutic target in immune-mediated diseases and cancer. Several studies have proved the protective effect of blocking the OX40/OX40L interaction in models of inflammatory disease like asthma, arteriosclerosis as well as in autoimmune diseases such as experimental autoimmune encephalomyelitis, diabetes, colitis, and collagen-induced arthritis (Croft, 2009; Kaur and Brightling, 2012).

In conclusion, expression of costimulatory molecules on DC is essentially involved in controlling T cell differentiation and the resulting immune response: immunity versus tolerance. No single costimulatory molecule is tolerogenic or immunogenic. It is rather the integration of several costimulatory molecules, interaction with soluble co-factors and the differentiation state of interacting T cells that dictates the immune response.

IL-10 – MEDIATOR OF TOLERANCE

One key soluble molecule that critically contributes to peripheral tolerance by modulation of DC costimulation is the immunosuppressive cytokine IL-10. IL-10/IL-10R-signaling is mediated by activation of the Jak/STAT pathway through tyrosine phosphorylation of Tyk2/Jak1, resulting in downstream activation and homodimerization of STAT3. These dimers are crucial transcription factors for regulation of anti-inflammatory genes (Finbloom and Winestock, 1995; Ding et al., 2003). In the context of DC maturation by several stimuli the most important pathways affected by IL-10 are PI3K/Akt/NF-κB-, MyD88/MAPK-, and Ras/Raf/MAPK-regulated signal transduction cascades (**Figure 5**). Early inhibition of the src kinase p56lyn and suppression of later events of this PTK-mediated activation (Ras, ERK, p38) are demonstrated for human monocytes and DC (Geng et al., 1994; Niiro et al., 1998; Sato et al., 1999). In addition, TLR-induced expression of the MyD88-dependent adaptor molecules IRAK4 and TRAF6 are post-transcriptional regulated by IL-10 and is associated with decreased phosphorylation of the MAPK p38 and JNK (Chang et al., 2009; Knödler et al., 2009). LPS- or TNF-α-induced activation of NF-κB is also blocked by IL-10 in versatile manner. Inhibition of the PI3K pathway by IL-10 is mediated by abolishment of Akt phosphorylation, abrogation of IκB degradation, blocking of inhibitor of κB kinase (IKK) activity and prevention of NF-κB translocation and DNA binding activity as demonstrated by several groups. In addition to IL-10-induced inhibition of NF-kB-subunit p65 translocation (Shames et al., 1998; Schottelius et al., 1999; Bhattacharyya et al., 2004), IL-10 initiates formation of p50 homodimers which are capable to inhibit transcriptional activity (Driessler et al., 2004).

Maturation of DC in presence of IL-10 induces two distinct subsets of tolerogenic DC (IL-10DC) characterized as $CD83^{high}CD80/CD86^{high}HLA\text{-}DR^{high}$and as $CD83^{low}CD80/CD86^{low}HLA\text{-}DR^{low}$ (**Figure 6A**). Expression of surface molecules associated with DC maturation like CD83, the lymph node homing receptor CCR7 and MHC class-II molecules is reduced on IL-10DC. In contrast, expression of inhibitory receptors such as immunoregulatory receptors like ILT2, ILT3, and ILT4 are increased on IL-10DC, which has been suggested to be involved in tolerance induction (Velten et al., 2004; Boks et al., 2010, 2012; Torres-Aguilar et al., 2010). The profile of cytokine release of IL-10DC differs according to protocols used for DC generation. Nevertheless, the amount of proinflammatory cytokines like IL-6 and TNF-α is similar as compared to classically matured DC. In contrast, IL-10 production is dramatically increased suggesting another possible mechanism resulting in tolerance induction (Velten et al., 2004; Boks et al., 2010, 2012; Gregori et al., 2010; Torres-Aguilar et al., 2010; Xiuling et al., 2010). Several studies showed that *in vitro* generated tolerogenic IL-10DC have an even higher capacity to induce iTreg than tissue resident immature DC (Steinbrink et al., 1997; Torres-Aguilar et al., 2010; Boks et al., 2012). Thus, a reduced expression of immunostimulatory molecules combined with a higher expression of inhibitory costimulators and the production of IL-10 may facilitate tolerogenic functions of IL-10DC.

The tolerogenic function of IL-10DC was demonstrated by induction of antigen-specific anergy in $CD4^+$ and $CD8^+$T cells (Kubsch et al., 2003). This anergic state is characterized by low T cell proliferation and decreased IL-2 and IFN-γ secretion (Steinbrink et al., 1999; Steinbrink, 2002) (**Figure 6B**). In addition, IL-10DC-primed anergic $CD4^+$ and $CD8^+$T cells exhibit suppressive capacity and are able to inhibit activated Th1, Th2, and Tc1 T cell responses (Steinbrink et al., 1997, 1999; Xiuling et al., 2010). Compared to tolerogenic tissue resident DC, IL-10DC are terminally differentiated and exhibit a stable phenotype. Hence, they are insensitive to conversion into immunostimulatory DC mediated by inflammatory factors. These properties provide the opportunity to use this tolerogenic DC subpopulation for induction of

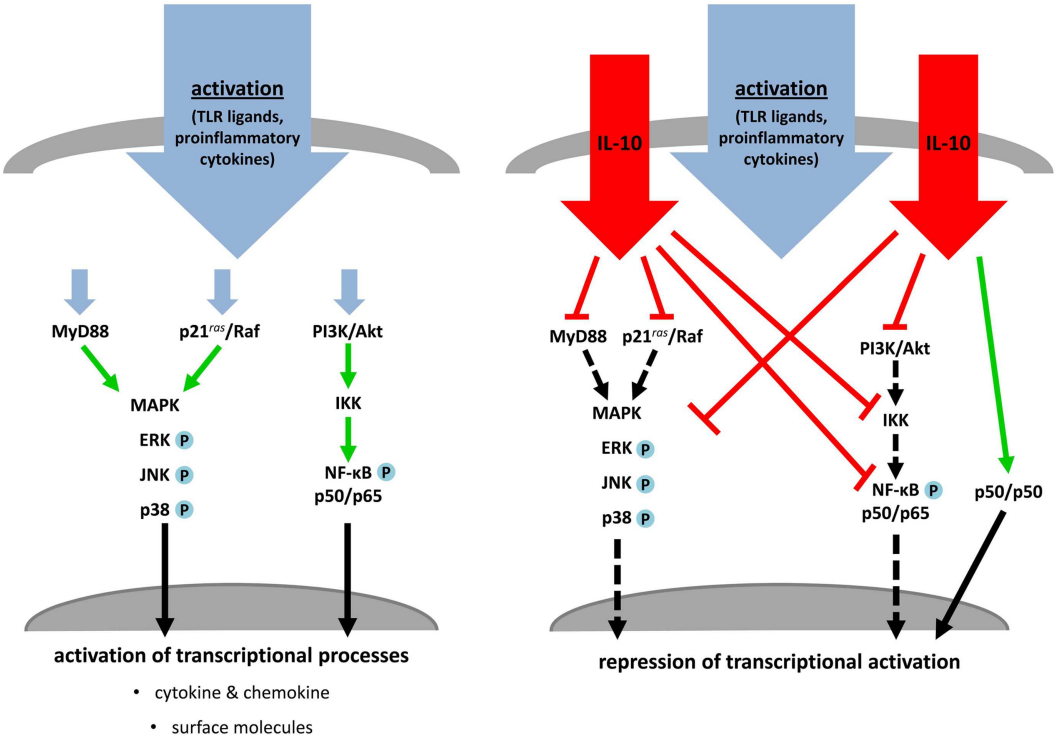

FIGURE 5 | IL-10 treatment blocks activation and maturation of dendritic cells and monocytes. TLR ligands or proinflammatory cytokines stimulate MAPK cascades, MyD88- and PI3K/NF-κB-dependent signaling, which induce transcriptional processes resulting in activation and maturation of stimulated APC (left). Simultaneous treatment of these activated immune cells with IL-10 inhibits key molecules of the named pathways. MAPK- and MyD88-dependent signaling and NF-κB activation are directly or indirectly prevented by IL-10. This leads to an abrogation of transcriptional and post-translational processes or induction of inhibitory transcriptional mechanisms (right).

tolerance in the context of autoimmunity. The inflammatory environment exhibits the capacity to induce maturation of immature DC leading to enhanced immune responses, while IL-10DC do not further respond to these signals (Steinbrink et al., 1999; Thurner et al., 1999).

Gregori et al. (2010) confirmed the *in vivo* existence of IL-10DC in peripheral blood of humans. This is in line with patients suffering from hyper-IgE syndrome due to defective STAT3-signaling, a central component of the IL-10-pathway. DC from these patients are insensitive to IL-10, and therefore, show reduced upregulation of inhibitory molecules (PD-L2, ILT3, and ILT4) in response to IL-10 correlating with an impaired capacity to induce Treg (Saito et al., 2011). Thereby a crucial role of tolerogenic IL-10DC for control of immune responses *in vivo* can be suggested. Modulation of immature DC with IL-10 might be a potential approach to develop novel DC-based vaccination strategies to control and limit harmful T cell responses by antigen-specific induction of potent Treg.

PROTOCOLS FOR GENERATION OF TOLEROGENIC AND IMMUNOGENIC DENDRITIC CELLS

Immunogenic DC are currently applied in various clinical studies to evoke anti-tumor immunity in cancer patients. Although immunological responses are often reported, these are mainly transient demonstrating that DC-based vaccines face several barriers limiting their effectiveness in clinical trials (Engell-Noerregaard et al., 2009). Also the use of tolerogenic DC has an important impact in clinical trials to suppress autoaggressive T cell activity in autoimmune diseases or unwanted responses to allergens. Whereas vaccination with immunogenic DC has been tested over the past decade (Schuler-Thurner et al., 2000; Jonuleit et al., 2001; Tuettenberg et al., 2006), less is known about the potential use of tolerogenic DC in the clinic. It was shown that injection of tolerogenic DC into healthy volunteers is feasible and safe (Dhodapkar et al., 2001). Also initial approaches in the treatment of patients with type 1 diabetes have been successfully performed (Giannoukakis et al., 2011) showing that this method will also be attractive for immunotherapeutic strategies in autoimmune diseases, allergy and transplant rejection. Thus, DC vaccination research has to answer crucial questions to improve the efficacy of vaccination strategies. However, just like for immunogenic DC in anti-cancer trials, several quality criteria have to be defined for tolerogenic DC. Characteristics of tolerogenic DC like their migratory behavior or their functional stability after application *in vivo* need to be addressed in detail.

Translation of DC immunology into clinic implies generation of large amounts of donor-specific DC under good manufacturing practice (GMP)-conditions. Peripheral blood contains two major DC subsets, plasmacytoid and conventional DC, both with

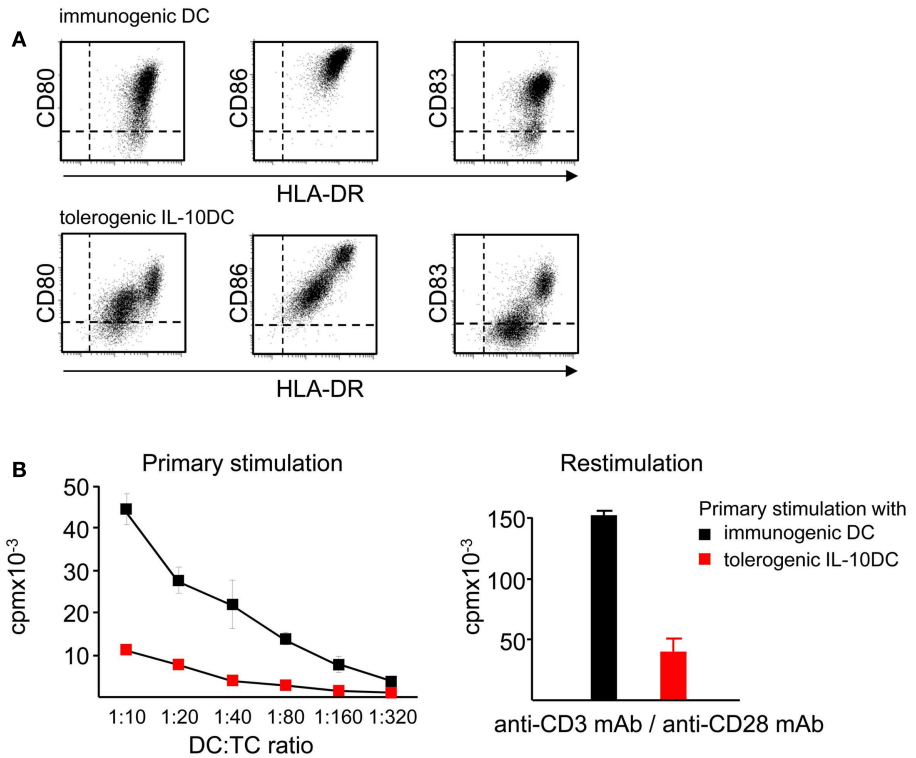

FIGURE 6 | IL-10 affects maturation and induces tolerogenic DC. **(A)** Presence of IL-10 during the maturation process of immature DC impairs the upregulation of costimulatory molecules like CD80 and CD86 resulting in the tolerogenic phenotype of IL-10DC. **(B)** Tolerogenic IL-10DC display a reduced T cell stimulatory capacity compared to immunogenic DC (left) and induce T cell anergy, displayed by reduced T cell proliferation after polyclonal restimulation (right).

low frequencies (1–2% of PBMC) (Macdonald et al., 2002). On account of these low frequencies only a few vaccination studies used directly isolated DC subsets from leukapheresis products (Hsu et al., 1996; Tel et al., 2013). The fact that tumor patients show reduced DC frequencies in peripheral blood further exacerbates isolation of DC subpopulations (Savary et al., 1998). Therefore, initial approaches to expand patients peripheral DC by systemically administering, i.e., Flt3 ligand [a growth factor for myeloid and lymphoid DC progenitor cells (Lyman and Jacobsen, 1998)] have been performed (Fong et al., 2001). As an alternative, $CD34^+$ hematopoietic stem cells isolated from blood or bone-marrow can be used for DC generation (Caux et al., 1997; Ueno et al., 2010). As $CD34^+$ cells are represented only in low frequencies in peripheral blood, these cells are commonly mobilized into the blood by administration of G-CSF. Since this protocol is based on proliferating stem cells the resulting DC are rather heterogeneous and additional steps to improve purity are required.

As shown recently, monocytes serve as a pool of DC precursors *in vivo* that are recruited under inflammatory conditions like a bacterial infection (Cheong et al., 2010). Therefore, monocytes constitute an ideal cell population with a high abundance in peripheral blood that give rise to homogenous DC populations (Romani et al., 1994; Sallusto and Lanzavecchia, 1994). Several protocols for isolation of monocytes from PBMC exist such as positive selection via CD14 (Thurner et al., 1999) or counterflow centrifugation elutriation (Figdor et al., 1982). A very simple technique is the isolation of monocytes by their adherence to plastic surfaces, exemplarily described in this section. Usually, whole blood or buffy coats comprise sufficient PBMC numbers for monocyte isolation in daily routine experiments. However, repetitive treatments of patients with DC require large DC numbers. In this case PBMC isolation from a leukapheresis is a feasibly option (Jonuleit et al., 2001).

Many different protocols for generation of DC have been used in clinical studies, mostly based on the common protocol using GM-CSF and IL-4 for DC generation from monocytes (Romani et al., 1996). But also culture of monocytes in presence of GM-CSF and IL-15 leads to differentiation of DC resembling Langerhans cells (Anguille et al., 2009) that are able to induce tumor-specific T cell responses (Dubsky et al., 2007). However, both DC populations were comparably efficient in induction of anti-tumor T cell responses *in vivo* (Mohamadzadeh et al., 2001; Romano et al., 2011; Schuler, 2011).

In the following we will describe two protocols for the generation of monocyte-derived DC, tolerogenic versus immunogenic. The protocol for terminal differentiated mature DC has been successfully used in many studies by several investigators as an immunotherapeutic drug to treat cancer (**Figure 7**) (Tuettenberg et al., 2006; Correll et al., 2010).

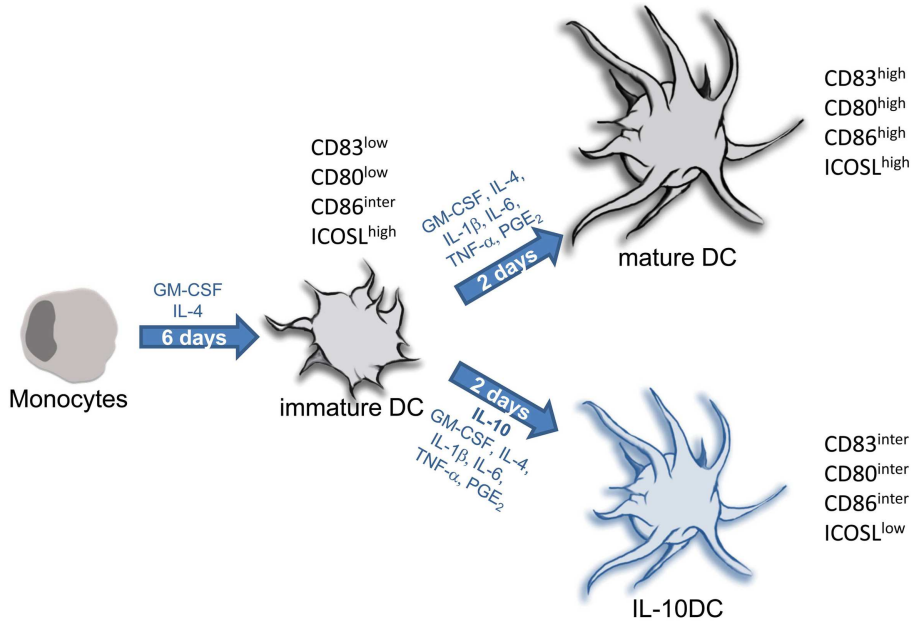

FIGURE 7 | Protocols for generation of tolerogenic and immunogenic dendritic cells from monocytic precursors. In the presence of IL-4 and GM-CSF isolated monocytes differentiate into immature DC within 5–7 days. Terminal differentiation into fully mature DC is induced upon stimulation with inflammatory factors (IL-1, IL-6, and TNF) together with PGE_2. This is a common protocol for generation of immunogenic DC used in numerous clinical trials for DC-based vaccination strategies in context of cancer. Maturation of DC carried out in presence of IL-10 provokes differentiation into tolerogenic IL-10DC.

ISOLATION OF PERIPHERAL BLOOD MONONUCLEAR CELLS

Buffy coats or blood samples should be stored always at room temperature (RT) to prevent agglutination of lymphocytes and granulocytes. For isolation of PBMC by performing density gradient centrifugation follow these steps:

1. Prepare 10–15 ml of separation medium in a 50-ml tube. Take care to obey manufactures instruction for storage of the separation medium as temperature affects density.
2. Carefully layer 25 ml of blood/buffy coat onto the separation medium.
3. Centrifuge at 300 *g* for 30 min. at RT without brake.
4. Four phases should be visible. Top to bottom: plasma phase, interphase with PBMC, separation media, erythrocytes/granulocytes.
5. Convey plasma (if needed) and PBMC into separate new 50 ml tubes. At this moment PBMC might be contaminated with thrombocytes. Several washing steps are required to increase purity of PBMC.
6. For washing add 1× PBS (without Ca^{2+}/Mg^{2+}) + 0.5 mM EDTA ad 50 ml to PBMC.
7. Centrifuge at 400 *g* for 7 min and 4°C.
8. Repeat washing until supernatant is clear and then determine the number of isolated PBMC.

Isolated PBMC can directly be used for generation of DC. If PBMC are suspended in culture media and kept at 4°C, they can be stored overnight in X-VIVO-15 or RPMI1640 + 2% heat-inactivated plasma until use. PBMC can be stored for a longer time period when suspended in human serum albumin + 10% DMSO and frozen in liquid nitrogen.

COLLECTION AND INACTIVATION OF PLASMA

Dependent on the culture medium, generation of DC from monocytes requires supplementation of culture media with autologous plasma. Plasma can be collected directly after density gradient centrifugation during PBMC isolation (see step 5 in Isolation of Peripheral Blood Mononuclear Cells). For inactivation of plasma proteins (e.g., complement factors), plasma is incubated in a water bath at 56°C for 30 min. The hereby degraded proteins are removed by centrifuging at 1500 *g* for 15 min. Note, first heat-inactivation, then centrifugation. Plasma can be stored at +4°C.

GENERATION OF IMMATURE DENDRITIC CELLS FROM MONOCYTES

In vitro generation of human DC from monocytes requires caution in terms of choosing the appropriate culture media. Several media meet the guidelines of GMP and can be used for DC generation. Successful studies have been performed using CellGro media (Cellgenix) or X-VIVO media (Lonza) (Schuler-Thurner et al., 2000; Royer et al., 2006; Schadendorf et al., 2006; Tuettenberg et al., 2006). Importantly, depending on local regulations for GMP media, there are major differences in the quality of generated DC. For example, X-VIVO-15 derived from Belgium is suited for generation of fully matured DC, whereas X-VIVO-15 produced in USA contains less serum proteins and generates more immature DC. Therefore, the particular objective dictates the particular media that should be used for DC generation. Likewise, RPMI1640 supplemented with autologous plasma can be employed (Thurner

et al., 1999). The use of FCS with xenogeneic proteins for supplementation should be avoided because residual FCS-proteins can potentially induce FCS-IgE responses in patients (Jonuleit et al., 2000a, 2001).

We suggest using the following protocol for generation of substantial yields of immature DC (**Figure 7**) in which we use X-VIVO-15 (Lonza, Belgium) as culture media.

- Preheat all media used through the protocol to 37°C.
- Prepare RPMI1640 (Gibco) with 1.5% autologous, heat-inactivated plasma.
- Prepare X-VIVO-15 with 1% autologous, heat-inactivated plasma.

1. Transfer 10–15 $\times$ 10^6 PBMC per well to a 6-well cell culture plate (polystyrene, wells treated for cell culture, e.g., Costar) in 2 ml of preheated RPMI1640 supplemented with 1% autologous plasma.
2. Incubate culture plates for 30–60 min at 37°C in an incubator. Place plates next to each other for optimal temperature adjustment. Monocytes start to produce an extracellular matrix that allows adherence to plastic surfaces.
3. Control grade of adherence under the microscope by slightly shaking the culture plate.
4. Carefully wash wells with preheated PBS and rinse off contaminating lymphocytes using a 10-ml pipette.
5. Adhered monocytes are cultured in 3 ml of preheated culture media, 400 IU/ml GM-CSF (growth factor for myeloid cells) and 1000 IU/ml IL-4 (prevents dominant differentiation of monocytes into macrophages). Cytokines in GMP-quality are available, i.e., from Cellgenix, Freiburg, Germany.
6. At day two and day four remove 1 ml of culture media from each well. Replace with 1 ml of preheated culture media supplemented with 800 IU/ml GM-CSF and 1000 IU/ml IL-4.
7. At day 6 immature DC can be harvested. Caution: immature DC are not terminally differentiated. Withdrawal of IL-4 supplementation leads to differentiation into macrophages.

TERMINAL DIFFERENTIATION OF IMMATURE DENDRITIC CELLS

The maturation process of DC is induced by imitating an inflammatory situation in the skin. This is simply performed by addition of proinflammatory cytokines usually produced during inflammation: IL-1β, IL-6, and TNF-α. In addition, prostaglandin-E2 (PGE_2) further accelerates DC maturation (Jonuleit et al., 1997; Steinbrink et al., 2000). Immature DC that are stimulated following this protocol do not produce IL-12p70, a cytokine involved in T cell polarization and NK cell activation that was thought to be crucial for proper anti-tumor T cell responses. However, clinical trials applying this protocol for DC maturation showed potent induction of cytotoxic tumor-reactive $CD8^+$ T cells and Th1 polarization in patients. Modified protocols for DC maturation employing TLR ligands or type I interferons enable IL-12 production in DC that likewise initiate anti-tumor T cell responses in patients (Schuler, 2011; Wieckowski et al., 2011; Hansen et al., 2013).

To terminally differentiate immature DC:

1. 10^6 immature DC are cultured in 3 ml preheated X-VIVO-15 supplemented with 1% plasma, 400 IU/ml GM-CSF, 1000 IU/ml IL-4, 1700 IU/ml IL-1β, 1000 IU/ml IL-6, 500 IU/ml TNF-α (i.e., Cellgenix), and 1 μg/ml PGE_2 (Cayman Chemical Comp, USA) in a fresh 6-well culture plate.
2. After 48 h DC are terminally differentiated and can be harvested. Mature DC are non-adherent, therefore contamination with adherent cells should be avoided.

Clinical use of DC requires the determination of quality control criteria (Romani et al., 1996; Vries et al., 2002). Fully mature DC exhibit high and homogeneous expression of CD83, CD80, CD86, MHC class-I and class-II analyzed by flow cytometry (**Figure 3A**). Also functional testing is required, for this, alloreactive T cells are stimulated with different ratios of mature DC (**Figure 2C**). Half maximal T cell proliferation provoked by fully matured DC is achieved at DC:T cell ratios of approximately 1:200.

Phenotypic stability: as mature DC are terminally differentiated this can easily be tested by performing a cytokine washout. For this, mature DC are harvested, washed and plated in a fresh 6-well plate in preheated culture medium in absence of additional cytokines. Terminally differentiated mature DC still conform to quality issues after 24–48 h of culture in absence of cytokines (Figdor et al., 2004).

GENERATION OF TOLEROGENIC IL-10-MODULATED DENDRITIC CELLS

As noted, tolerogenic properties of immature DC are strengthened by modulation with IL-10 (Steinbrink et al., 1997). IL-10 is a very sensitive cytokine; its biological activity is preserved best by storage at −80°C and is often lost within 24 h after thawing. For generation of IL-10DC the following protocol is suggested.

1. Incubate 15 $\times$ 10^6 PBMC per well of a 6-well-plate in 2 ml RPMI1640 + 1% plasma for 30 min in an incubator.
2. Rinse off non-adherent cells by washing wells with 1× PBS.
3. Subsequently, remaining adherent cells are cultured in 2 ml X-VIVO-15 + 1% plasma overnight.
4. On day 1, replace media with 3 ml fresh X-VIVO-15 supplemented with 400 IU/ml GM-CSF, 150 IU/ml IL-4, and 1% plasma.
5. On day 3, replace 1 ml media with 1ml fresh X-VIVO-15 supplemented with 800 IU/ml GM-CSF, 150 IU/ml IL-4, and 1% plasma.
6. On day 6, immature DC can be harvested.
7. 10^6 immature DC are cultured in 3 ml X-VIVO-15 supplemented with 1% plasma, 400 IU/ml GM-CSF, and 150 IU/ml IL-4. For differentiation into IL-10DC 5 ng/ml IL-1β, 5 ng/ml TNF-α (e.g., Miltenyi Biotec, Germany), 50 IU/ml IL-6, 1 μg/ml PGE_2, and 20 ng/ml IL-10 (e.g., Schering-Plough Corporation) are added.
8. Stable tolerogenic IL-10DC can be harvested 48 h later.

In conclusion, a successful immunotherapeutic strategy will include a combination of DC vaccination with additional therapies targeting other immune cell populations as well as the tumor or tumor micromilieu itself. The combination of DC-modulating agents with additional therapies such as antibody treatment to inactivate or deplete Treg could possibly increase the potential of DC-based immunotherapy. Some clinical studies using Treg-depletion in tumor patients followed by DC vaccination showed

that elimination of Treg enhances the magnitude of tumor-specific T cell responses. Finally, these issues need to be addressed in comparative clinical studies to determine optimal vaccine characteristics. DC vaccination can then be put to the ultimate test in randomized clinical trials. The same is true for the use of tolerogenic DC in terms of treating an over activated immune system in context of autoimmunity. Also here, application of tolerogenic DC in combination with modulation of proinflammatory mediators could possibly synergize in action.

Importantly, the described protocol for generation of immunogenic DC is commonly used for initiation of anti-melanoma responses. It can be suspected that a different target tumor could require a different maturation protocol or even a different protocol for DC generation. Therefore, analyses addressing the impact of various protocols for DC generation and maturation on the resulting T effector cell populations are of importance and could improve DC-based therapeutic success.

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Dendritic cells enhance the antigen sensitivity of T cells

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Naive T cells continuously migrate between the circulatory system and lymphoid organs, where they make dynamic contacts with rare dendritic cells (DCs) that strategically form an extensive dendrite network. In such a scenario, T cells spend most of their time quickly scanning the antigenic content of multiple DCs. These interactions provide the basis for efficient adaptive responses by increasing the probability of encounters between rare antigen-specific T cells and those DCs presenting the respective cognate antigens. In the absence of foreign antigen, however, T cells show different degrees of functional sensitivity toward TCR stimulation. Scanning of MHC/self-peptide complexes by naive T cells in the absence of infection is not without consequences but it increases their subsequent response toward antigenic challenge. This indicates that TCR sensitivity in naive T cells is tuned depending on the MHC/self-peptide signals they integrate from the environment even before T cells encounter cognate antigen. DCs have emerged as key components in providing MHC/self-peptide complexes and increasing the sensitivity of T cells toward subsequent TCR triggering. In the absence of cognate antigen, DCs maintain a tonic TCR signaling and license T cells for immune synapse (IS) maturation resulting in enhanced T cell responses toward a subsequent antigen stimulation. This review discusses recent findings on this subject and highlights the importance of the DC pool size for optimal T cell awareness to foreign antigen.

Keywords: dendritic cells, T cells, tonic TCR signaling, self-MHC, antigen sensitivity

INTRODUCTION

Bone marrow (BM)-derived T cell precursors seed the thymus, where they differentiate into mature T cells. During this process, those T cells bearing appropriate α/β T cell receptors (TCR) are positively selected in order to ensure MHC restriction. In addition, those harmful T cells recognizing MHC/self-peptide complexes (referred to as self-MHC hereafter) with high affinity are mostly purged from the repertoire by negative selection. This process of T cell selection in the thymus warrants that peripheral T cells are MHC restricted, so they are able to recognize infected cells, but react only weakly to self-MHC and thus autoimmunity is minimized (Klein et al., 2009).

Once naive T cells exit the thymus, they recirculate between secondary lymphoid organs via blood or lymph. Upon encounter of cognate antigen presented by MHC on dendritic cells (DCs), T cells are primed and differentiate into potent effector cells with the ability to leave the systemic circulation and infiltrate inflamed sites. After the threat is resolved, memory CD4 and CD8 T cells remain patrolling the body and act as sentinels for fast responses against secondary infections. Different memory T cell populations have been described, some of them recirculating between lymphoid organs and others being present at peripheral tissues where the initial infection took place (Sallusto et al., 2004; Harty and Badovinac, 2008; Wakim and Bevan, 2010; Jiang et al., 2012). Therefore, naive, effector, and memory T cells all encounter antigen-presenting cells (APCs) such as DCs in different environments, i.e., lymphoid and non-lymphoid organs.

Dendritic cells are BM-derived APCs that are crucial for initiating T cell responses (Steinman and Cohn, 1974; Jung et al., 2002). One of their hallmarks is to excel in antigen presentation on MHC-I and -II to CD8 and CD4 T cells, respectively (signal 1), provide costimulatory signals (signal 2), and promote the differentiation of naive T cells into specialized effector cells via the provision of key cytokines (signal 3; Sporri and Reis e Sousa, 2005; Heath and Carbone, 2009; Joffre et al., 2009; Segura and Villadangos, 2009; Kurts et al., 2010). It is commonly accepted that the capacity of DCs to provide signals 1, 2, and 3 simultaneously makes them specially suited to promote priming of naive T cells. In addition, DCs are located in the T cell areas of lymphoid organs, or easily migrate into them upon activation, forming an extensive network of dendrites thus providing a topographical context in which DCs and T cells interact (Lindquist et al., 2004). This may be an important differential feature of DCs, since other professional APCs such as B cells also express high levels of costimulatory molecules and produce a variety of T cell growth factors, but are not located in the T cell area under normal conditions.

During the steady state, T cells frequently contact DCs in secondary lymphoid organs. There are at least two important consequences of these frequent contacts: (1) they increase the likelihood for encounters between extremely low frequencies of antigen-specific naive T cells and the few DCs presenting the respective cognate antigen; (2) self-MHC recognition on DCs in the absence of cognate antigen induces a basal, tonic TCR signaling that augments the antigen sensitivity of T cells (**Box 1**). This review focuses on recent developments by which self-MHC recognition on DCs prior to an encounter with foreign antigen induces tonic TCR signaling thereby increases the awareness of T cells for subsequent encounters with their cognate antigen. Finally,

BOX 1 | Summary of self-MHC recognition, tonic TCR signaling and antigen sensitivity.

Antigen sensitivity is the capacity of T cells to respond to TCR stimulation via cognate MHC/antigen recognition to become activated and undergo proliferation. The higher the sensitivity, the lower the amount of MHC/antigen recognition required to trigger full T cell activation. T cells can undergo different states of antigen sensitivity depending on the cues they integrate from the environment. A key cue is the recognition of *MHC/self-peptide complexes* (referred to as self-MHC), which induces a basal level of TCR activation resulting in increased sensitivity toward cognate antigen (Stefanova et al., 2003; Hochweller et al., 2010). This basal activation of the TCR complex is also referred to as *tonic TCR signaling* and is exemplified by low levels of CD3ξ phosphorylation. Thus, self-MHC recognition increases the awareness of T cells and licenses them to respond to lower amounts of cognate antigen.

When does self-MHC recognition increase the antigen sensitivity of T cells? There are two stages during which self-MHC recognition increases the T cell antigen sensitivity: *prior to* and *concomitant to* recognition of foreign antigen:

Self-MHC recognition in the absence of cognate antigen. DCs and T cells continuously interact in secondary lymphoid organs. Self-MHC recognition by T cells results in tonic TCR signaling and increased T cell responsiveness toward a subsequent encounter with cognate antigen. The nature of the self-peptide(s) is presently unknown.

Concomitant recognition of self- and foreign-antigen bound to MHC. The sole recognition of MHC/foreign-peptide complexes is inefficient to trigger naive T cell activation. Co-recognition of self-MHC complexes dramatically increases T cell responsiveness (Krogsgaard et al., 2005). The same self-MHC complexes that drive positive selection in the thymus have been shown to increase the antigen sensitivity during concomitant recognition of foreign antigen (Ebert et al., 2009; Lo et al., 2009).

we discuss some key questions in this field that remain to be answered.

T CELLS FREQUENTLY CONTACT DCs IN SECONDARY LYMPHOID ORGANS IN THE STEADY STATE

The frequent contacts between T cells and DCs provide a structural basis for the uniqueness of DCs in T cell priming. Elegant *in vivo* two-photon microscopy experiments have provided important insights into the kinetics of T cell priming (for reviews, see Bousso and Robey, 2003; von Andrian and Mempel, 2003; Cahalan and Parker, 2005; Cahalan and Gutman, 2006; Germain et al., 2008; Kastenmuller et al., 2010).

In the absence of cognate antigen, T cells and DCs move along networks of reticular fibroblasts (Bajenoff et al., 2006), with T cell motility appearing to be otherwise random (Miller et al., 2002, 2004a; Textor et al., 2011). The average speed of naive CD4 and CD8 T cells in the absence of antigen has been reported to vary between about 6 μm/min (Skokos et al., 2007) and 18 μm/min (Textor et al., 2011), with most reports showing an average speed of about 10 μm/min (Miller et al., 2002, 2004a; Bousso and Robey, 2003; Hugues et al., 2004; Mempel et al., 2004; Shakhar et al., 2005). These variations may likely be due to differences in the T cell clonality, technical issues, as well as the depth of imaging in the lymph node (LN) which has been shown to significantly impact T cell speed (Worbs et al., 2007). In the absence of cognate antigen, it has been estimated that the mean transit time in LNs is about 10 h for CD4 T cells and about 20 h for CD8 T cells, with considerable variation depending on the particular LN. Of this time, about one-third is spent interacting with MHC molecules on DCs (Mandl et al., 2012), with the majority of the contacts between T cells and DCs lasting between 3 and 5 min (Miller et al., 2004a,b; Mandl et al., 2012). These interactions are highly dynamic, as CD4 T cells undergo 160–200 contacts with DCs during their transit time in the LNs, whereas CD8 T cells undergo about 300 contacts (Mandl et al., 2012). On the other side, each DC is contacted by about 500 CD8 T cells (Bousso and Robey, 2003) or 5000 CD4 T cells (Miller et al., 2004a) per hour. Thus, T cells frequently scan the surface of DCs during their transit through secondary lymphoid organs in the absence of foreign antigen. It is generally accepted that these frequent contacts serve as a "finding needle in the haystack" function: otherwise impossible interactions can proceed between extremely rare antigen-specific T cells and DCs presenting that particular antigen. Regarding the kinetics of T cell priming, different laboratories using intravital two-photon microscopy have reached similar conclusions: following recognition of cognate antigen on DCs, T cells undergo activation in three distinct and sequential phases (Hugues et al., 2004; Mempel et al., 2004; Miller et al., 2004a,b). Within 8 h of access to the T cell zone, T cells slow down their mean speed to about 4 μm/min in average engaging serial encounters with DCs bearing cognate antigen. By sampling the antigen dose during this initial phase, T cells become activated and make the decision of whether they enter the next phase of T cell activation (Henrickson et al., 2008). This phase is characterized by taking place over a longer period of time (about 12 h) in which an arrest of T cell mobility is observed with prolonged interactions with DCs, which may last longer than 1 h. Consequently, T cell speed is halved during this phase. Coinciding with T cell proliferation, T cells disengage from these stable contacts with DCs and enter the final phase of T cell activation by resuming their motile behavior serially interacting with different DCs. Integration of signals derived from serial encounters with DCs bearing cognate antigen has been shown to increase the effector function of T cells (Celli et al., 2005). Following activation of CD4 T cells, naive CD8 T cells undergo directional rather than random migration toward DC–CD4 T cell conjugates via a CCL3 and CCL4 gradient, thereby increasing the likelihood of receiving help to increase their cytolytic and recall responses (Castellino et al., 2006). A similar process has been observed during alternative cross-priming whereby activated NKT cells attract naive CD8 T cells to the relevant DCs via CCL17 (Semmling et al., 2010). In summary, T cells frequently sample the surface of DCs in a highly dynamic fashion during their transit through secondary LNs in the absence of cognate antigen. Upon encounter with cognate antigen, T cells change their kinetic behavior and undergo intense interactions with antigen-bearing DCs.

Besides increasing the likelihood for T cells finding the DC presenting the respective cognate antigen, the frequent interactions between DCs and T cells in the steady state (absence of cognate antigen) have in addition two other major consequences: (1) recognition of self-antigen on DCs outside the thymus results in peripheral tolerance, i.e., deletion of self-reactive T cells thereby

minimizing autoimmunity (Kurts et al., 1997, 2001; Probst et al., 2003), and (2) recognition of self-MHC on DCs induces a tonic TCR signaling that promotes the sensitivity of T cells toward their cognate antigen (Stefanova et al., 2002; Hochweller et al., 2010). These two major functions of steady-state DCs seem at first contradictory. We have proposed that the affinity of TCR/self-MHC recognition dictates the final T cell outcome: high-affinity interactions lead to T cell deletion, whereas those of weaker affinity promote T cell antigen sensitivity (Garbi et al., 2010).

DENDRITIC CELLS ARE REQUIRED TO MAINTAIN THE ANTIGEN SENSITIVITY OF NAIVE T CELLS

By analyzing CD5 expression as a surrogate marker of TCR triggering, it has recently been shown that most of the tonic TCR signaling of naive T cells occurs in the secondary lymphoid organs (Mandl et al., 2012). Most contacts between T cells and DCs in the LN take place for about 5 min. For CD4 T cells, these contacts are highly dependent on MHC-II expression by the DC, because absence of MHC-II results in shorter interactions of about 2 min (Mandl et al., 2012). Pioneering work in Germain's laboratory showed that self-MHC recognition by T cells in the absence of cognate antigen resulted in basal activation of the TCR complex and increased antigen sensitivity of T cells toward subsequent encounters with their cognate antigen (Stefanova et al., 2002). The requirement of DCs for tonic TCR signaling and maintenance of the antigen sensitivity in T cells was described in transgenic CD11c.DOG mice, in which DCs express the human diphtheria toxin receptor (DTR) and thus can be depleted by single or repetitive administrations of diphtheria toxin (DT; Hochweller et al., 2008). In these mice, naive CD4 and CD8 T cells isolated after DT application show a marked hypoproliferative response against a variety of antigens presented by professional APCs, including cognate peptide, superantigen (Hochweller et al., 2010), and anti-TCRβ antibody (**Figure 1A**). These results indicate that DC–T cell interactions in the steady state in the absence of cognate antigen are required to maintain the sensitivity of naive T cells for their cognate antigen. Similar results have been obtained in other transgenic mouse strains such as CD11c.DTR (Hochweller et al., 2010), and the recently described CD11c.LuciDTR (**Figure 1B**) that expresses luciferase and DTR under the CD11c promoter (Tittel et al., 2012). The proliferative response to anti-CD3ε antibody is, however, not compromised in T cells from DC-depleted mice (Birnberg et al., 2008; **Figure 1C**). Although at present we cannot explain why T cells from DC-depleted mice are able to respond normally to anti-CD3ε stimulation, but not to activation with MHC/antigen or anti-TCRβ antibody, differences in the binding affinities or in the ability of anti-TCRβ and anti-CD3ε antibodies to cross-link different TCR complexes may contribute to explain this paradox.

Tuning of the T cell antigen sensitivity is a dynamic process that depends on fast interactions with DCs. Antigen sensitivity is lost very quickly after disruption of cell–cell contacts (within 15 min; Stefanova et al., 2002), and it is regained also very promptly, within 30 min of reintroduced DC–T cell contacts (Hochweller et al., 2010). The loss of antigen sensitivity is not associated to decreased viability of T cells following DC depletion. Both the frequency and the numbers of viable T cells is not altered in DC-depleted mice (Hochweller et al., 2010), which is consistent with findings that mice constitutively lacking DCs do not present reduced T cell counts (Birnberg et al., 2008; Ohnmacht et al., 2009).

Studies using DC depletion have demonstrated that DCs are required to maintain the sensitivity of T cells for subsequent challenges with their cognate antigen. Both splenic $CD8^+$ and $CD8^-$ DCs are equally suited for tuning the T cell's antigen sensitivity (Hochweller et al., 2010). B cells are also able to maintain T cell responsiveness *in vitro*, although due to anatomical restrictions *in vivo*, naive T cells will only seldomly interact with B cells at the

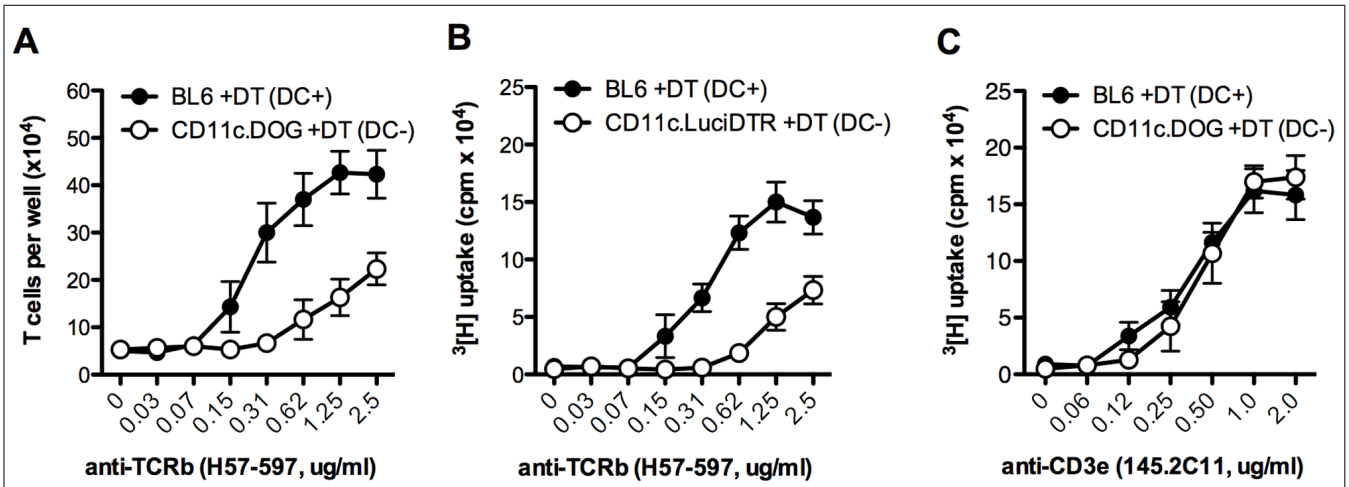

FIGURE 1 | CD4 T cell proliferation in mice lacking DCs. 5×10^4 MACS-purified CD4 T cells from the indicated mouse strain were activated for 4 days with the specified concentration of plate-bound anti-TCRβ (clone H57-597) or anti-CD3ε (145.2C11) antibodies. Proliferation was quantified either by the number of T cells per well at the end of the experiment **(A)**, or by incorporation of ^{3}[H]-thymidine for the last 9 h of the experiment **(B,C)**. Results are expressed as mean ± SEM ($n = 3$ mice). Shown is one representative of three experiments. Similar results were obtained with BL6 mice treated with DT or with the respective transgenic mice treated with PBS.

borders between the T and B cell zones. However, not all APCs can do it; macrophages are not able to promote T cell antigen sensitivity (Hochweller et al., 2010), and whether this is due to differences in the expression level of self-MHC or other molecules is still an open question. A central question was how many DCs are required to maintain the T cell sensitivity? Using mixed BM chimeras in which a graded percentage of DCs expressed DTR, we showed that depletion of only half of the DC compartment already resulted in partial loss of antigen sensitivity. The activation status of the remaining DCs was not altered regarding expression of MHC-I and -II, and costimulatory molecules (N. Garbi, unpublished data), suggesting that the level of DC activation does not play a key role in the maintenance of T cell antigen sensitivity. Thus, minor alterations in the size of the DC compartment seem to have an impact on the T cell responsiveness. This is particularly important in view of the rapid turnover of DCs in the lymphoid organs of mice. Depending on the methodology used, the half-life of cDCs in the spleen has been estimated to be between about 2 days (Kamath et al., 2002) and 7 days (Liu et al., 2007), the whole splenic DC compartment is replaced about 150 or 45 times, respectively, during the lifespan of a laboratory mouse.

DEPENDENCE ON SELF-MHC RECOGNITION AND COSTIMULATORY MOLECULES

Seminal work by the group of Germain, demonstrated that recognition of self-MHC class II by CD4 T cells promoted the sensitivity of naive CD4 T cells against a subsequent challenge with cognate antigen (Stefanova et al., 2002, 2003). This work demonstrated that in the steady state, the TCR is actively integrating cues from self-MHC recognition leading to a basal activation of proximal TCR signaling events, specifically CD3z phosphorylation (Stefanova et al., 2002). A subsequent study showed that self-MHC-II recognition was required to promote CD4 T cell antigen sensitivity also *in vivo* (Fischer et al., 2007). DCs were later identified as the cells providing self-MHC recognition to CD4 and CD8 T cells resulting in increased T cell antigen sensitivity toward subsequent challenges (Garbi et al., 2010; Hochweller et al., 2010). Therefore, the requirement of DCs to maintain T cell antigen sensitivity is molecularly based on recognition of self-MHC. Indeed, interaction of DCs and T cells resulted in a specific increase in the basal phosphorylation of ZAP70-associated CD3ξ, although the total levels of ZAP70 and CD3ξ were not altered (Hochweller et al., 2010). Thus, self-MHC recognition on DCs induces tonic TCR signaling that is critical to maintain antigen sensitivity. It is presently unclear whether more upstream events in TCR signaling are also affected by lack of DC–T cell interaction, for instance recruitment of the NCK adaptors into the TCR complex, which is known to modulate the TCR antigen sensitivity (Roy et al., 2010).

The loss in antigen sensitivity is not accompanied by changes in the expression of molecules known to modulate TCR signaling such as TCRβ, CD3ε, CD3ξ, CD4, and CD8α (Hochweller et al., 2010), or in global gene expression (Hochweller and Garbi, unpublished data), suggesting specific defects in signaling events rather than in expression patterns. This is supported by the rapid loss of antigen sensitivity after disruption of DC–T cell contacts (∼15 min; Hochweller et al., 2010) and rapid reconstitution upon contact reintroduction (∼30 min; Stefanova et al., 2002). In addition, T cells from DC-depleted mice proliferate normally in response to TCR-independent stimuli such as ConA or PMA/ionomycin stimulation, indicating that they do not have a global defect in cell cycle entry.

The requirement for self-MHC recognition on DCs to promote T cell responsiveness toward a subsequent antigenic challenge is reminiscent of recent data showing that self-MHC-II recognition at the time of foreign antigen recognition also increases the response of CD4 T cells to their cognate antigen (Ebert et al., 2009; Lo et al., 2009) in what has been defined as the pseudodimer model (Krogsgaard et al., 2005, 2007). The nature of the MHC class I/peptide complex required to maintain CD8 T cell antigen sensitivity is less clear. Our results show that CD8 T cells require prior self-MHC recognition on DCs to maximally respond to a subsequent antigenic challenge (Hochweller et al., 2010). In analogy to the pseudodimer model for CD4 T cell activation, simultaneous recognition of MHC class I molecules loaded with foreign stimulating peptide and with endogenous non-stimulating peptides strongly increases the sensitivity to the former (Purbhoo et al., 2004; Cebecauer et al., 2005; Yachi et al., 2005, 2007; Anikeeva et al., 2006). However, as opposed to CD4 T cells, all tested MHC class I-binding peptides served as coagonists (Yachi et al., 2005, 2007), suggesting that it is the interaction between CD8 coreceptor and MHC class I/endogenous peptide what is required to amplify responses against cognate antigens and not the specific TCR-MHC/self peptide recognition observed for CD4 T cells (Gascoigne, 2008). This hypothesis is supported by the finding that the CD8 coreceptor, but not CD4 is required to increase sensitivity of T cells at high density of peptide ligands (Purbhoo et al., 2004). However, as for the maintenance of CD4 T cell antigen sensitivity, it remains unknown whether specific MHC class I/endogenous peptide complexes need to be recognized prior to foreign antigen challenge for maximal responses.

Altered peptide ligands (APLs) bound to MHC have been shown to partially activate the TCR complex (Evavold et al., 1993). However, the outcome of these partial TCR activation dramatically differs from the tonic TCR signaling induced by self-MHC recognition discussed in this review. APLs often result in (1) partial T cell activation leading to functional T cell anergy in response to subsequent encounter with cognate antigen, or (2) TCR antagonism when recognized simultaneously with cognate antigen (Sloan-Lancaster and Allen, 1996). Although some endogenous self-peptides have been shown to function as APL for a given TCR clone (Evavold et al., 1995), self-MHC ligands inducing tonic signaling do not induce T cell activation (as defined by the "quiescent" state of naive T cells *in vivo*) but increase their sensitivity toward subsequent encounters with cognate antigens. Although presently unknown, the biochemical basis for the difference between self-ligands inducing T cell anergy (APLs) and those inducing productive tonic TCR signaling may reside in the affinity for the TCR.

Thus, self-MHC recognition tunes T cell responsiveness toward foreign antigen in two different contexts: first, exclusive self-MHC recognition in the absence of foreign antigen results in tonic TCR signaling and enhanced T cell responsiveness to a subsequent challenge with cognate antigen; second, as defined in the pseudodimer

model, concomitant recognition of MHC molecules loaded with self- and foreign- peptides leads to increased sensitivity to the later.

Interestingly, it is the same ligands driving positive selection in the thymus that increase the CD4 T cell responsiveness toward cognate antigen when recognized simultaneously in the periphery (Ebert et al., 2009; Lo et al., 2009). We proposed that a similar mechanism is in place to promote responsiveness to subsequent antigenic challenge, i.e., it is the recognition in the periphery of the ligands inducing positive selection in the thymus that results in tonic TCR signaling and increased T cell antigen sensitivity (Garbi et al., 2010). Although this hypothesis is not formally proven yet, Stefanova et al. (2002) demonstrated that recognition of the same MHC class II restriction element that drives positive selection of AND TCR transgenic CD4 T cells is required to maintain their antigen responsiveness in the periphery. Whether this finding can be generalized to other TCR specificities is still an open issue, but strongly suggests that the selecting MHC class II haplotype is required and that the mere interaction between MHC-II and the CD4 coreceptor is not sufficient to maintain antigen sensitivity (Stefanova et al., 2002).

Presently, it is still unclear whether other molecular cues between DCs and T cells participate in promoting antigen sensitivity in addition to self-MHC recognition. MHC-deficient DCs are able to partially maintain T cell responsiveness, albeit to a much lower degree than their MHC-sufficient counterparts (Hochweller et al., 2010). DCs express large amounts of costimulatory molecules such as CD80 and CD86 in the steady state. Because activation of their receptor CD28 synergizes TCR engagement of cognate antigen to bolster T cell proliferation, it is tempting to speculate that CD28 ligation may also synergize with self-MHC recognition to promote tonic TCR signaling. In addition, other mechanisms may also be involved. In this context, non-MHC-dependent contact of T cells to DCs induces a transient semi-activation of the former resulting in enhanced T cell responses to subsequent cognate antigen in a process known as "adhesion-induced T cell priming" (Revy et al., 2001). However, this phenomenon is not specific to interaction with DCs because adhesion to other cell types, immobilized ligands or even glass had a similar effect (Randriamampita et al., 2003).

ARE DENDRITIC CELLS REQUIRED TO MAINTAIN THE ANTIGEN SENSITIVITY OF OTHER T CELL POPULATIONS: EFFECTOR, MEMORY, AND REGULATORY T CELLS?

Presently it is unknown whether effector or memory T cells in the steady state are dependent on DC-induced tonic TCR signaling to increase their sensitivity against a subsequent challenge with cognate antigen. During infection, memory CD8 T cells interact with DCs in lymphoid and non-lymphoid sites resulting in antigen-specific reactivation (Belz et al., 2007; Wakim et al., 2008). However, further experiments are needed to determine whether effector/memory T cells also depend on constant self-MHC recognition on DCs in the absence of infection to increase their sensitivity against a subsequent antigen encounter.

In the different context of simultaneous recognition of self- and cognate-antigen, effector T cells seem to be less dependent on self-MHC recognition than their naive counterparts for antigen-specific responses (Yachi et al., 2007). Based on those findings, we hypothesize that effector/memory T cells are also less dependent on recognition of self-MHC in the steady state to increase their sensitivity to further cognate antigenic challenge.

There is some correlative evidence that DCs regulate the size of the Treg compartment in a positive manner. In mice depleted for DCs or lacking DCs constitutively, a reduced frequency of Tregs by a factor of approximately 2–3 has been reported in the spleen, LNs, and/or blood (Darrasse-Jeze et al., 2009; Bar-On et al., 2011). However, in other reports, no differences or very small differences in the number of Tregs in the spleen and/or LNs were reported in mice constitutively lacking DCs (Birnberg et al., 2008; Ohnmacht et al., 2009). In addition, DC depletion did not result in decreased suppressive function of splenic Treg cells (Birnberg et al., 2008). Following depletion of DCs for 2 days in CD11c.DOG, we did not observe any alteration in the number of Treg cells, suppressive capacity or phenotype in the spleen (**Figure 2** and unpublished data). Our results and those by Birnberg et al. suggest that DCs are not required to maintain the suppressive capacity of Tregs. Therefore, further studies are required to investigate the apparently contradictory results on the role of DCs in the maintenance of Treg homeostasis.

DENDRITIC CELLS LICENSE T CELLS FOR IMMUNE SYNAPSE FORMATION

Following TCR signaling in response to recognition of foreign antigen, T cell surface molecules and scaffolding protein are redistributed and enriched in the contact zone between T cells and APCs, resulting in the generation and maturation of the IS. The IS is characterized by a central enrichment of TCR and CD3 molecules termed central supramolecular activation cluster (cSMAC) that is surrounded by a further cluster formed by LFA-1, also called peripheral SMAC (Grakoui et al., 1999). Initial TCR triggering results in the so-called inside-out signaling leading to activation of LFA-1 (Kinashi, 2005). In turn, activated LFA-1 binds to ICAM-1 molecules on the APC promoting firm T cell-APC adhesion (Lim et al., 2011) and further TCR/CD3 signaling events (Davis and Dustin, 2004; Fooksman et al., 2010).

Naive CD4 T cells isolated from DC-depleted mice fail at developing a mature IS following recognition of their cognate antigen (Hochweller et al., 2010), indicating that the tonic TCR signaling resulting from self-MHC recognition is also required for licensing T cells for IS maturation. The key question here is whether an impaired IS maturation is the consequence or the reason for defective TCR signaling and T cell proliferation. In other words, do hyporesponsive T cells fail to mount a mature synapse due to defective inside-out signaling resulting in impaired TCR signal transduction and proliferation?, or is the TCR signaling cascade itself defective and, consequently, there is lack of LFA-1 activation and IS formation? These questions remain to be elucidated yet.

MODEL OF LOCATION-DEPENDENT T CELL ANTIGEN SENSITIVITY

As discussed earlier, the antigen sensitivity of naive T cells is continuously fine-tuned depending on whether or not T cells

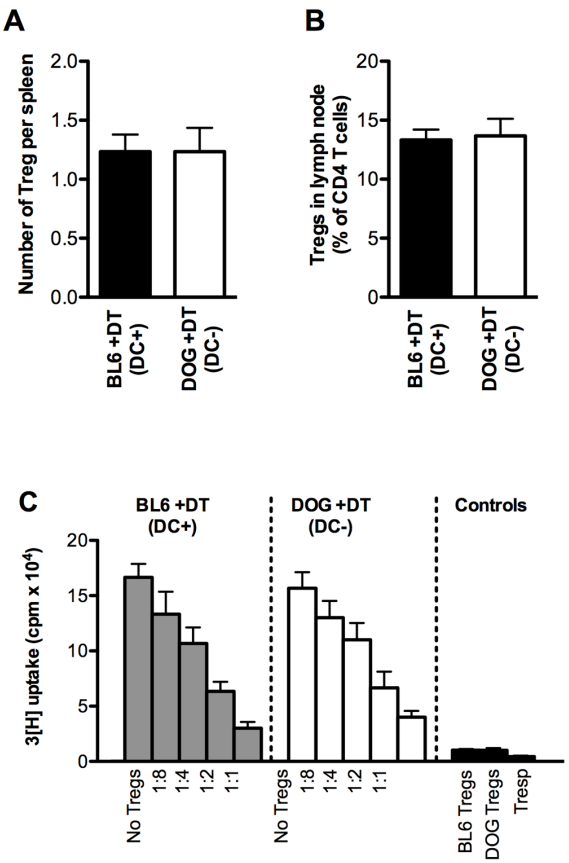

FIGURE 2 | Treg numbers and function from mice lacking DCs. (A) Absolute numbers of Treg cells in the spleen of mice with (DC^+) or without (DC^-) DCs 2 days after DT administration. Tregs were identified as live $CD4^+$ $FoxP3^+$ lymphocytes by flow cytometry. **(B)** Frequency of Tregs in skin-draining lymph nodes of mice with (DC^+) or without (DC^-) DCs two days after DT administration. **(C)** Standard *in vitro* assay for suppressive capacity of Treg cells isolated from the spleen of mice with (DC^+; gray bars) or without (DC^-; white bars) DCs 2 days after DT administration. Live $CD4^+CD25^{high}$ Tregs were pooled from three mice and sorted using flow cytometry. Live $CD4^+CD25^-$ responder T cells were sorted using flow cytometry from the spleen of untreated BL6 mice. Responder T cells were stimulated in the presence of irradiated APCs, anti-CD3ε antibody (clone 145.2C11, 2 μg/ml) and titrated amounts of Treg cells for 4 days. Proliferation of responder cells was assayed by ^{3}[H]-thymidine for the last 9 h of the experiment. Results are expressed as mean ± SEM. For **(A,B)**, $n = 3$ mice; For **(C)**, $n = 3$ wells. Shown is one representative of three experiments.

interact with DCs. Self-MHC recognition on DCs results in a rapid increase in the sensitivity of the TCR for a subsequent antigenic challenge, whereas lack of self-MHC recognition leads to a rapid loss of sensitivity (Stefanova et al., 2002; Hochweller et al., 2010). Both of these processes take place within minutes following initiation or disruption of DC–T cell interaction, thus the loss of T cell responsiveness to cognate antigen caused by reduced interactions is quickly reverted after reintroduction of DC–T cell contacts. Naive T cells continuously recirculate between lymphoid organs and the systemic circulation where they spend only about 30 min (Pabst, 1988). In the blood, where self-MHC recognition on DCs is very unlikely, CD4 T cells show reduced tonic TCR signaling and responsiveness to TCR stimulation (Stefanova et al., 2002). Consequently, it has been shown recently that most of the tonic TCR signaling in the steady state takes place within the secondary lymphoid organs (Mandl et al., 2012). It is therefore crucial that naive T cells recover quickly their TCR responsiveness upon re-entering lymphoid organs and interacting with DCs to ensure optimal responses against foreign antigens. Indeed, the state of T cell hyporesponsiveness is completely reverted 30 min after reintroducing DC–T cell interaction (Hochweller et al., 2010).

Thus, T cells appear to go through several rounds of normal and hyporesponsive states toward cognate antigen depending on their location at a given time: they are fully responsive in the lymphoid organs, where they can be primed against invading antigens, whereas they remain hyporesponsive in the blood where priming is not supported mainly due to anatomical restrictions. Presently, it is difficult to understand the physiological relevance of intermittently loosing TCR antigen sensitivity each time that T cells enter the systemic circulation. It may serve as a transient "metabolic rest" facilitating T cells to increase their tonic TCR signaling and antigen sensitivity upon re-entering lymphoid organs, where they have to be fully aware of minute amounts of foreign antigen displayed by DCs at the initial stages of an infection.

In addition, self-MHC recognition during the steady state also affects other responses mediated by T cells. Recently, Hünig's group has shown that the proliferative response of human T cells to the superagonist CD28 TGN1412 antibody is also dependent on tonic TCR signaling maintained by MHC scanning (Romer et al., 2011; Hunig, 2012). Similarly, it has been shown that naive CD8 T cells require self-MHC recognition in order to become proliferative in response to IL-2 and IL-15 (Cho et al., 2010). Therefore, self-MHC recognition induces tonic TCR signaling that is required not only for increasing TCR sensitivity to cognate antigen, but also for optimizing responses against other TCR-independent stimuli.

INHIBITING DENDRITIC CELL APOPTOSIS LEADS TO AN INCREASE IN DENDRITIC CELL FREQUENCY AND T CELL HYPERACTIVATION

Self-MHC recognition on DCs results in enhanced T cell antigen sensitivity and optimal proliferation in response to cognate antigen. As discussed here, a decrease in DC numbers results in hyporesponsive T cells that fail to proliferate to a normal level. Just a twofold decrease in the numbers of DCs already results in partially reduced T cell proliferation (Hochweller et al., 2010). Interestingly, the opposite also seems to apply: an increase of about threefold in the frequency of DCs results in T cell hyperactivation and autoimmunity (Chen et al., 2006). Enforced expression of the baculoviral antiapoptotic p35 protein by DCs, resulted in DC accumulation and chronic T cell hyperactivation leading to multiorgan infiltration and production of autoantibodies (Chen et al., 2006). MHC-II and CD40 expression, hallmarks of DC activation, were unaltered in that study, suggesting that T cell hyperactivation was a result of increased DC frequency rather than activation due to increased half-life. Thus, DC homeostasis in the absence of foreign cognate T cell antigen is critical to ensure optimal T cell responses to subsequent challenges with cognate antigen:

whereas too few DCs result in reduced antigen sensitivity of T cells, a sustained increase in the number of DCs apparently leads to T cell hyperactivation and autoimmunity. These findings are summarized in **Figure 3** and highlight the importance in maintaining the correct size of the DC pool to promote healthy T cell responses.

CONCLUSIONS AND OPEN QUESTIONS

There is mounting evidence that self-MHC recognition in the periphery is critical for several processes including: (i) maintenance of tonic TCR signaling and T cell antigen sensitivity, which are critical for optimal responses to subsequent challenge with cognate antigen; (ii) synergism at the time of cognate antigen recognition leading to increased T cell responses; (iii) increased TCR-independent T cell proliferative responses to various stimuli such as superagonist CD28 TGN1412, IL-2, and IL-15. The former two are mediated by self-MHC recognition on DCs, whereas the role of DCs in providing self-MHC for the TCR-independent responses is not clear yet.

Despite these advances several open questions are remaining. Amongst these, the following are central to understand the molecular mechanisms of DC-induced tonic TCR signaling:

(1) Characterization of the signaling events induced by self-MHC recognition on DCs resulting in increased T cell antigen sensitivity. It is clear that self-MHC recognition induces tonic TCR signaling by partial CD3ξ phosphorylation. The finding that the maturation of the IS is compromised in DC-less T cells, opens the possibility that beyond tonic TCR signaling, integrin (such as LFA-1) activation is impaired following stimulation with cognate antigen, leading to deficient IS maturation and thus reduced T cell proliferative responses.

(2) Are there other molecular events in DC–T cell interactions that contribute to maintenance of the T cell antigen sensitivity? Hypothetically, costimulatory molecules such as CD80 and CD86 may participate in the tonic T cell signaling by partially activating CD28 in the absence of cognate antigen. Costimulation plays a key role in enhancing the proliferative response to TCR stimulation. Whether this also applies to basal TCR signaling promoted by self-MHC recognition is unclear.

(3) What is the nature of the self-MHC ligands required to induce tonic TCR signaling? We have previously proposed that these are the same ligands that induce positive selection in the thymus, but it needs to be demonstrated.

(4) Do memory T cells require tonic TCR signaling for enhanced responses to antigenic rechallenge? Different subtypes of memory T cells reside in lymphoid and extralymphoid compartments. DCs have been shown to interact with memory T cells and to be required for maximal T cell

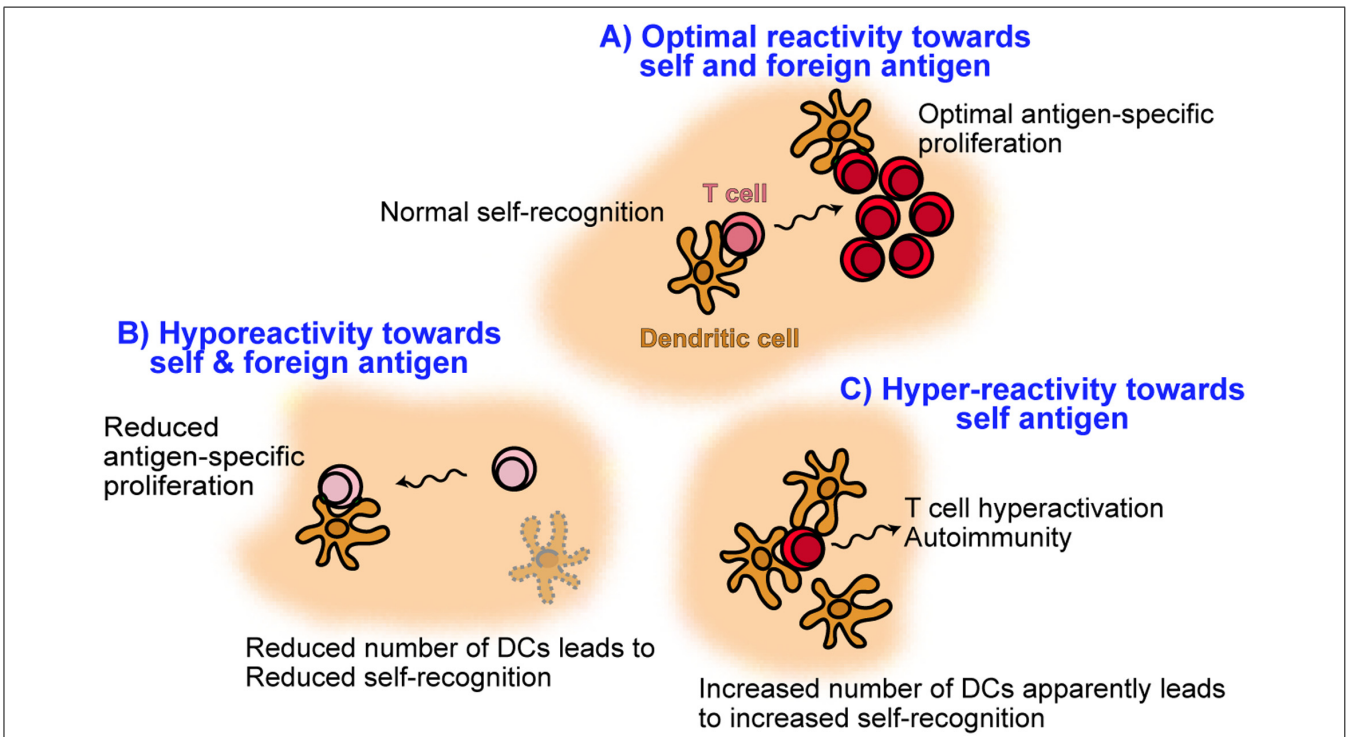

FIGURE 3 | Model illustrating the role of dendritic cell frequency on promoting healthy T cell responses. Under normal DC homeostasis **(A)**, naive T cells recognize self-MHC on DCs resulting in tonic TCR cell signaling and increased antigen sensitivity. As a result, subsequent foreign antigen challenge leads to optimal T cell activation and proliferation. However, upon conditions of reduced self-MHC recognition on DCs such as DC depletion **(B)**, T cells undergo reduced tonic TCR signaling and decreased antigen sensitivity. These T cells become hyporesponsive and are not able to undergo strong proliferation in response to antigenic challenge. On the other hand, an increase in the number of DCs **(C)** apparently results in T cell hyperactivation, possibly due to increased self-MHC recognition, and autoimmunity.

restimulation following antigenic rechallenge both in lymphoid organs and in extralymphoid organs (Zammit et al., 2005; Wakim et al., 2008). However, it remains open whether or not memory T cells also require tonic T cell signaling for increased antigen sensitivity.

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The regulation of immune responses by DC derived Type I IFN

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Our immune system bears the tremendous task of mounting effective anti-microbial responses whilst maintaining immunoregulatory functions to avoid autoimmunity. In order to quickly respond to pathogens, Dendritic cells (DC) are armed with pattern recognition receptors (PRRs), allowing them to recognize highly conserved pathogen-associated molecular patterns (PAMPs) that are uniquely expressed by invading microbes. PRR activation can trigger DCs to release the pleiotropic cytokine, Type I interferons (IFN), which facilitates various biological functions in different immune cell types. In this review, we will discuss the classical PRR-induced Type I IFN response in DCs as well as describe a novel mechanism for Type I IFN induction by the tumor-necrosis factor receptor superfamily (TNFRSF) members, TNFR-1 and lymphotoxin-β receptor (LTβR). While PRR activation during viral infection, produces large amounts of Type I IFN in a relative short period of time, TNFRSF-induced Type I IFN expression is modest with gradual kinetics. Type I IFN can exert pro-inflammatory effects, but in some cases it also facilitates immune-regulatory functions. Therefore, DCs are important regulators of immune responses by carefully modulating Type I IFN expression.

Keywords: lymphotoxin, tumor necrosis factor, toll-like receptors, dendritic cells

INTRODUCTION

Dendritic cells (DCs) play a critical role in bridging the innate immune response with the adaptive immune system. DCs reside throughout the body, particularly enriched in secondary lymphoid organs (SLOs), intestinal lamina propria, and the epidermal layer of the skin, and they constantly sample antigen to maintain balance between immunity and tolerance. Infection triggers pattern recognition receptors (PRR) activation, promoting DC maturation, and the production of inflammatory cytokines including TNF-α, interleukin (IL)-1, IL-6, IL-12, and Type I interferons (IFN). Upon activation fully mature DCs migrate to SLOs where they interact with naive T cells, resulting in the activation of the adaptive immune response. DCs can be broadly categorized into conventional DCs (cDCs) and plasmacytoid DCs (pDCs). cDCs are comprised of different subsets governed by differential expression of CD4 and CD8α (Villadangos and Schnorrer, 2007; Hashimoto et al., 2011). cDCs are highly efficient at presenting external antigens through the major histocompatibility complex (MHC) class I molecule to CD8+ T cells for the generation of cytotoxic T lymphocytes (CTLs), a process known as cross-presentation that plays an important role in pathological contexts including autoimmune disease, allograft rejection, and tumor immunity (Carbone and Bevan, 1990; Den Haan et al., 2000; Pooley et al., 2001). pDCs are also capable of capturing, processing, and presenting antigens to T cells (Sapoznikov et al., 2007; Young et al., 2008), but in comparison to cDCs, they possess more restricted antigen uptake and lower levels of co-stimulatory molecules and MHC (Colonna et al., 2004; Dalgaard et al., 2005; Young et al., 2008). Instead, pDCs patrol between blood vessels and the lymphatic system, and when they encounter viruses, they are capable of secreting very large amounts of Type I IFN in a short period of time as a strategy for viral control (Siegal, 1999; Colonna et al., 2004). Therefore, together cDCs and pDCs provide both distinct and overlapping contributions towards host defense against pathogens.

IFNs were first discovered to be an important anti-viral factor that interferes with viral replication in mammalian cells. There are three classes of IFN (Type I, II, and III) categorized based on their structural homology and the specific receptor with which they associate (Platanias, 2005). The Type I IFN family is very diverse and includes a single IFN-β member, numerous IFN-α variants (13 in human and 14 in mouse), and the lesser known IFN-ε, -κ, -ω, and -δ (Sen, 2001). Despite this diversity, all Type I IFNs bind exclusively to the interferon-α receptor (IFNAR). Upon receptor activation, IFNAR1 and IFNAR2 dimerize and phosphorylate the Janus Kinase family members TYK2 and JAK1. Activated TYK2 and JAK1 phosphorylate STAT1 and STAT2, and together they bind IRF9 to form a trimeric transcription factor, ISGF3. ISGF3 translocates into the nucleus and interacts with the ISRE elements to activate IFN-related gene transcription (Platanias, 2005). Type I IFNs are secreted by various cell types including fibroblasts, epithelial cells, innate immune cells, and lymphocytes, and they represent a key initiating factor against viral infections. In this review, we will describe the conventional mechanism of Type I IFN production by cDCs, and for brevity will restrict ourselves to virus infection scenarios. In addition, we will discuss a novel mechanism of Type I IFN induction that is triggered by tumor-necrosis factor receptor superfamily (TNFRSF) members such as TNFR-1 and lymphotoxin-β receptor (LTβR), independent of PRR

activation. Finally, we will contextualize the versatile role of Type I IFN in tuning T cell responses in different contexts such as responses to replicating pathogens as opposed to cell-associated protein antigens.

PRR-INDUCED TYPE I IFN IN RESPONSE TO VIRAL INFECTION

Our immune system is in a constant evolutionary battle with pathogens that has played out through millennia. In order to combat viral infections, the immune system relies on detection tools, in particular PRR, that elicit potent Type I IFN expression.

TOLL-LIKE RECEPTORS

Thus far, 13 mammalian TLRs have been identified (10 in human and 12 in mice) and each TLR recognizes a specific class of microbial pathogen-associated molecular patterns (PAMPs) that triggers distinct responses (Casanova et al., 2011). TLRs belong to the IL-1 receptor family, and all of them contain a common cytoplasmic domain referred to as the toll-interleukin-1 receptor (TIR) domain. Upon activation, the TIR domain recruits TIR-associated adapter molecules including MyD88, TRIF, TRAM, and TIRAP that mediate various downstream signaling pathways. TLRs are mainly expressed in immune cells such as neutrophils, macrophages, DC, and some lymphocytes, while non-immune cells such as fibroblasts and intestinal epithelial cells express a more restricted sets of TLRs (Reynolds et al., 2010; Akira, 2011). The majority of TLRs are found on the cell membrane with the exception of TLR3, 7–9, and 11–13, which are expressed within intracellular endosomes. These TLRs are associated with the detection of viral, bacterial, and parasitic nucleic acids (Meylan and Tschopp, 2006; Stetson and Medzhitov, 2006), and their sub-cellular compartmentalization allows the immune system to distinguish self from non-self antigens (Barton et al., 2006). Under certain circumstances however, host nucleic acids can be mistaken as non-self resulting in autoimmune diseases. For example, in the case of systemic lupus erythematosis, complexes of autoreactive antibodies and host nucleic acids can trigger the activation of endosomal TLRs in pDCs, resulting in uncontrolled production of Type I IFN and disease pathology (Elkon and Stone, 2011).

Conventional DCs and pDCs express different sets of TLRs that facilitate their specialized function. Human pDCs lack TLR3 but express high levels of TLR7, TLR8, and TLR9, and they are responsible for the production of large amounts of Type I IFN in response to viral infections (Hornung et al., 2002). The signaling pathway downstream of TLR7, 8, and 9 is mediated through the key adaptor molecule MyD88 (Takeda and Akira, 2005). Upon activation, MyD88 recruits IRAK4 and IRAK1 to the receptors, and through interaction of their death domains, IRAK1 becomes phosphorylated. Activated IRAK1 further recruits and activates the constitutively expressed IRF7 in pDC, resulting in the production of Type I IFN (Coccia et al., 2004; Asselin-Paturel and Trinchieri, 2005; Honda et al., 2005).

In contrast to pDCs, the role of TLR7 and TLR8 in cDCs predominantly initiates a DC maturation program that leads to the production of IL-12 rather than Type I IFN (Ito, 2002; Larangé et al., 2009). Type I IFN production by cDC is primarily triggered through the activation of TLR3 that recognizes viral-derived double stranded RNA and TLR4 that recognizes the gram negative bacterial cell wall component lipopolysaccharide. TLR3 is the only TLR that exclusively recruits Trif to mediate signaling, while TLR4 signals through both MyD88 and Trif (Weighardt et al., 2004). Trif is the key adaptor molecule that mediates Type I IFN expression downstream of both receptors, and upon activation, Trif binds the TNF receptor-associated factor 3 (TRAF3) and TRAF6, which recruits RIP1 that induces NFκB activation. Conversely, TRAF3 becomes polyubiquitinated to the lysine at position 63 of the ubiquitin molecule (K63-linkage). K63-linked TRAF3 is essential for the recruitment of TBK1, IKKε, and IRF3, ultimately leading to IRF3 phosphorylation (Oganesyan et al., 2005; Tseng et al., 2010; Häcker et al., 2011). Phoshporylated IRF3 subsequently homodimerizes and translocates into the nucleus. Together IRF3 and NFκB form the transcription factors required for the expression of IFN genes (**Figure 1**). Taken together, pDCs and cDCs have unique roles in response to infection. The expression of different PRRs and differences in the signaling that mediate Type I IFN production in pDCs versus cDCs conspire to contain infection by triggering a systemic antiviral state and efficiently priming T cell activation.

RIG-I OR RIG-I LIKE RECEPTORS

In contrast to TLRs which are primarily expressed on innate immune cells, RIG-I are ubiquitously expressed in the cytoplasm of all nucleated cells. Instead of actively sensing viral particles, RIG-I are triggered when cells become infected. RIG-I and the RIG-I like receptors, such as the melanoma differentiation antigen 5 (MDA5) belong to a family of DExD/H box RNA helicases. The N-terminal region of RIG-I is characterized by two caspase recruitment domains (CARD), and the C-terminal region contains RNA helicase activity (Yoneyama and Fujita, 2009). RIG-I recognizes double stranded RNA by the RNA helicase domain, and through a CARD–CARD interaction, RIG-I recruits the CARD-containing adaptor MAVS (also known as VISA, IPS-1, or CARDIF) to mediate downstream events (Yoneyama and Fujita, 2009). Upon activation, MAVS localizes on sub-cellular compartments including the mitochondrial membrane and peroxisomes. Signaling through the peroxisomal-localized MAVS leads to rapid induction of antiviral genes but independent of Type I IFN production (Dixit et al., 2010). In contrast, mitochondrial MAVS produces a slower kinetic that results in Type I IFN expression. Using the mitochondria membrane as a scaffold and powered by the mitochondrial membrane potential (Koshiba et al., 2011), MAVS interacts with TRAF3, TBK1, and IKKε to form a "signalosome" that phosphorylates IRF3 and IRF7 for Type I IFN production (Kawai et al., 2005; Seth et al., 2005). *In vivo* models have shown that RIG-I-deficient mice, despite having intact TLR signaling, succumb to infection by vesicular stomatitis virus, Newcastle disease virus, and Sendai virus (Kato et al., 2005). TLRs and RIG-I signaling complement each other to provide complete coverage across various types of viruses, and both detection systems are geared toward rapid production of Type I IFN that leads to a systemic antiviral state and the control of viral infection.

INDUCTION OF TYPE I IFN BY THE TNFR FAMILY

The TNFRSF is a very diverse family of receptors comprising of 29 different family members that provide critical signals regulating

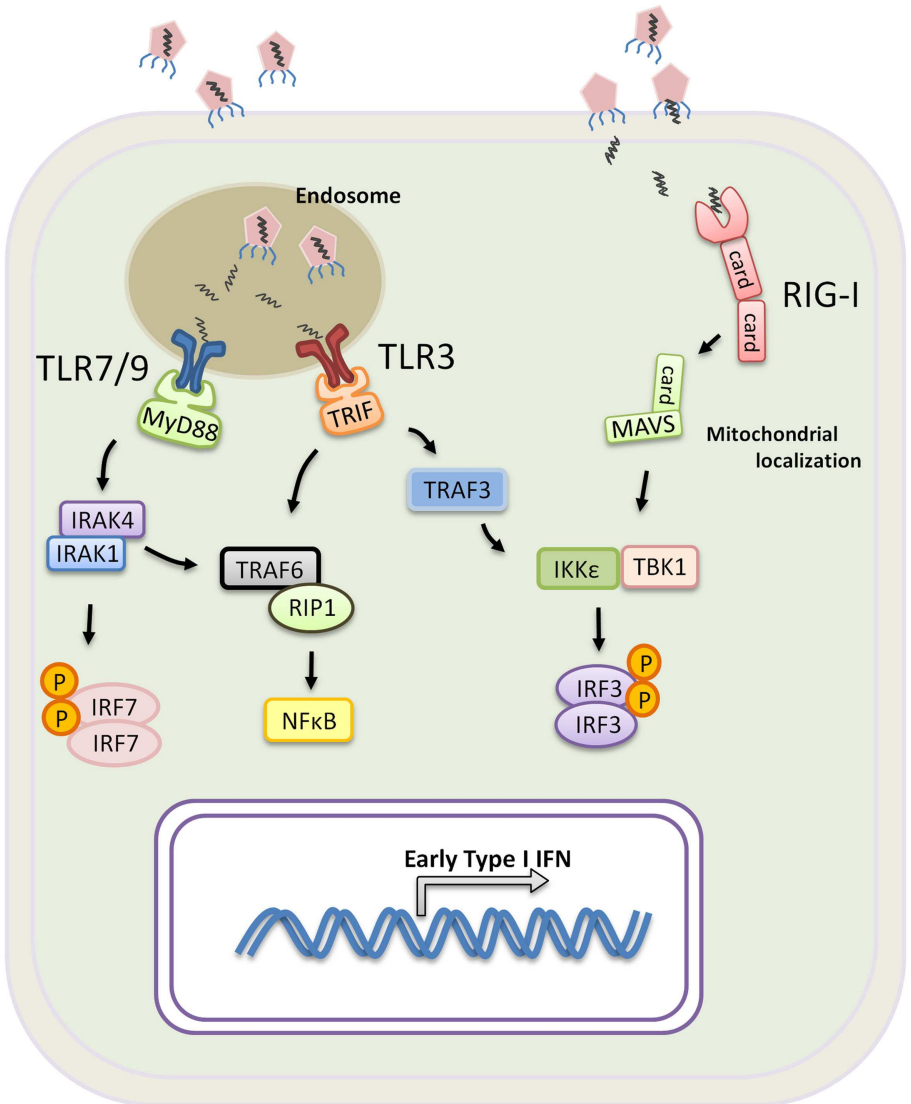

FIGURE 1 | Induction of Type I IFN by PRR during a viral infection. TLR3, 7, and 9 are mainly expressed within the endosomes of innate immune cells. Virus or virus-infected cells are taken up by macrophages or DCs, and the viral nucleic molecules are exposed upon endosomal acidification. Activation of TLR7 and 9 requires signaling through MyD88 and recruitment of IRAK4, IRAK1, and IRF7. IRF7 becomes phosphorylated and translocates into the nucleus upon dimerization resulting in transcription of Type I IFN genes. TLR3 signals exclusively through Trif which binds TRAF6 and recruits RIP1 for NF-κB activation. Trif also binds TRAF3 leading to TRAF3 K63-linked ubiquintination, facilitating the recruitment of TBK1, IKKε and IRF3 for IRF3 phosphorylation. Phosphorylated IRF3 homo-dimerizes and translocates into the nucleus for transcription of Type I IFN genes. RIG-I or RIG-I like receptors are expressed in all nucleated cells, and they recognizes viral RNA found in the cytoplasm. Upon activation, RIG-I recruits MAVS through the CARD domain interaction, and, analogous to TRIF, MAVS further binds IKKε, TBK1 and IRF3 to promote IRF3-activation and Type I IFN expression.

numerous physiological functions including inflammation, lymphoid organ development, and adaptive immune responses (Force et al., 1995; Sarin et al., 1995; Lin and Stavnezer, 1996). More recently, it has been shown that TNFR-1 and LTβR activation can trigger Type I IFN expression in macrophages and in cDCs respectively (Yarilina et al., 2008; Summers-deLuca et al., 2011). The production of Type I IFN by TNFR family members in the absence of PRR signaling signifies a novel mechanism of Type I IFN induction that may be critical during immune responses to non-replicating antigen such as tumor antigens and self-antigens.

TNFR-1 AND TYPE I IFN

TNF-α was first discovered to be an endotoxin-induced serum factor that exerts cytotoxic effects against sarcoma cells in mice (Carswell et al., 1975), and it has since been recognized as a pleiotropic cytokine that regulates numerous biological functions. Macrophages are major producers of TNF-α in both acute immune responses and chronic inflammatory diseases. TNF-α induces inflammation by affecting many different cell types throughout the body due to the ubiquitous expression of two distinct receptors, TNFR-1 and TNFR-2. Most biological functions are

mediated through TNFR-1, while TNFR-2 has been shown to potentiate TNFR-1 induced cell death (Li et al., 2002). TNF-α stimulation in primary human and mouse macrophages induces the phosphorylation of IRF1 and IRF3 leading to the production of Type I IFN and the secretion of chemokines, such as CXCL10 and CXCL11 (Yarilina et al., 2008). Activated T cells express the chemokine receptor CXCR3 that binds to CXCL9, CXCL10, and CXCL11, resulting in their recruitment into sites of inflammation, and numerous studies have recognized the critical role of CXCR3 activating chemokines in various immune responses such as autoimmune mediated skin inflammation and allograft rejection that lack overt PRR activation (Flier et al., 2001; Meyer et al., 2001; Zhao et al., 2002). What is important to note is that the modest and gradual kinetics of the TNFR-1 induced Type I IFN response described above contrast with the more rapid and robust Type I IFN induced by PRR mediated responses such as what occurs during infection – see **Figure 2** (Sakaguchi et al., 2003; Honda et al., 2005; Seth et al., 2005).

LTβR AND TYPE I IFN

LTβR is expressed on stromal cells, DCs, macrophages, and high endothelial venules, while the ligand LTαβ is found on lymphoid tissue inducer cells, B cells and activated T cells. LTβR signaling in stromal cells is required for the development of SLOs (Murphy et al., 1998; Mebius, 2003), and it also plays an important role in the homeostatic maintenance of specific subsets of cDCs in the spleen (Kabashima et al., 2005; Wang et al., 2005; De Trez et al., 2008). More recently, our lab as well as others have examined the role of LTβR in DC: T cell cross-talk (Summers-DeLuca et al., 2007; León et al., 2012). During an immune response, activated CD4+ helper T cells up-regulate CD40L and LTαβ which binds to the corresponding receptors CD40 and LTβR respectively on cDCs. Both CD40 and LTβR signaling provide "help" for cDCs so that they may optimally cross-present antigen for CD8+ T cell activation. Using an immunization model of cell-associated protein antigen, we found that LTβR signaling in cDCs is required for optimal CD8+ T cell clonal expansion while CD40 signaling was required

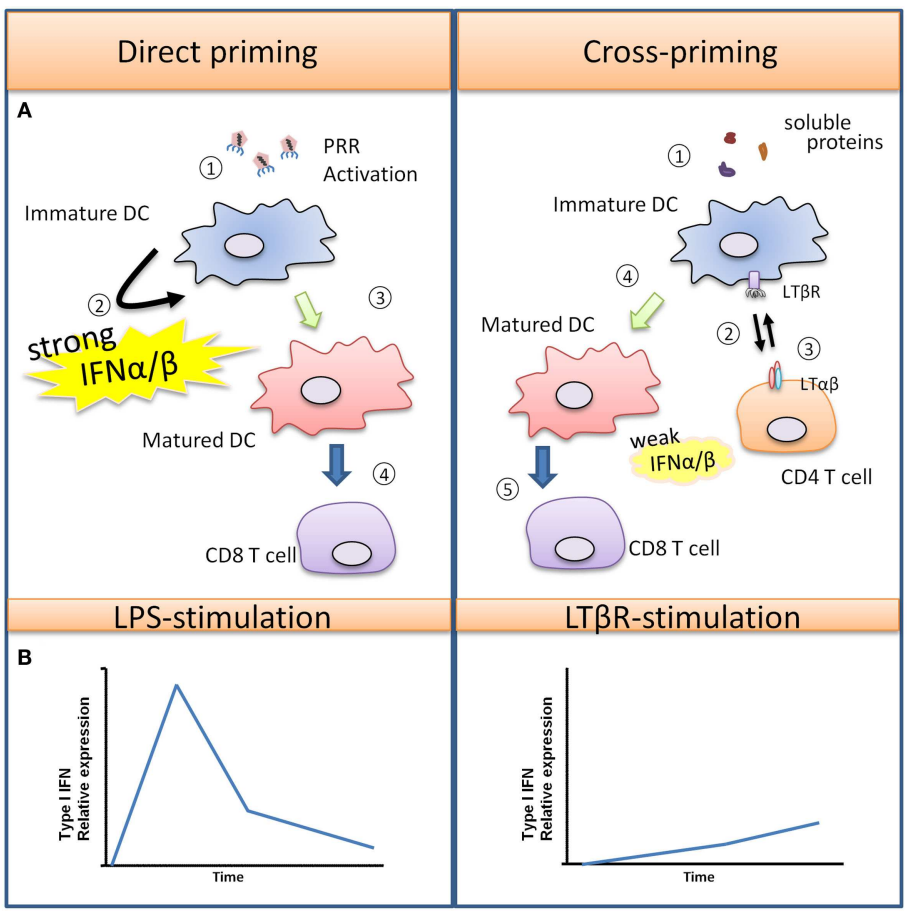

FIGURE 2 | Type I IFN promotes T cell priming during viral infection versus soluble antigens. (A) Viruses can trigger PRR activation on immature DCs, leading to DC maturation and production of various pro-inflammatory cytokines and a large quantity of Type I IFN. In this scenario, DCs are strongly activated, and they are capable of directly interacting with CD8+ T cells for the generation of virus-specific CTLs. In the case of soluble antigens, DCs are poorly activated due to the absence of PRR-stimulus. Semi-mature DCs must first interact with helper CD4+ helper T cells which rapidly up-regulate "help signals" CD40L and LTαβ upon activation. DC-intrinsic LTβR and CD40 activation promotes DC maturation, with LTβR signaling producing a modest amount of Type I IFN for a sustained period that facilitates CD8+ T cell expansion. **(B)** The differences in kinetics and magnitude of Type I IFN induced by PRR or TNFSFR are illustrated graphically here.

for CD8+ T cell effector functions. Since Benedict and colleagues demonstrated that LTβR signaling could induce Type I IFN in stromal cells, independent of PRR activation (Schneider et al., 2008), we also explored whether direct LTβR stimulation leads to Type I IFN induction in DCs. We found that, not unlike the scenario for TNFR-1, LTβR stimulation induced a modest level of Type I IFN that gradually increased over time. Furthermore, LTβR signaling was required for maximal Type I IFN production in the context of LPS (TLR4) co-stimulation (Summers-deLuca et al., 2011).

Interestingly, the modest level of Type I IFN induced by LTβR signaling in cDCs was important for optimizing CD8+ T cell clonal expansion *in vitro*, since low amounts of exogenous IFN-α can rescue CD8+ T cell expansion in the absence of LTβR signaling. Hence, Type I IFN produced by LTβR or TNFR-1 may facilitate optimal T cell recruitment and/or clonal expansion (**Figure 2**). Given that some autoimmune diseases are characterized by a "Type I IFN signature" (Lande et al., 2007; Sozzani et al., 2010; Elkon and Stone, 2011), it will be of interest to determine the relevancy of these PRR-independent signals for the induction of Type I IFN in the context of autoimmunity.

THE MANY ROLES OF TYPE I IFN

Thus far, we have described two very different mechanisms for Type I IFN induction in DCs and monocytes. PRRs detect viral nucleic acids, to produce a robust Type I IFN response for viral clearance, and this is particularly the case for pDCs. Conversely, TNFRSF (TNFR-I, LTβR) induce a modest but prolonged expression of Type I IFN that acts in a co-stimulatory manner to potentiate T cell-mediated immunity (**Figure 2B**). Therefore, the difference in level, period, and duration of Type I IFN produced by DCs likely dictate the pleiotropic effects of this cytokine resulting in pro-inflammatory or pro-regulatory immune functions.

PRO-INFLAMMATORY FUNCTION OF TYPE I IFN

Type I IFN is generally regarded as a pro-inflammatory cytokine in numerous immune settings such as autoimmune diseases like psoriasis and systemic lupus erythematosus (Nestle et al., 2005; Lande et al., 2007; Elkon and Stone, 2011), allograft rejection in transplantation (Tovey et al., 1996), and immunity against tumors (Diamond et al., 2011; Fuertes et al., 2011). Type I IFN has robust pro-inflammatory effects that can act in both an autocrine and paracrine manner on immune cells to modulate their functions. During an immune response with minimal PRR activation, DCs cannot reach maximal immunogenic status, and Type I IFN overcomes this hurdle by promoting DC maturation (Le Bon and Tough, 2008; Longhi et al., 2009; Axtell et al., 2013). IFNAR activation in DC triggers NFκB and p38 mitogen-activated protein kinase (MAPK) activation, resulting in the up-regulation of MHC class I and class II as well as co-stimulatory molecules B7-H1 and B7-H2 (Pollara et al., 2006). In addition, blood circulating monocytes, when differentiated into DCs in the presence of Type I IFN, upregulate the chemokine receptor CCR7 thus allowing them to migrate more efficiently into SLOs (Parlato, 2001). In human CD4+ T cells, Type I IFN can complement IL-12 to drive TH-1 differentiation, where IFNAR-mediated STAT2 phosphorylation recruits and activates STAT4, a transcription factor that potentiates IL-12R signaling (Farrar et al., 2000; Gautier et al., 2005). Moreover, Type I IFN signaling through STAT4 has also been implicated in the induction of IFN-γ in natural killer cells and T cells, particularly in the absence of STAT1 (Nguyen et al., 2000). Various studies have reported that Type I IFN acts as a potent third signal that promotes antigen cross-priming (Curtsinger et al., 2005; Le Bon et al., 2006; Le Bon and Tough, 2008). IFNAR activation in CD8+ T cell can induce chromatin remodeling through histone acetelyation, promoting transcription of many genes required for clonal expansion and production of effector molecules (Agarwal et al., 2009). Lastly, Type I IFN prolongs the CD8+ T cell expansion phase in response to cross-presented antigen, and it enhances the responsiveness of antigen specific CD8+ T cells to IL-2 and IL-15 for increased survival (Le Bon et al., 2006). Hence, Type I IFN can independently act on DCs, CD4+ T cells, and CD8+ T cells through very different mechanisms that facilitate inflammatory immune responses.

ANTI-INFLAMMATORY ROLE OF TYPE I IFN

Many experimental and clinical settings use Type I IFN as a treatment to quiet inflammatory conditions, suggesting that Type I IFN can exert immunoregulatory functions. In particular, IFN-β has been shown to be an effective therapeutic treatment for collagen-induced arthritis (Van Holten et al., 2004; Adriaansen et al., 2006), relapsing-remitting multiple sclerosis (MS) (Weinstock-Guttman et al., 2008) and autoimmune familial Mediterranean fever (Tweezer-Zaks et al., 2008; Guarda et al., 2011). The anti-inflammatory functions of Type I IFN, particularly in MS, have been characterized by numerous studies, and yet the exact mechanism remains unclear. Blood-derived DCs become activated upon IFNAR activation, however astrocytes and microglia in the central nervous system (CNS) down-regulate MHC-II in response to Type I IFN (Satoh et al., 1995; Hall et al., 1997). T cell recruitment into the CNS may require Type I IFN to induce relevant CXCR3 attracting chemokines, however other studies have also showed that prolonged IFN-β treatment in MS patients down-regulates the expression of cell adhesion molecules such as VCAM-1 and ICAM-1 in brain endothelial cells, resulting in reduced immune cell infiltration into the CNS (Corsini et al., 1997; Defazio et al., 2000). In addition, IFN-β can prevent leukocyte egress from lymph nodes by down-regulating the sphingosine 1-phosphate receptor-1 (S1P1) (Shiow et al., 2006; Gao et al., 2009), and S1P receptor agonists are used to treat MS (Kataoka et al., 2005; Chun and Hartung, 2010; Choi et al., 2011; Galicia-Rosas et al., 2012). IFN-β or IFNAR1 deficient mice have been shown to produce an enhanced number of antigen specific CD8+ T cells when immunized with a DNA-based vaccine, suggesting that Type I IFN is also required to control T cell proliferation (Dikopoulos et al., 2005). Recent studies showed that IFN-induced STAT1 activation negatively regulates the expression and function of the oncogene c-myc in CD8+ T cells which is important for homeostatic proliferation (Gil et al., 2006, 2012). Furthermore, other studies have also shown that IFNAR or IFN-β deficient mice exhibit lower numbers of IL-10 producing T cells, which may also explain the increased CD8 T cell expansion in the absence of IFNAR signaling (Dikopoulos et al., 2005; Bochtler et al., 2008). It is important to point out, however, that scenarios where IFNAR signaling is completely absent may

lead to different effects on shaping T cell responses than situations where the levels/kinetics of Type I IFN production have been altered.

CONCLUSIONS

Type I IFN is a pleiotropic cytokine that affects many different cell types with a wide range of effects. The modulation of the innate and adaptive immune responses relies on a finely tuned rheostat of Type I IFN production. The amount of IFN produced through a particular time frame during the course of an immune reaction will likely dictate very different outcomes. Therefore, understanding the mechanisms underlying Type I IFN induction by DCs is crucial for providing future groundwork in developing therapeutics that quiet autoimmunity or promote pathogen and tumour clearance.

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Interleukin-2 production by dendritic cells and its immuno-regulatory functions

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Dendritic cells (DCs) are uniquely potent antigen presenting cells that acquire microbial products and prime adaptive immune responses against pathogens. Furthermore, DCs also play a key role in induction and maintenance of tolerance. Although numerous studies have assessed the diverse functions of DCs, many unanswered questions remain regarding the molecular mechanisms that DCs use to achieve immunoregulation. While not widely regarded as a significant provider of T-cell growth factors, DCs have previously been identified as a potential source of IL-2 cytokine. Recent research indicates that microbes are the most effective stimuli to trigger IL-2 production in DCs by activating the calcineurin/NFAT signaling pathway. Herein we describe recent insights into the production and function of IL-2 cytokine and IL-2 receptor in DCs early after stimulation through pattern recognition receptors. These findings clarify how DCs fine-tune effector and regulatory responses by modulating IL-2 production in both tolerance and immunity.

Keywords: IL-2, dendritic cell, LPS, dectin-1, calcineurin, NFAT

INTRODUCTION

The emergence of multicellular organisms during evolution has been accompanied by the concomitant development of the innate immune system, which serves to protect the host from pathogen colonization. Co-evolution of multicellular systems and their associated microbiomes over millions of years has produced a sophisticated host receptor repertoire and signaling networks that respond to specific perturbations in homeostasis. This ability to sense the environment is best demonstrated by dendritic cells (DCs), which have developed the capacity to respond to ligation of specific pathogen recognition receptors (PRRs) in order to trigger adaptive immune responses. Among the many different responses induced by PRR ligation in DCs, we have found that specific agonists can stimulate translocation of the transcription factor "nuclear factor of activated T-cells" (NFAT) to the DC nucleus both *in vitro* and *in vivo*, resulting in the activation and regulation of gene expression (Granucci et al., 2001; Zanoni et al., 2009). Interestingly, one of the gene products regulated by NFAT in DCs is the interleukin (IL)-2 (Granucci et al., 2001), which is particularly crucial to the functioning of the immune system in both immune homeostasis and immunity (Malek, 2008). In the late 1970s, Kendall A. Smith and Steven Gillis described IL-2 cytokine as a T-cell growth factor produced by T lymphocytes (Smith, 1988). In the subsequent 40 years, the wider role played by IL-2 in regulating the adaptive immune response has been fully recognized. Although previous studies of this cytokine have focused primarily on T-cells, new findings have highlighted that IL-2 is also produced by other cell types and in various different settings. For these reasons, IL-2 can be defined as a pleiotropic cytokine with different functions extending from driving T-cell proliferation, supporting the activation of natural killer (NK) cells to the induction of pro-apoptotic gene transcription (Malek, 2008). Indeed, the many diverse functions of IL-2 are well demonstrated by the spontaneous development of autoimmune and inflammatory bowel diseases in mice that are deficient for IL-2 or the IL-2 receptor (IL-2R; O'Shea et al., 2002). Notably, adoptive transfer of IL-2-sufficient T-cells or administration of IL-2 cytokine can significantly ameliorate disease in these animals (Malek and Castro, 2010).

In the myeloid immune compartment, IL-2 gene transcription is inducible in DCs but not in macrophages or in other phagocytic cells (Granucci et al., 2001). The initial finding that IL-2 production by DCs is a major outcome of PRR ligation raised the question of whether it could contribute to adaptive immune responses. In this review we will summarize the current knowledge about the functional role of IL-2 as an innate cytokine.

INDUCTION OF IL-2 TRANSCRIPTION IN DCs

Kinetic transcriptional profiling has shown that developmentally synchronized DCs grown in medium containing GM-CSF produce IL-2 within 4–6 h of stimulation with alive Gram-negative *Escherichia coli* bacteria. Interestingly, the kinetic of the IL-2 mRNA expression and of IL-2 protein production were similar. In contrast, macrophages are not able to produce IL-2 under these conditions (Granucci et al., 2001). Thus, the narrow window of transcription and secretion of IL-2 is unique to DCs.

Because of the crucial role played by DCs in initiating and regulating immune responses, it is key to clarify the kinetics of IL-2 production as well as the stimuli which elicit this cytokine and the signaling machinery which support IL-2-secretion by DCs. Indeed, DCs are well established, as critical mediators of antimicrobial immunity, and it would appear that microbial products are the

most potent stimuli for IL-2 production by myeloid DCs. In this regard, Granucci et al. (2003) showed that LPS, peptidoglycan, and CpG ligands for Toll-like receptors (TLRs) were able to trigger variable levels of IL-2 release. In contrast, pro-inflammatory cytokines including TNF-α, IFN-α, and IL-1β failed to stimulate IL-2 production by DCs. Interestingly, myeloid DCs were also shown to produce very high amounts of IL-2 in response to zymosan or following challenge with live yeast *Saccharomyces cerevisiae* (Granucci et al., 2003). Immature DCs exhibit marked phagocytic activity, but phagocytosis of inert particles *per se* does not constitute a trigger for IL-2 by DCs (Granucci et al., 2003). In contrast, blockade of DC phagocytic activity by treatment with Latrunculin B inhibits antigen uptake and IL-2 production by these cells (Rogers et al., 2005) but does not impair the production of IL-12p40 and IL-10 in response to zymosan. This study also investigated the intracellular signaling pathway that leads to IL-2 production in DCs, showing that murine bone marrow-derived and splenic DCs grown in GM-CSF-enriched medium were able to produce large amounts of IL-2 cytokine via Syk kinase signaling and receptor dectin-1 binding. Dectin-1, also known as CLEC7A, is a C-type lectin receptor expressed in myeloid cells and specifically binds 1–3 β-glucan on the cell wall of fungi (Sancho and Reis e Sousa, 2012). The main feature of this PRR is that it expresses a hemITAM motif, which recruits Src kinase and Syk kinase (**Figure 1**). Syk then recruits CARD9 to the membrane and activates the canonical NF-κB signaling pathway through the CARD9/Blc10/Malt-1 complex. Dectin-1 can also activate the non-canonical NF-κB pathway through RelB. The pro-inflammatory response triggered by NF-κB signaling, with subsequent increases in IL-23p19, TNF-α, and IL-6 production, is counterbalanced by a regulatory response characterized by the production of IL-2 and IL-10 (Sancho and Reis e Sousa, 2012). In this context, IL-2 is likely to be transcribed through the NFAT transcriptional gene program (Granucci et al., 2001; Goodridge et al., 2007). The binding of 1–3 β-glucan to dectin-1 induces the dephosphorylation of Src kinases through the surface molecule CD45. Once activated, Src kinases phosphorylate the ITAM motif of dectin-1, which then forms a docking site for Syk. Phospholipase C (PLC)γ-2 is subsequently activated by Syk to stimulate increases in intracellular Ca^{2+} flux, culminating in the translocation of NFAT into the nucleus (**Figure 1**). Thus, uptake of fungal particles via dectin-1 is reminiscent of the synapse formed between T-cells and antigen presenting cells (Goodridge et al., 2011). This is due to the exclusion of CD45 from the phagocytic synapse when particles are recruited to the phagocytic cup, which facilitates prolonged dectin-1 signaling via Syk and subsequent NFAT translocation.

The molecules involved in NFAT signaling were primarily described in T-cells about 20 years ago. In contrast, NFAT expression in myeloid cells has been identified only recently (Muller and Rao, 2010). Furthermore we have shown that NFAT controls the development and homeostasis of the myeloid compartment (Fric et al., 2012). In resting DCs, NFAT remains phosphorylated in the cytoplasm, and the translocation of NFAT between cytoplasm and nuclei is tightly regulated by a number of protein kinases and phosphatases. The NFAT family of transcription factors comprises five members; NFAT1 (NFATc2 or NFATp), NFAT2 (NFATc or NFATc1), NFAT3 (NFATc4), NFAT4 (NFATx or NFATc3), and

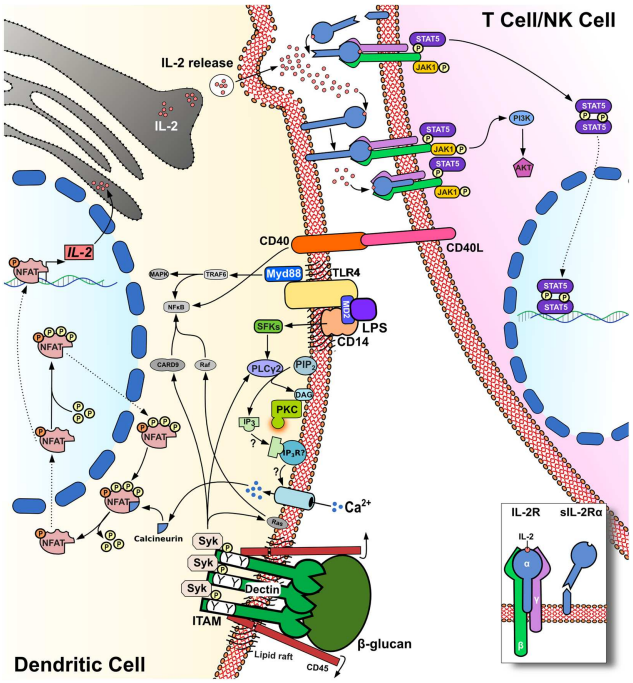

FIGURE 1 | IL-2 release by DCs stimulation of PRRs triggers the calcineurin/NFAT pathway via PLC-γ2 and intracellular Ca^{2+} flux. NFAT dephosphorylation by calcineurin leads to IL-2 transcription and release, which is increased in response to CD40/CD40L interactions. CD25 (IL-2Rα) can capture IL-2 release in the synaptic cleft on the surface of DCs for presentation in *trans* to adjacent T-cells.

NFAT5 (TonEBP; Muller and Rao, 2010; Buxade et al., 2012). In 2009, it was reported that smooth LPS could trigger Ca^{2+} flux through CD14, independently of TLR4 ligation. LPS signaling through this pathway drives NFAT1 translocation and regulates various transcripts that control DC survival and apoptosis (Zanoni et al., 2009; **Figure 1**). LPS-induced Ca^{2+} mobilization was dependent on PLCγ-2 and required CD14 localization in lipid rafts. More recently, CD14 has also been shown to play an essential role for the internalization of TLR4, which is endocytosed in response to LPS stimulation via Syk and PLCγ-2 (Zanoni et al., 2011).

Re-phosphorylation of NFAT in the nucleus is also regulated by several kinases; CK1, GSK3, and DYRK. The phosphorylation status of NFAT in the cytoplasm of DCs can be further modulated by Leucine-rich repeat kinase 2 (also known as dardarin), a recently described negative regulator of NFAT1 signaling in myeloid cells (Liu et al., 2011). Calcineurin is a key calcium-dependent phosphatase, which mediates NFAT signaling (Jain et al., 1993; McCaffrey et al., 1993). Calcium flux stimulates calcineurin to dephosphorylate NFAT, thereby promoting NFAT translocation into nuclei. In T-cells, activation of this cascade induces gene transcription and leukocyte activation, thereby driving the production of NFAT-dependent cytokines including IL-2, IL-4, GM-CSF, and TNF-α (Rao et al., 1997).

More recently, mice that conditionally lack NFAT or calcineurin in myeloid cells were also studied and a key role for calcineurin/NFAT signaling in the innate immune compartment

has been demonstrated (Greenblatt et al., 2010). Indeed the stimulation of myeloid cells with live fungi or with fungal cell wall extracts such as curdlan or zymosan results in the activation of a number of genes regulated through NFAT. In macrophages exposed to zymosan, transcription targets include genes which encode IL-10, IL-12, cyclooxygenase-2 (Cox-2), and members of the early growth response (Egr) family of transcription factors, Egr2 and Egr3 (Goodridge et al., 2007). In bone marrow-derived DCs, zymosan was similarly found to trigger IL-2, IL-10, and IL-12p70 production in an NFAT-dependent manner. Importantly, the levels of IL-2 released in response to β-glucan particles were comparable to the known levels of IL-2 normally detected in T-cell cultures in response to lectins (Slack et al., 2007). The intracellular levels were detected in response to zymosan particles at the same time points as in response to LPS (Rogers et al., 2005). Since zymosan also activates TLR2 and Myd88 signaling, the IL-2 production detected by these assays was found to be partially dependent on Myd88 expression. Intriguingly, IL-2 was seemingly co-produced with IL-10 in a Myd88-independent manner (Rogers et al., 2005). Zymosan-stimulated cytokine production was also found to depend at least in part on TLR2 expression (Goodridge et al., 2007). In DCs, ERK activation is required for maximal production of IL-2, which is Myd88-independent but does require Syk (Slack et al., 2007). These data clearly show that the Syk-dependent pattern recognition is extremely important for TLR-independent anti-fungal immune responses.

IMMUNO-REGULATORY ROLE OF DC-PRODUCED IL-2

The role played by DC-derived IL-2 in immune responses has been difficult to interpret since many other innate and adaptive cell types can produce large quantities of this cytokine *in vivo* (Boyman and Sprent, 2012). Indirect evidence of a contribution from DC-derived IL-2 to host protection comes from the observation that DCs infected with murine cytomegalovirus (MCMV) lose the ability to produce IL-2 in response to LPS (Andrews et al., 2001). The finding that MCMV inhibits IL-2 production implies that DC production of this cytokine is detrimental to the pathogen and may therefore contribute to protective immunity. It is possible then that one strategy employed by MCMV to escape immune surveillance is by decreasing DC potential for T-cell priming (Andrews et al., 2001). In addition, it is known that IL-2 supports T-cell proliferation, drives the production of cytokines including IFN-γ and TNF-α, and regulates the homeostatic survival of lymphoid lineage cells. Recent investigations of how regulatory T-cells (Tregs) are induced and maintained have now demonstrated how IL-2 contributes to both tolerance and immunity (Cheng et al., 2011).

Several roles have been proposed for IL-2 cytokine produced by DCs. One hypothesis is that DC-derived IL-2 can enhance NK cell functions, and accordingly, cross talk between NK cells and IL-2-producing DCs increases IFN-γ production by the NK cells (Zanoni et al., 2005). DCs appear then to provide important stimuli for the priming and cytotoxic function of NK cells (Granucci et al., 2008). Notably it has been observed that IL-2-deficient DCs are impaired in their ability to stimulate the proliferation of allogeneic T-cells (Granucci et al., 2001). In mixed lymphocyte reactions, DCs primed with bacteria were used to stimulate allo-reactive $CD4^+$ and $CD8^+$ T-cells. These experiments confirmed that IL-2-deficient DCs were poor antigen presenting cells compared with wild-type DCs. Similarly, when T-cell proliferation was tested by flow cytometry, very few cycling T-cells were detected in co-cultures stimulated by IL-2-deficient DCs (Granucci et al., 2001). These data clearly indicate that IL-2 produced by DCs significantly affects T-cell activation in mice. In humans, the production of IL-2 by activated human DCs has been shown (Feau et al., 2005) and more recently the activation of human T-cells by DC-produced IL-2 was also revealed using an *in vitro* IL-2-secretion assay (Wuest et al., 2011). IL-2 also regulates important aspects of Treg biology including the thymic development and suppressive function of these cells (Cheng et al., 2011). This has led to the evaluation of potential therapeutic strategies that employ IL-2 cytokine to expand Tregs (Yamazaki et al., 2007). Guiducci et al. (2005) reported that DCs impaired in IL-2 production fail to expand Tregs *in vivo*, suggesting an important role for DC-derived IL-2 in the regulation of Treg biology. It has also been reported that IL-2 produced by DCs enhances CD40/CD40L interaction thus promoting Treg expansion (Guiducci et al., 2005). When DCs are loaded with cognate antigens and are co-cultured with T-cells IL-2-secretion may be detected in the immune synapse (Wuest et al., 2011). In the synaptic cleft, IL-2 released by DCs has been found to bind in an autocrine manner to the α-subunit of the IL-2R (CD25). This subunit is expressed on the surface of DCs and presents IL-2 in *trans* to the other two signaling chains of the IL-2R on T-cells (**Figure 1**). The IL-2-related cytokine IL-15 can signal in a similar fashion (Stonier and Schluns, 2010), and it is possible that DC secretion of IL-2 in the synaptic cleft may provide an efficient and previously unexpected "*signal 3*" for T-cells, including T effector and Tregs. Other than transpresentation, it might be also possible that DC IL-2R is important for autocrine maturation (Mnasria et al., 2008) and eventually may function as decoy receptor. It remains to be determined how the IL-2-secretion that follows PRR ligation in DCs can lead to either the activation of effectors or regulatory T-cell functions.

INNATE IL-2 AS A POTENTIAL NEW TARGET IN CLINICAL STUDIES

We have shown that early IL-2 production by DCs in response to PRR ligation exerts important functions in both innate and adaptive leukocytes. The potential key role for the early produced IL-2 in innate responses requires tight regulation at the transcriptional level to control the absolute levels of this cytokine in the microenvironment and in the immune synapse. Immune homeostasis may depend in part on the ongoing production of IL-2, which exerts a critical influence on the differentiation and function of Tregs (Malek, 2008). Indeed, in a recent observational cohort study, daily sub-cutaneous administration of low-dose IL-2 was found to be associated with Treg expansion and a reduction in clinical manifestations of graft-versus-host disease (Koreth et al., 2011). More importantly, drugs such as cyclosporin A (CsA) and tacrolimus (FK506; Flanagan et al., 1991; McCaffrey et al., 1993) potently block the calcineurin/NFAT signaling pathway and are widely used to prevent transplant rejection and autoimmune diseases, but these drugs also increase risk of infection. Intriguingly, treatment with CsA can also increase susceptibility to *Candida*

albicans infection in mice that lack conventional lymphocytes (Greenblatt et al., 2010) suggesting that the drug must also act on non-lymphoid cell types.

The newly uncovered functions of IL-2 might therefore explain how drugs that modify the calcineurin/NFAT pathway impact on immune homeostasis and induce side effects in treated patients. Indeed, the ability of DCs to produce IL-2 and other NFAT regulated proteins is impaired upon such treatment (Goodridge et al., 2007). The quantitative and qualitative effects of DC-derived IL-2 thus constitute a new level of regulation of the immune response after microbial encounter and open new paths for identification of new therapeutic targets.

EARLY IL-2 PRODUCTION IN MYELOID DCs IN DIFFERENT TISSUES

- In the skin there are dermal DCs, epidermal DCs, and Langerhans cells (LCs) with very different functional capabilities (Kaplan, 2010a,b). Murine LCs stimulated with LPS *in vitro* and analyzed by flow cytometry display IL-2 production by intracellular staining. IL-2 production was abrogated by CsA treatment (Granucci et al., 2003).
- In the spleen, both $CD8^+$ and $CD8^-$ subsets of $CD11c^+$ DCs were found to produce IL-2 following injection with LPS, zymosan, or bacteria (Granucci et al., 2003).
- Genomic profiling of DCs and macrophages has confirmed that IL-2 transcription following PRR activation is an exclusive feature of DCs (Granucci et al., 2001).
- Human myeloid DCs produce IL-2 in response to LPS via a mechanism that depends on CD40L and IL-15 (Feau et al., 2005).

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Layers of dendritic cell-mediated T cell tolerance, their regulation and the prevention of autoimmunity

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The last decades of Nobel prize-honored research have unequivocally proven a key role of dendritic cells (DCs) at controlling both T cell immunity and tolerance. A tight balance between these opposing DC functions ensures immune homeostasis and host integrity. Its perturbation could explain pathological conditions such as the attack of self tissues, chronic infections, and tumor immune evasion. While recent insights into the complex DC network help to understand the contribution of individual DC subsets to immunity, the tolerogenic functions of DCs only begin to emerge. As these consist of many different layers, the definition of a "tolerogenic DC" is subjected to variation. Moreover, the implication of DCs and DC subsets in the suppression of autoimmunity are incompletely resolved. In this review, we point out conceptual controversies and dissect the various layers of DC-mediated T cell tolerance. These layers include central tolerance, Foxp3$^+$ regulatory T cells (Tregs), anergy/deletion and negative feedback regulation. The mode and kinetics of antigen presentation is highlighted as an additional factor shaping tolerance. Special emphasis is given to the interaction between layers of tolerance as well as their differential regulation during inflammation. Furthermore, potential technical caveats of DC depletion models are considered. Finally, we summarize our current understanding of DC-mediated tolerance and its role for the suppression of autoimmunity. Understanding the mechanisms of DC-mediated tolerance and their complex interplay is fundamental for the development of selective therapeutic strategies, e.g., for the modulation of autoimmune responses or for the immunotherapy of cancer.

Keywords: DC, tolerance, Foxp3, Treg, CD103, autoimmunity, infection

INTRODUCTION

Tolerance can be defined by the complete absence or partial inhibition of a potentially harmful adaptive immune response. Immunological tolerance operates continuously in order to protect mammals not only from the deleterious attack of self tissues, but also from the rejection of semi-allogeneic offspring and from uncontrolled immune responses against foreign antigens (Goodnow et al., 2005; Trowsdale and Betz, 2006; Erlebacher, 2010; Berod et al., 2012). Therefore, although tolerance is a prerequisite for the existence of mammal species, excessive tolerance can become similarly life threatening, e.g., by circumventing cancer immunosurveillance, or by dampening pathogen-specific immunity and thereby causing lethal or chronic infections (Zou, 2006; Belkaid, 2007; Berod et al., 2012). Thus, a fine-tuned balance between tolerance and immunity allows for the specific attack and clearance of dangerous pathogens and transformed cells, whereas harmless self tissues, commensal microorganisms, food- and environmental antigens remain ignored. The risk of tolerance dysregulation and autoimmunity may, however, be the evolutionary price of having a highly specialized immune system. Indeed, a recent study identified pathogens as the major driving force for the local genetic adaptation of humans, suggesting co-evolution between microbes and the human immune system (Fumagalli et al., 2011). Strikingly, gene loci that confer susceptibility for autoimmune diseases including type I diabetes, colitis, and rheumatoid arthritis, represent prominent targets for this pathogen-driven selection (Fumagalli et al., 2011). Thus, although such alleles may have evolved for better pathogen control, they likely also contribute to autoimmunity, exemplifying that tolerance and immunity are two sides of a double-edged sword.

Dendritic cells (DCs) populate virtually all tissues and serve as sentinels equipped to detect potential threats. Rapidly, they induce responses in order to protect the host. These include innate immune cell activation and recruitment, inflammation, orchestration of adaptive immunity, and immunological memory. Thus, DCs are "natural adjuvants" bridging innate and adaptive immunity and have clinically been exploited for almost a century to prevent life-threatening infections by vaccination. Additionally, by integrating environmental signals, DCs critically balance tolerance and immunity (Banchereau and Steinman, 1998; Hawiger et al., 2001; Probst et al., 2005).

DC-mediated tolerance can be segregated into at least four layers: central tolerance, Foxp3$^+$ regulatory T cells (Tregs), anergy/deletion and feedback regulation. Additionally, interactions between these layers need to be considered. Although insufficient

insights into the regulation of individual layers is still a limiting factor in resolving the complexity of DC-mediated tolerance, central aims of this review are (1) to dissect the multiple layers of DC-mediated tolerance according to current knowledge; (2) to discuss whether tolerance mechanisms are inherent to or acquired by particular DC subsets and how individual layers of tolerance are regulated by inflammation; and (3) to examine the role of DCs for the suppression of autoimmunity.

DCs AND FOXP3$^+$ TREGS: GUARDIANS OF T CELL IMMUNITY

DCs are heterogeneous with respect to subset composition, localization and function. The DC network across the tissues of different mammal species was recently classified into five conserved subsets. These subsets share common developmental requirements, gene expression patterns, and/or functional specializations and comprise: two types of conventional DCs (CD8α-like cDCs and CD11b-like cDCs), plasmacytoid DCs (pDCs), monocyte-derived DCs (Mo-DCs), and Langerhans cells (LCs) (reviewed in Geissmann et al., 2010; Guilliams et al., 2010a,b; Bar-on et al., 2011b; Belz and Nutt, 2012). Although this simplified model helps to understand parallels between mice and men, markers to identify DC subsets differ substantially between species. In mice, CD8α-like cDCs comprise lymphoid organ-resident CD8α$^+$CD103$^{+/-}$CD11b$^-$DEC-205$^+$DNGR-1$^+$ cDCs and non-lymphoid tissue migratory CD8α$^-$CD103$^+$CD11b$^-$DEC-205$^+$DNGR-1$^-$ cDCs (Sancho et al., 2008; Ginhoux et al., 2009; Del Rio et al., 2010; Edelson et al., 2010). Both subsets will be summarized as CD8α/CD103$^+$ cDCs in the following. CD11b-like cDCs (later called CD11b$^+$ cDCs) include lymphoid organ-resident and non-lymphoid tissue migratory CD11b$^+$ cDCs, both being heterogeneous and comprising further subgroups (Ginhoux et al., 2009; Guilliams et al., 2010b; Henri et al., 2010). While CD11b$^+$ cDCs are more specialized in innate responses and CD4$^+$ T cell immunity, CD8α/CD103$^+$ cDCs are efficient at cross-presenting exogenous antigens (Guilliams et al., 2010b). The intestinal lamina propria is the only known site populated by CD11b$^+$ Mo-DCs in the steady state and it further contains a CD103$^+$CD11b$^+$ migratory cDC subset with unique developmental and functional characteristics (Jaensson et al., 2008; Bogunovic et al., 2009; Ginhoux et al., 2009; Schulz et al., 2009; Helft et al., 2010).

One critical component of DC-mediated tolerance is the interaction of DCs with immunosuppressive CD4$^+$ Tregs (Tarbell et al., 2004). Foxp3 was identified as a master regulator of CD4$^+$ Treg function through transcriptional control of around 1100 target genes (Fontenot et al., 2003; Hori et al., 2003; Khattri et al., 2003; Apostolou et al., 2008). However, Foxp3 itself is dispensable for Treg lineage commitment and thus for part of the Treg-specific transcriptome (Lin et al., 2007; Lahl et al., 2009). Foxp3 is highly restricted to CD4$^+$TCRαβ$^+$ Tregs in mice with the exception of its transient expression by a minor population of non-Tregs (Komatsu and Hori, 2007; Liston et al., 2007; Sakaguchi et al., 2008; Kim et al., 2009; Wolf et al., 2010; Mayer et al., 2012; Miyao et al., 2012). Moreover, Foxp3 is expressed by few CD8$^+$ T cells that were recently shown to expand after allogeneic bone marrow transplantation (Mayer et al., 2011; Robb et al., 2012). Of note, in humans additional markers like CD45RA and CD45RO help to discriminate Foxp3$^+$ Tregs from activated T cells (Sakaguchi et al., 2010).

Foxp3$^+$ Tregs comprise two major subsets, natural and induced Tregs. Both subsets are devoted to immune suppression and most likely act in a complementary fashion by extending T cell receptor diversity (Haribhai et al., 2011). Natural Foxp3$^+$ Tregs develop as distinct T cell lineage in the thymus in a DC-independent fashion (Ohnmacht et al., 2009). In contrast, DCs critically control induced Foxp3$^+$ Treg generation from Foxp3$^-$ T cells in the periphery. Additionally, DCs tailor the peripheral homeostasis of both natural and induced Foxp3$^+$ Tregs (see "DCs and Foxp3$^+$ Tregs"). Requirements for natural and induced Foxp3$^+$ Treg development, as well as their immunosuppressive activities, have previously been reviewed (Curotto De Lafaille and Lafaille, 2009; Huehn et al., 2009; Josefowicz and Rudensky, 2009; Sakaguchi et al., 2009; Ohkura and Sakaguchi, 2010; Berod et al., 2012; Hsieh et al., 2012). However, these issues are far from being completely solved.

TOLEROGENIC DCs

The term "tolerogenic" is increasingly being used to characterize certain DC functions or DC subsets. However, its definition can be quite intuitive. For example, tolerogenicity has been attributed to DCs solely based on the expression of one or more immunosuppressive molecules. Conversely, reduced expression of immunostimulatory molecules has similarly been regarded as a tolerogenic property. Other definitions include the capacity of a DC to generate Tregs or its inability to generate a T cell response. Whether these T cell responses are measured *in vitro* or *in vivo* is up to the scientist's definition. Moreover, the use of *in vitro* generated or *ex vivo* isolated DCs adds an additional level of complexity. Finally, various protocols have been developed to generate "tolerogenic" DCs by pharmacologic treatment (reviewed in Maldonado and Von Andrian, 2010). These examples illustrate that the term "tolerogenic DC" is vaguely defined and requires refinement.

Figure 1 outlines different models of DC-mediated tolerance. Classically, DCs are believed to retain an immature/semi-mature steady state in order to primarily induce tolerance (Steinman et al., 2003). The triggering of diverse pattern recognition receptors, cytokine receptors or co-stimulatory receptors (e.g., through infections) lead to DC maturation (**Figure 1A**). Maturation is characterized by increased densities of MHC: peptide complexes and co-stimulatory molecules like CD80/CD86 on the surface of a DC, as well as the release of inflammatory cytokines and chemokines (Steinman et al., 2003). Matured DCs are considered to be potent stimulators of immunity (**Figure 1A**). However, as the mere expression of known co-stimulatory molecules like CD80/CD86 is not indicative of an immunogenic function, this classical view has been refined (Reis E Sousa, 2006). In this new model, maturation is rather seen functionally and results in the generation of immunogenic DCs, e.g., involving the licensing of DCs by CD4$^+$ T cell help (Reis E Sousa, 2006). Thus, DC-mediated tolerance can be classically described by a situational fate decision where the DC either achieves functional maturation and becomes immunogenic, or fails to mature and promotes tolerance (**Figure 1A**).

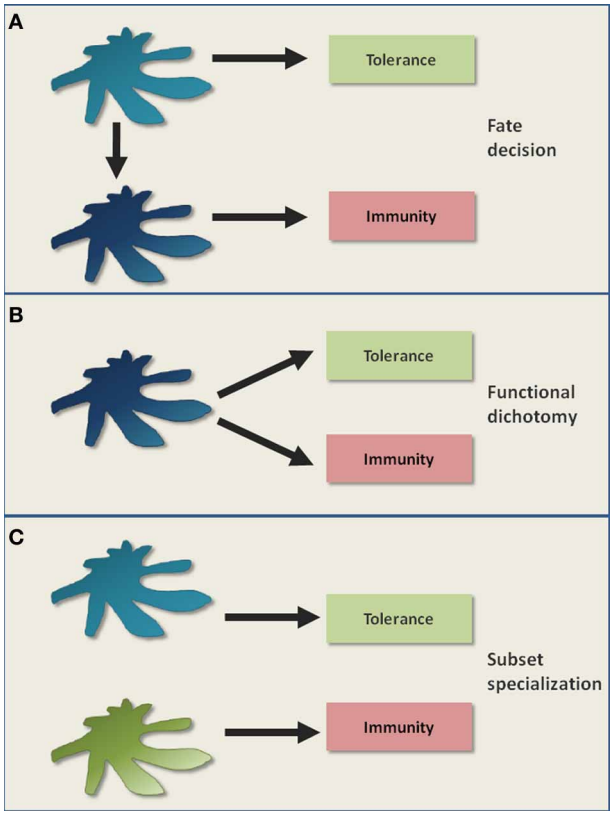

FIGURE 1 | Classical models of DC-mediated tolerance. It is a long standing controversy how DCs mediate immunological tolerance. **(A)** One classical view is that the maturation status of DCs acts as a switch, determining the decision for either tolerance in the steady state (immature/semimature DCs) or for immunity upon inflammation (mature DCs). **(B)** The subsequent discovery of immunosuppressive regulatory T cells that can be activated by both immature and mature DCs, raised the possibility for functional dichotomy. This means that a DC is capable of potentially executing both tolerogenic and immunogenic immune responses as parallel events. **(C)** Certain DC subsets were proposed to be inherently more potent in tolerance induction compared to other DC subsets. In this model, a division of labor between DC subsets regulates tolerance versus immunity.

A key question remains how matured DCs can distinguish between pathogen-specific T cells and T cells autoreactive to ubiquitous self antigens. In fact, the maintenance of self tolerance may become even more important during inflammatory conditions than during the steady state in order to prevent autoimmunity. Taking this into consideration, one can postulate that even matured immunogenic DCs continue to promote tolerance. In this model, DCs possess a functional dichotomy by promoting tolerance and immunity as parallel events (**Figure 1B**), although it fails to explain the abrogation of tolerogenic DC functions upon inflammation, as observed in certain experimental settings (Hawiger et al., 2001; Laffont et al., 2010).

An alternative model is the preferential induction of tolerance by specialized DC subsets (Del Rio et al., 2010; Lutz et al., 2010; Matta et al., 2010; Scott et al., 2011). Here, a division of labor between DC subsets might regulate tolerance versus immunity (**Figure 1C**). Tolerogenicity may be acquired either inherently or by environmental conditioning of certain DC subsets. However, if certain DC subsets are specialized at inducing tolerance in the steady state, it is still not clear why the *in vivo* elimination of these DC subsets so far failed to elicit autoimmunity (Kaplan et al., 2005; Hildner et al., 2008; Swiecki et al., 2010; Takagi et al., 2011). Therefore, although these different models help to describe certain findings, they fail to fully describe DC-mediated tolerance. Instead, tolerance might be better understood as various interacting and differentially regulated layers that will be dissected in the following paragraphs and in **Figure 2**.

LAYERS OF DC-MEDIATED TOLERANCE

DCs AND CENTRAL TOLERANCE

The thymus produces T cells with random specificities. This creates the problem that potentially harmful autoreactive T cells are readily generated. For this reason, the thymic elimination of such autoreactive T cells (so called negative selection) represents an essential component of tolerance (**Figure 2A**). Negative selection is thought to involve DCs (Kyewski et al., 1986; Brocker et al., 1997; Volkmann et al., 1997). The majority of thymic DCs localize to the medulla or cortico-medullary junction and comprise CD8α/CD103$^+$ cDCs (∼50% of total DCs), pDCs (∼35%) and CD11b$^+$ cDCs (∼15%) (Wu and Shortman, 2005). Consistent with DC localization, the thymic medulla is the primary site for negative selection. However, negative selection can also occur at a very early developmental stage in the cortex, with involvement of both cortical thymic epithelial cells (cTECs) and rare cortical DCs (McCaughtry et al., 2008). Most CD8α/CD103$^+$cDCs seem to derive locally from thymic T cell precursors, whereas pDCs and CD11b$^+$ cDCs likely originate from the circulation, thereby importing peripheral antigens for negative selection (Wu and Shortman, 2005; Bonasio et al., 2006; Li et al., 2009; Hadeiba et al., 2012).

Additionally, non-hematopoietic medullary epithelial cells (mTECs) express and directly present tissue-specific antigens under the control of AIRE (Anderson et al., 2002; Kyewski and Klein, 2006). On the one hand, it was elegantly demonstrated via independent approaches that this DC-independent type of antigen presentation can be sufficient to mediate the negative selection of T cells specific for particular model antigens (Luckashenak et al., 2008; Hinterberger et al., 2010). On the other hand, this may largely depend on the type, localization and concentration of the respective antigen. Indeed, mTEC-mediated negative selection was proposed to be incomplete and to require hematopoietic antigen presenting cells such as medullary DCs which cross-present mTEC-derived antigens (Barclay and Mayrhofer, 1981; Gallegos and Bevan, 2004; Kurobe et al., 2006). This may explain the thymic predominance of the CD8α/CD103$^+$ cDC subset which is highly efficient at cross-presentation (Dresch et al., 2011). Thus, DCs likely contribute to central tolerance [(Klein et al., 2011); **Figure 2A**], although the role of individual DC subsets remain to be established.

DCs AND PERIPHERAL TOLERANCE

Several mechanisms of peripheral tolerance operate after T cells have completed their thymic development and have entered the

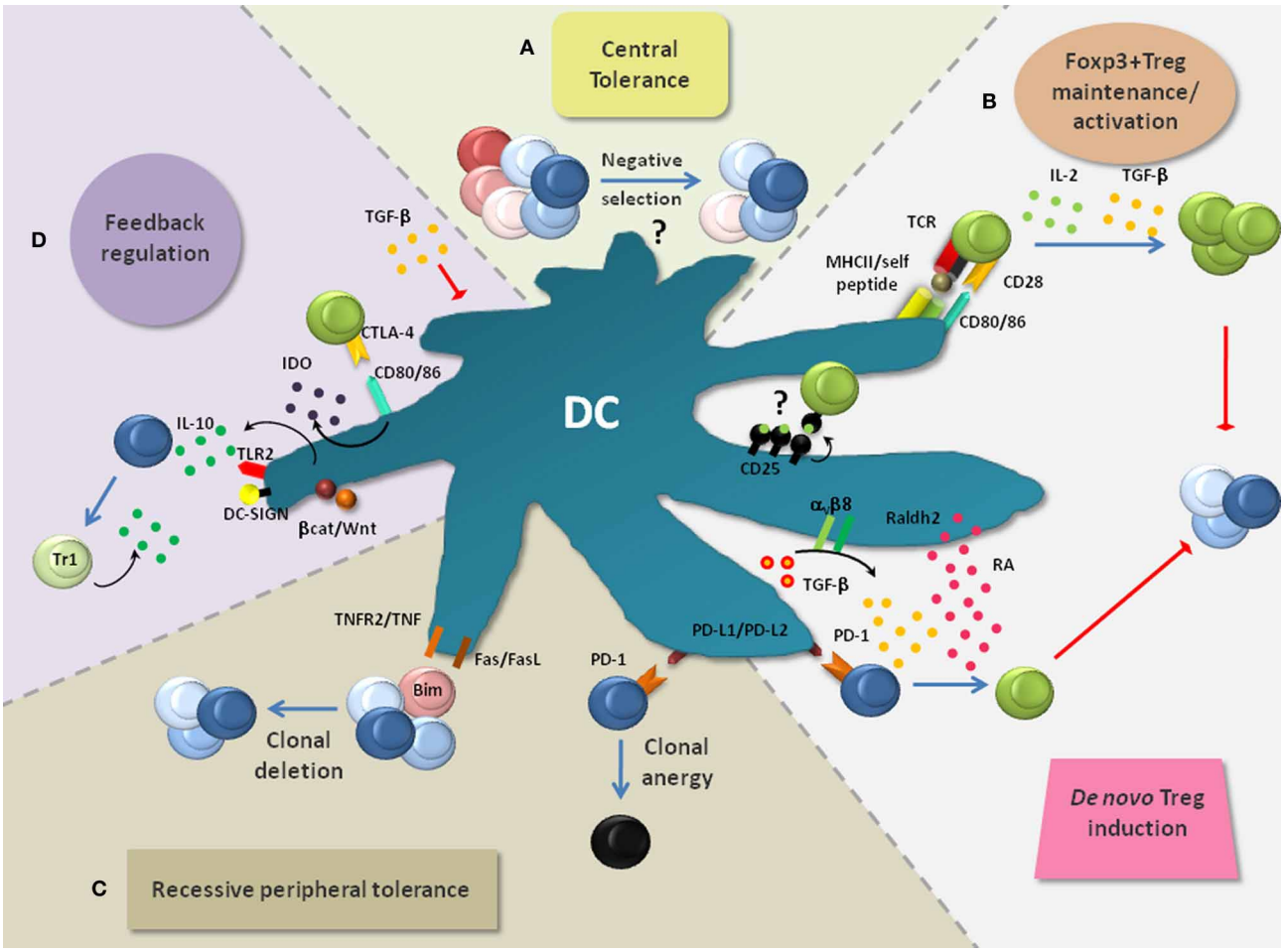

FIGURE 2 | Layers of DC-mediated tolerance. DCs promote tolerance via multiple layers, thereby rendering the term "tolerogenic DC" highly indefinite. **(A)** DCs are implicated in the negative selection of self-reactive T cells and thus in central tolerance, although this is still a subject of intense research. **(B)** DCs are critically involved in the *de novo* generation, homeostasis and activation of Foxp3$^+$ Tregs which play a non-redundant role in the suppression of lethal autoimmunity. Foxp3$^+$ Treg activation by DC-associated MHC-class-II: peptide complexes and CD80/CD86 is crucial to activate the immunosuppressive properties of Foxp3$^+$ Tregs and to induce their expansion. Additionally, IL-2 and TGF-β, which can be produced by DCs, are critically involved in the maintenance of Treg function and proliferation. DC-derived TGF-β can also act on Foxp3$^-$ T cells to convert them into Foxp3$^+$ Tregs. This process is enhanced by retinoic acid (RA) which is generated in DCs from retinaldehyde by the enzyme Raldh2. PD-1 ligands (PD-L1 and PD-L2) were also implied in Foxp3$^+$ Treg induction. **(C)** Recessive peripheral tolerance is established in a T cell-intrinsic manner following instructive encounters with DCs. Antigenic activation of Foxp3$^-$ T cells can result in their functional inactivation (clonal anergy). This partly depends on the triggering of PD-1 on T cells via its ligands PD-L1 and PD-L2. Similarly, signaling via CTLA-4 can induce clonal anergy (not depicted). Another outcome of recessive tolerance can be clonal T cell deletion which depends on interactions via TNF/TNFR2, FasL/Fas or induction of the pro-apoptotic factor Bim. **(D)** Multiple mechanisms can result in the feedback inhibition of DCs. Foxp3$^+$ Tregs bind the co-stimulatory molecules CD80/CD86 on DCs via CTLA-4. This interaction leads to the production of indolamine-2,3-dioxygenase (IDO) and in the suppression of DC maturation (not depicted). IDO causes the apoptosis of conventional T cells and activates Foxp3$^+$ Tregs. TGF-β produced and activated by DCs can directly inhibit DC functions. Additionally, signaling via TLRs (e.g., TLR2), DC-SIGN and the Wnt/β-catenin pathway can result in the production of IL-10 by DCs. IL-10 potently inhibits DC maturation/function and induces IL-10-producing T_R1 cells, thereby creating a regulatory feedback loop.

periphery. One is dominant, cell-extrinsic and exerted by Foxp3$^+$ Tregs (**Figure 2B**). In contrast, so-called recessive peripheral tolerance is characterized by a T cell-intrinsic mode of action (Sakaguchi et al., 2008). Examples for recessive tolerance are clonal deletion that results from activation-induced cell death, and clonal anergy which represents a state of functional unresponsiveness (**Figure 2C**). Finally, negative feedback regulation contributes to peripheral tolerance and will also be considered (**Figure 2D**).

DCs and Foxp3$^+$ Tregs

Interactions between Foxp3$^+$ Tregs and DCs comprise the maintenance/activation of Foxp3$^+$ Tregs and their *de novo* differentiation (**Figure 2B**). In this section, both aspects will be pointed out. Additionally, putative differential regulation upon inflammation and the involvement of DC subsets are discussed.

Foxp3$^+$ Treg maintenance/activation. DCs frequently form contacts with Foxp3$^+$ Tregs which *in vitro* depend on both

antigen and LFA-1 (Tarbell et al., 2004; Sakaguchi et al., 2009). Thereby, Foxp3$^+$ Tregs compete with conventional T cells for forming interactions with DCs (**Figure 2B**). Consequently, the clustering of Foxp3$^+$ Tregs around DCs physically inhibits the activation of conventional T cells. Furthermore, Foxp3$^+$ Tregs become activated in response to much lower antigen concentrations than conventional T cells even if immature DCs present the antigens (Takahashi et al., 1998; Tarbell et al., 2004; Sakaguchi et al., 2008). Once activated, Foxp3$^+$ Tregs proliferate (**Figure 2B**), enrich at sites of specific antigen encounter, become highly suppressive and may infiltrate inflamed tissues in order to locally confer transient or long-term immune regulation (Yamazaki et al., 2003; Samy et al., 2005; Lahl et al., 2009; Wheeler et al., 2009; Rosenblum et al., 2011). Accordingly, the loss of MHC-class-II expression on DCs results in decreased Treg proliferation and markedly reduced numbers of Foxp3$^+$ Tregs (Darrasse-Jeze et al., 2009). Besides antigenic stimulation, Foxp3$^+$ Treg homeostasis involves CD28-derived co-stimulatory signals (**Figure 2B**). In non-obese diabetic (NOD) mice, CD28- or CD80/CD86 deficiency causes drastically reduced numbers of Tregs which results in the onset of autoimmune diabetes (Salomon et al., 2000). Additionally, IL-2- and TGF-β-induced signals were shown to maintain Foxp3 expression, Treg suppressive function, and/or survival [(Marie et al., 2005; Setoguchi et al., 2005; Sakaguchi et al., 2008); **Figure 2B**]. Treg homeostasis could involve IL-2 trans-presentation by CD25-expressing DCs [(Wuest et al., 2011); **Figure 2B**]. Moreover, DCs (especially CD8α/CD103$^+$ cDCs and intestinal CD103$^+$CD11b$^+$ cDCs) are potent producers of bioactive TGF-β [(Annacker et al., 2005; Coombes et al., 2007; Travis et al., 2007; Yamazaki et al., 2008; Paidassi et al., 2011); **Figure 2B**]. Thus, DCs guide the homeostasis of Foxp3$^+$ Tregs via multiple mechanisms.

The importance of DCs for the homeostasis of Foxp3$^+$ Tregs can be further exemplified by conditions which alter the numbers of DCs *in vivo*. DC-deficient ΔDC mice, CD11c: DTA mice and DT-treated CD11c-DTR bone marrow chimeras all harbor decreased numbers of Foxp3$^+$CD25$^+$ Tregs with reduced Foxp3 expression (Darrasse-Jeze et al., 2009; Ohnmacht et al., 2009; Baron et al., 2011a). This extends to humans with DC deficiency syndromes that are accompanied by reduced numbers of Tregs (reviewed in Collin et al., 2011). Vice versa, an expansion of the mouse DC compartment with exogenous FLT3L induces Treg proliferation (Darrasse-Jeze et al., 2009; Swee et al., 2009).

Are particular DC subsets specialized for the maintenance of Tregs? Given that all DC subsets express significant amounts of MHC-class-II in the immature steady-state, Treg activation seems to be an inherent function of DCs. However, certain DC subsets may more effectively provide additional factors such as IL-2 or active TGF-β. Interestingly, pDCs have been recently shown to control the homeostasis of intestinal Foxp3$^+$ Tregs (Takagi et al., 2011). The underlying mechanisms remain to be explored. Foxp3$^+$ Treg homeostasis is likely enforced by inflammation which e.g., causes the increased expression of MHC-class-II, CD80, CD86, and IL-2 (Oldenhove et al., 2003; Yamazaki et al., 2003; Banerjee et al., 2006; O'Sullivan et al., 2011). In summary, DCs critically regulate the homeostasis and activation state of Foxp3$^+$ Tregs.

De novo induction of Foxp3$^+$ Tregs. A second layer of DC/Treg interaction is based on the finding that conventional T cell activation by particular DC subsets can lead to the *de novo* generation of Foxp3$^+$ Tregs. This specialization is presumably acquired by DC conditioning and depends on the ability of DCs to produce and/or activate latent TGF-β [(Chen et al., 2003; Paidassi et al., 2011); **Figure 2B**]. Moreover, DC-derived retinoic acid (RA) enhances the TGF-β-mediated generation of Foxp3$^+$ Tregs [(Hill et al., 2008; Mucida et al., 2009); **Figure 2B**]. RA production in DCs is regulated by the expression of the retinaldehyde-converting enzymes Raldh1 or Raldh2 (Manicassamy and Pulendran, 2009). GM-CSF, RA and TLR signaling (especially via TLR2) represent putative Raldh2-inducing factors within tissues (Manicassamy et al., 2009; Yokota et al., 2009; Guilliams et al., 2010a; Jaensson-Gyllenback et al., 2011). Moreover, β-catenin-dependent signals have been shown to induce TGF-β, Raldh1 and Raldh2 in intestinal DCs (Manicassamy et al., 2010). Additionally, the uptake of apoptotic DCs may imprint Foxp3$^+$ Treg-inducing capacity (Kushwah et al., 2009, 2010). As another example, PD-1 ligation on activated T cells was implied in DC-mediated Treg induction [(Fukaya et al., 2010); **Figure 2B**]. The contribution of these individual mechanisms for Treg induction and the overall role of induced Foxp3$^+$ Tregs for tolerance are still under investigation. A recent study suggests that induced Foxp3$^+$ Tregs make an important contribution to self tolerance (Haribhai et al., 2011). Moreover, Rudensky and colleagues reported a role for iTregs in suppressing Th2 inflammation at mucosal surfaces (Josefowicz et al., 2012).

Which DC subsets are capable of inducing Foxp3$^+$ Tregs? So far, only mesenteric lymph node CD8α/CD103$^+$ and CD103$^+$CD11b$^+$ cDCs, splenic CD8α/CD103$^+$ cDCs and subcutaneous lymph node CD11b$^+$ cDCs were described to possess endogenous Foxp3$^+$ Treg inducing activity (Coombes et al., 2007; Sun et al., 2007; Yamazaki et al., 2008; Guilliams et al., 2010a; Azukizawa et al., 2011). Lung CD8α/CD103$^+$ and CD11b$^+$ cDC subsets display Raldh2 activity, but have not been functionally tested yet (Guilliams et al., 2010a). In line with these findings, migratory lamina propria CD103$^+$CD11b$^+$ cDCs were shown to mediate oral tolerance and imprint gut homing properties to T cells including induced Foxp3$^+$ Tregs (Jaensson et al., 2008; Schulz et al., 2009; Cassani et al., 2011; Hadis et al., 2011). Furthermore, antigen delivery to CD8α/CD103$^+$ cDCs via DEC-205 or DNGR-1 can induce Foxp3$^+$ Tregs in the absence of inflammation, yet provokes immunity after co-administration of adjuvants (Kretschmer et al., 2005; Yamazaki et al., 2008; Joffre et al., 2010). This functional dichotomy of CD8α/CD103$^+$ cDCs is consistent with the reported abrogation of Treg-inducing functions upon inflammation (Laffont et al., 2010; Hackl et al., 2011). However, DC stimulation by pathogens (e.g., via TLR2) may augment the production of RA and thereby Treg induction (Manicassamy et al., 2009). Indeed, certain infections are known to be accompanied by an expansion of Foxp3$^+$ Tregs (Belkaid, 2007; Berod et al., 2012). However, it is still controversial whether this involves genuine *de novo* induction or expansion of pre-existing Foxp3$^+$ Tregs. Once induced, Foxp3$^+$ Tregs were shown to expand heavily even

during immunogenic conditions (Kretschmer et al., 2005). This may reflect the previously discussed increased homeostasis of Foxp3$^+$ Tregs by matured *vs*. steady-state DCs (see "Foxp3+ Treg maintenance/activation"). Nevertheless, the suppressive activity of Foxp3$^+$ Tregs can be negatively regulated (e.g., by certain inflammatory cytokines), possibly to prevent excessive tolerance (Anz et al., 2010).

One can ask the question why only few DC subsets are endowed with Foxp3$^+$ Treg-inducing capacity. This may relate to anatomical localization. For example, mucosal surfaces like the intestine are heavily challenged with microbes. This may lead to the constant triggering e.g., of TLR2 and β-catenin-dependent pathways in intestinal compared to splenic DCs (Manicassamy et al., 2009, 2010). Alternatively, DC subsets could be differentially sensitive toward imprinting signals. Moreover, the stability of imprinting could differ between DC subsets. All of these factors may crystallize toward subset specialization within a dedicated organ (e.g., tolerogenic CD103$^+$CD11b$^+$ cDCs *versus* immunogenic CD11b$^+$ Mo-DCs in the steady state intestine), yet may at the same time explain variability between tissues (e.g., intestine *versus* skin). Markers that distinguish natural from induced Foxp3$^+$ Tregs would greatly help to clarify the importance of individual DC subsets for Treg induction. According to recent studies, the previously reported candidate Helios seems to be less specific for natural Tregs than originally thought (Thornton et al., 2010; Akimova et al., 2011; Gottschalk et al., 2012).

Recessive peripheral tolerance

Recessive tolerance is a T cell intrinsic process. In the absence of infection, trauma or necrosis, DCs maintain a functionally immature state and continuously process and present self antigens (Reis E Sousa, 2006). Although such recognition of self antigen by T cells can lead to their expansion, they may remain functionally impaired due to clonal anergy (**Figure 2C**). This process partially depends on CTLA-4 and PD-1 expression by activated T cells [(Nishimura et al., 1999; Freeman et al., 2000; Greenwald et al., 2001; Keir et al., 2006; Fife et al., 2009); **Figure 2C** and not depicted]. Antigen presentation by DCs can also result in activation-induced cell death (Kurts et al., 1997; Adler et al., 1998; Sakaguchi et al., 2008; Mueller, 2010). This clonal deletion involves Fas-, Bim- or TNF-dependent apoptosis in responding T cells [(Hughes et al., 2008; Hutcheson et al., 2008; Weant et al., 2008; Luckey et al., 2011); **Figure 2C**]. Apoptotic immune cells or tissue cells were shown to actively enforce this process, partly through suppression of NFκB signaling in DCs (Liu et al., 2002; Rothlin et al., 2007). Notably, steady state DCs can be sufficient to induce immunity in particular settings (Mayerova et al., 2004). It has been discussed that the decision between peripheral tolerance and immunity may be based on only subtle differences that determine how many T cells survive an initial wave of clonal expansion (Reis E Sousa, 2006). The nature of these signals is still a matter of intensive investigation.

Skin, lung, and splenic, if not all CD8α/CD103$^+$ DCs are believed to critically maintain CD8$^+$ T cell tolerance by cross-presenting cell-associated antigens (Del Rio et al., 2007; Qiu et al., 2009; Henri et al., 2010; Desch et al., 2011). Indeed, in the absence of co-stimulation, antigen targeting to steady state CD8α/CD103$^+$ DCs via an antigen-coupled αCD103 antibody was recently shown to promote CD8$^+$ T cell tolerance and protection from allergic airway inflammation (Semmrich et al., 2012). Similarly, mice harboring cross-presentation deficient DCs accumulate autoreactive CD8$^+$ T cells (Luckashenak et al., 2008). However, clonal deletion can turn into potent immunity upon DC maturation (Hawiger et al., 2001; Bonifaz et al., 2002; Semmrich et al., 2012). Similarly, co-stimulation of CD28 via CD80/CD86 antagonises clonal anergy (Harding et al., 1992). Thus, these mechanisms maintain peripheral tolerance in the steady state, but seem reversible upon inflammation. This pattern of regulation seems opposite to the homeostasis of Foxp3$^+$ Tregs.

Negative feedback regulation

DCs can also enforce tolerance by initiating negative regulatory feedback loops. Foxp3$^+$ Tregs represent one prominent example (**Figure 2D**). Once activated by DCs, Foxp3$^+$ Tregs suppress the production of FLT3L by still incompletely characterized cell populations, resulting in the strictly regulated development of new DCs from their precursors (Kim et al., 2007; Liu et al., 2009; Schildknecht et al., 2010). In the steady state, the cellular sources for FLT3L were recently described to be widespread, including hematopoietic and non-hematopoietic cells, yet their relative contribution to DC development can vary between different tissues (Miloud et al., 2012). In addition to restricting DC numbers, Foxp3$^+$ Tregs negatively regulate DC function. This can be achieved by simply outcompeting naïve T cells for interaction (**Figure 2B**). Furthermore, the binding of CTLA-4 to CD80/CD86 leads to the suppression of DC maturation [(Onishi et al., 2008); **Figure 2D**]. Accordingly, the relief from Treg-mediated suppression can cause the maturation of DCs (Schildknecht et al., 2010). By suppressing DC maturation, Foxp3$^+$ Tregs in turn promote recessive peripheral tolerance (see "Recessive peripheral tolerance"). Another consequence of the CTLA-4-CD80/86 interaction is the release of indolamine-2,3-dioxygenase (IDO) which converts tryptophan into toxic metabolites (**Figure 2D**). These induce the apoptosis of conventional T cells (Grohmann et al., 2002). Additionally, IDO enforces the suppressive function of Foxp3$^+$ Tregs (Sharma et al., 2009). Notably, the disruption of CTLA-4 signals can abrogate the suppressive function of Tregs *in vivo* (Sakaguchi et al., 2008; Wing et al., 2008). This underlines the importance of CTLA-4-mediated feedback inhibition for the suppressive activity of Foxp3$^+$ Tregs. Foxp3$^+$ Tregs can also secrete TGF-β which results in the *de novo* generation of Foxp3$^+$ Tregs and in the suppression of DC functions (**Figures 2B** and **D**). TGF-β can be additionally provided by activated T cells or by DCs themselves (Coombes et al., 2007; Rubtsov and Rudensky, 2007; Travis et al., 2007).

The important question remains whether Foxp3$^+$ Tregs preferentially engage such regulatory loops in particular DC subsets. Given that FLT3L is a universal growth factor for most DCs and their progenitors, Foxp3$^+$ Tregs probably globally control DC homeostasis (Merad et al., 2008; Kingston et al., 2009). Similarly, the targets of CTLA-4 (CD80/CD86) are expressed by most DC subsets. However, it cannot be excluded that Tregs are more potent inhibitors of particular DC subsets by still undefined mechanisms. Interestingly, Treg-dependent DC inhibition does

not affect MHC-class-II expression, possibly in order to preserve Foxp3$^+$ Treg homeostasis as discussed above (Onishi et al., 2008; Darrasse-Jeze et al., 2009).

IL-10 represents another example for feedback regulation. This cytokine inhibits DC maturation and function. Additionally, IL-10 induces IL-10-producing Foxp3$^-$ T_R1 cells (**Figure 2D**), thus amplifying negative regulation by so-called infectious tolerance (O'Garra and Vieira, 2007; Barnes and Powrie, 2009). DCs produce IL-10 upon activation e.g., through DC-SIGN-, TLR2- or Wnt/β-catenin-dependent signaling pathways [(Dillon et al., 2006; Manicassamy et al., 2010; Hajishengallis and Lambris, 2011); **Figure 2D**]. This may especially protect tissues from immune pathology that are continuously challenged with microbial ligands triggering these receptors. This is supported by IL-10-deficient mice which spontaneously suffer from severe colitis (Kuhn et al., 1993). In conclusion, DCs make use of several mechanisms to induce negative regulatory loops which reinforce tolerance. While some of these mechanisms might prevail in the steady state, other loops may primarily serve to counter-regulate inflammation at specific anatomical locations.

ANTIGEN PRESENTATION

DCs acquire exogenous antigens by diverse mechanisms. These include the ingestion of particles (phagocytosis), the engulfment of extracellular fluid (macropinocytosis), and receptor-mediated endocytosis. Exogenous antigens typically gain access to the MHC-class-II presentation pathway. However, these can also be shuttled to the MHC-class-I pathway, a process referred to as cross-presentation (reviewed in Kurts et al., 2010). In contrast, endogenous antigens are classically presented on MHC-class-I molecules.

Since most of the so far dissected mechanisms of tolerance involve antigen recognition, the mode of antigen presentation to T cells also needs to be considered as a component of tolerance. Major insights into such details have been gained by the possibility to target antigens to particular DC subsets *in situ* via the use of antibody conjugates (reviewed in Caminschi and Shortman, 2012). For example, antigen targeting to splenic CD11b$^+$ cDCs (via 33D1) or to CD8α/CD103$^+$ cDCs (via DEC205) revealed DC-intrinsic specializations for distinct MHC presentation pathways. CD11b$^+$ cDCs efficiently form MHC-class-II: peptide complexes whereas CD8α/CD103$^+$ cDCs potently cross-present peptides on MHC-class-I molecules (Dudziak et al., 2007). In contrast, steady-state pDCs express low levels of MHC-class-II (Wu and Shortman, 2005). Consequently, CD11b$^+$ cDCs may be more efficient at activating existing CD4$^+$Foxp3$^+$ Tregs compared to CD8α/CD103$^+$ cDCs and pDCs, especially when the antigen is limited. In contrast, CD8α/CD103$^+$ cDCs efficiently generate active TGF-β, and suboptimal antigen recognition enhances the *de novo* generation of Foxp3$^+$ Tregs (Yamazaki et al., 2008). Thus, differences in the antigen uptake capacity and/or expression of the antigen presentation machinery can result in specializations for certain tolerance mechanisms.

The type of uptake receptor triggered on a particular DC can further modulate the resulting immune response. This can be exemplified by the human C-type lectin DC-specific intercellular adhesion molecule-3 grabbing non-integrin (DC-SIGN). This endocytic receptor capable of signaling via Raf-1 is expressed predominantly on human cDCs, yet lacks functionally related homologues in mice. DC-SIGN binds both self antigens and various pathogens including mycobacteria. Mice expressing human DC-SIGN on CD11c$^+$ DCs were shown to have reduced immunopathology during mycobacterial infection when compared to wildtype mice (Schaefer et al., 2008). Accordingly, human DC-SIGN$^+$ DCs from these infected mice produced significantly less IL-12p40 than wildtype DCs. Additionally, the targeting of DC-SIGN via an antigen-coupled antibody enhances cross-presentation about 1000-fold when compared to unspecific antigen uptake (Tacken et al., 2011). These examples illustrate that the mode of antigen uptake can strongly influence the resulting immune response.

Another important factor is the kinetics of antigen presentation. It has been suggested that persistent antigen presentation may rescue T cells from deletion after their initial priming (Reis E Sousa, 2006). Indeed, antigen targeting to DNGR-1 was shown to induce potent CD4$^+$ T cell-dependent antibody responses as a result of persistent antigen presentation (Caminschi and Shortman, 2012). In contrast, poor antibody responses are seen when targeting DEC205 or Clec12A, although all three receptors are expressed by CD8α/CD103$^+$ cDCs (Lahoud et al., 2011). Other parameters that can regulate the kinetics of antigen presentation may include the half-life of MHC: peptide complexes on the DC surface, the half-life of DCs themselves and the migration of peripheral DCs into secondary lymphoid organs. Notably, inefficient antigen presentation can result in ignorance as a passive type of tolerance (Mueller, 2010). Thus, the mode, efficiency and kinetics of antigen presentation are important components of tolerance.

DCs AND AUTOIMMUNITY

We have depicted how DCs control multiple layers of tolerance (section "Layers of DC-mediated tolerance"). Consequently, it could be expected that the disruption of DC-mediated tolerance layers or the elimination of DCs that execute such programmes lead to the breakdown of self tolerance. This complex issue can only be briefly discussed (also from a technical point of view) in this section and is summarized in **Figure 3**.

DISRUPTION OF TOLERANCE LAYERS

Foxp3$^+$ Tregs are an important component of DC-dependent tolerance (**Figure 2**). Indeed, loss-of-function mutations of Foxp3 in mice and men, or the ablation of Foxp3$^+$ cells in transgenic mice, all lead to lethal autoimmunity (Brunkow et al., 2001; Gambineri et al., 2003; Kim et al., 2007; Lahl et al., 2007; Sakaguchi et al., 2008). Due to the disrupted negative feedback loop between Foxp3$^+$ Tregs and DCs, FLT3L accumulates and DCs expand in Foxp3$^+$ Treg-deficient mice [(Liu et al., 2009); **Figure 3C**]. Additionally, DCs mature (see "Negative feedback regulation") and prime autoreactive T cells (**Figure 3C**). Notably, these include tumor-specific T cells (Klages et al., 2010). Moreover, Foxp3$^+$ Tregs increase the threshold for foreign-specific immunity which may occur both at the priming and effector phase (Baru et al., 2010; Haque et al., 2010; Suttner et al., 2010; Arnold et al., 2011;

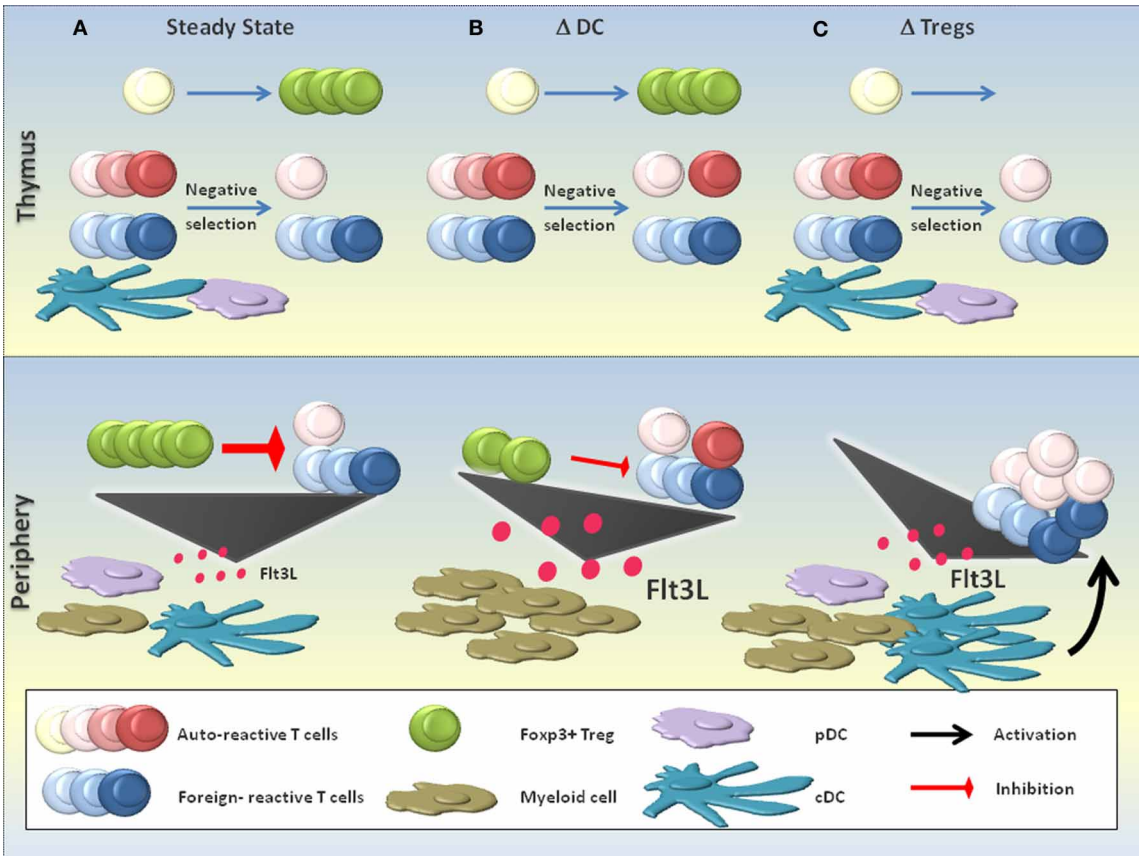

FIGURE 3 | DCs in the suppression of autoimmunity. The role of DCs in the suppression of autoimmunity is complex and a matter of discussion. **(A)** In the steady state, DCs are believed to contribute to the negative selection of autoreactive T cells. Few autoreactive T cell clones that escape negative selection can be efficiently controlled by Foxp3$^+$ Tregs in the periphery, a process that critically depends on the continuous activation and homeostasis of Tregs by DCs. Equilibrium between the production of the myeloid growth factor FLT3L and its consumption maintains a constant number of DCs and other myeloid cells. **(B)** DC-deficient (ΔDC) mice may exhibit defective negative selection, resulting in the increased seeding of the periphery with self-reactive T cells. Additionally, the absence of peripheral DCs impairs Foxp3$^+$ Treg homeostasis and activation, resulting in a substantial reduction of Treg numbers and probably also functionality. The massive accumulation of FLT3L due to the absence of DCs as key consumers of this growth factor leads to a myeloproliferative syndrome. Signs of increased autoreactivity are detectable in ΔDC mice, yet the absence of DCs most likely prevents the activation of self-reactive T cells and thus the full precipitation of systemic autoimmunity. **(C)** The absence of functional Foxp3$^+$ Tregs (ΔTregs; e.g., in scurfy mice) is compatible with a normal negative selection, yet provokes severe systemic autoimmunity due to the defective negative regulation of DCs and autoreactive T cells. An increased level of FLT3L in the absence of Foxp3$^+$ Tregs expands the numbers of DCs and other myeloid cells.

Blankenhaus et al., 2011; Dietze et al., 2011; Hadis et al., 2011; Navarro et al., 2011).

TGF-β is another key molecule in tolerance. This cytokine has important immunoregulatory functions, e.g., the maintenance of Foxp3$^+$ Tregs and the suppression of DC functions (**Figure 2**). A failure of DCs to activate latent TGF-β through the $\alpha_v\beta_8$ integrin results in autoimmunity and colitis (Travis et al., 2007). Moreover, the disruption of TGF-β signaling specifically in DCs renders mice susceptible to experimental autoimmune encephalomyelitis (Laouar et al., 2008). Similarly, the DC-intrinsic disruption of other global inhibitory proteins (e.g., Blimp-1, A20) or the artificial engagement of stimulatory pathways (e.g., Tim-1, CD70) can precipitate immune dysregulation (Keller et al., 2008; Kim et al., 2011; Kool et al., 2011; Xiao et al., 2011). Thus, interfering with DC-mediated tolerance mechanisms can clearly induce autoimmunity (**Figure 3C**).

DC ABLATION

Various mouse models with constitutive DC deficiency are available. Mice deficient in certain transcription factors involved in DC differentiation (like RelB, E2-2 or Batf3) lack specific DC populations, whereas other mutant mice lack several DC subsets (Belz and Nutt, 2012). For example, mice that lack FLT3L, an essential growth factor for DC progenitors, cDCs and pDCs, have severely reduced numbers of DCs (Kingston et al., 2009). Although these models have helped to unveil several aspects of DC development, they often have the main drawback that the transcription factors or growth factors deleted are not DC specific and might therefore affect other immune cell populations. In the last years, two mouse models have been developed in which the suicide gene diphtheria toxin A is expressed in CD11c$^+$ cells (ΔDC mice, CD11c: DTA mice). This leads to the constitutive ablation of DCs (Birnberg et al., 2008;

Ohnmacht et al., 2009). Moreover, CD11c-DTR mice allow for the inducible depletion of DCs by administration of diphtheria toxin (Jung et al., 2002). By using these mouse models, a role for DCs in the maintenance of tolerance has been suggested. Indeed, increased proportions of Th1 and Th17 cells, hypergammaglobulinemia and/or autoantibody formation have been independently detected in ΔDC-, CD11c:DTA-, FLT3L$^{-/-}$- and inducible DC-depleted mice (Birnberg et al., 2008; Darrasse-Jeze et al., 2009; Ohnmacht et al., 2009). In support of these findings, ΔDC mice exhibit defective negative selection [(Ohnmacht et al., 2009); **Figure 3B**]. Furthermore, humans with DC, monocyte, B and NK lymphoid (DCML) deficiency can develop autoimmunity (Collin et al., 2011). Altogether, these studies suggest that DC deficiency can result in increased autoreactivity (**Figure 3B**). However, both ΔDC and CD11c:DTA mice develop a myeloproliferative syndrome that has been explained by the accumulation of FTL3L due to the absence of cDCs (**Figure 3B**). For this reason, the observed abnormalities of adaptive immune cells have been attributed to the myeloproliferative disease rather than to a primary autoimmune disorder (Birnberg et al., 2008; Bar-on et al., 2011a).

The connection between myeloproliferative disease (as a consequence of DC ablation) and autoimmunity is an intriguing question. FLT3L$^{-/-}$ mice resistant to FLT3L-dependent myeloproliferative disease display features of increased autoreactivity similar to ΔDC and CD11c:DTA mice (Darrasse-Jeze et al., 2009). This extends to humans with DCML deficiency who can develop autoimmune manifestations in the absence of myeloproliferation (Collin et al., 2011). Based on these data, one can argue that increased autoreactivity is not necessarily a consequence of myeloproliferation. Furthermore, the transfer of CD4$^+$CD25$^+$ Tregs can prevent autoreactivity in DT-treated CD11c-DTR chimeras (Darrasse-Jeze et al., 2009). Foxp3$^+$ Treg dysfunction may even contribute to myeloproliferation given that Foxp3$^+$ Tregs suppress FLT3L production (Liu et al., 2009). Bar-On et al. use the lack of autoreactivity in CD80/86$^{-/-}$:CD11c:DTA mixed bone marrow chimeras as a supportive argument (Bar-on et al., 2011a). However, one could envisage that CD80/CD86-deficient DCs may sufficiently provide MHC-class-II-dependent signals to Foxp3$^+$ Tregs and stimulate their suppressive functions (Sakaguchi et al., 2008; Darrasse-Jeze et al., 2009). Thus, CD80/86$^{-/-}$ DCs may inhibit autoreactivity when compared to DC-deficient settings in which Foxp3$^+$ Tregs experience virtually no antigenic stimulation. This does not necessarily conflict with reduced Foxp3$^+$ Treg numbers in both scenarios.

More recently, mouse models that allow the depletion of specific DC subsets have been generated to examine the role of particular DC subsets in tolerance and immunity. Langerin (CD207)-positive LCs are located in the epidermis and are believed to take up and transport self antigens to the skin-draining lymph nodes (Waithman et al., 2007). Both inducible (Langerin-DTR) and constitutive (Langerin-DTA) LC depletion models have been developed. However, no obvious onset of skin-specific or systemic autoimmunity has been reported in these LC-ablated mice (Bennett et al., 2005; Kaplan et al., 2005; Kissenpfennig et al., 2005). This is also not the case when the skin of LC-deficient mice is infected with Leishmania major, suggesting that Langerhans cells do not play a major role in suppressing autoimmunity (Kautz-Neu et al., 2011).

Similarly, the contribution of CD8α/CD103$^+$ DCs to tolerance has been examined. Although CD103 is not a universal marker for Foxp3$^+$ Treg-inducing DCs (Azukizawa et al., 2011), CD8α/CD103$^+$ and CD103$^+$CD11b$^+$ cDCs have prominent steady-state regulatory functions across tissues (Bonifaz et al., 2002; Coombes et al., 2007; Henri et al., 2010). Yet, Batf3$^{-/-}$ mice which constitutively lack CD8α/CD103$^+$ DCs, have a normal steady state phenotype (Edelson et al., 2010). Autoimmunity is also not induced upon infections in these mice. In fact, the absence of CD8α/CD103$^+$ cDCs results in defective immunity e.g., against influenza, poxvirus, salmonella and West Nile Virus (Hildner et al., 2008; Bogunovic et al., 2009; Beauchamp et al., 2010; Ho et al., 2011). In this case it is thus likely that other DC subsets can compensate for the loss of CD8α/CD103$^+$ cDCs to maintain tolerance. Additionally, mice that lack intestinal CD103$^+$CD11b$^+$ cDCs do not spontaneously develop colitis or autoimmunity. However, severe colitis is induced in these mice upon challenge with dextrane sodium sulphate (Varol et al., 2009). Conversely, an increase in the proportion of CD103$^+$CD11b$^+$ cDCs by FLT3L treatment results in an increase of Foxp3$^+$ Tregs and amelioration of TNF-driven ileitis (Collins et al., 2011). Thus, CD103$^+$CD11b$^+$ cDCs may balance intestinal immunity in specific situations, but seem to lack a unique tolerogenic function.

Finally, pDCs need to be mentioned. Although pDCs are implied in the pathogenesis of autoimmune diseases like systemic lupus erythematosus, they have also been suggested to promote self tolerance via negative selection and Foxp3$^+$ Treg homeostasis (Tian et al., 2007; Matta et al., 2010; Hadeiba et al., 2012). BDCA2-DTR and SiglecH-DTR mice have recently become available to selectively deplete pDCs. Following DT administration, these mice were so far not reported to develop autoreactivity, even after infection with murine cytomegalovirus or vesicular stomatitis virus (Swiecki et al., 2010; Takagi et al., 2011). Whether this is a matter of depletion efficiency and kinetics, or points towards a dispensable role of pDCs in maintaining tolerance, remains to be resolved (also discussed in "Technical aspects of DC ablation"). In conclusion, although there is definite evidence that DCs substantially contribute to prevent autoimmunity by acting at different layers of tolerance (**Figure 2**; section "Disruption of tolerance layers"), no DC subset has been identified till date which is required to maintain self tolerance in a non-redundant fashion. Constitutive pDC-deficient mice and mice that specifically eliminate CD11b$^+$ cDCs remain to be developed in this regard. Moreover, recent studies suggest that certain technical and conceptual aspects need to be considered to resolve these findings.

TECHNICAL ASPECTS OF DC ABLATION

Depletion efficiency and side effects

The DC depletion efficiency as well as secondary effects induced by DC depletion can influence the outcome of experiments and their interpretation. One example is represented by ΔDC and CD11c: DTA mice both of which re-activate DTA expression in CD11c$^+$ DCs. ΔDC mice lack cDCs, pDCs, and LCs and develop autoimmunity (Ohnmacht et al., 2009). In contrast, CD11c: DTA

mice possess pDCs and LCs, but lack cDCs and autoimmunity (Birnberg et al., 2008). This could point toward a critical role of pDCs and LCs in tolerance induction. However, as mentioned, constitutive LC-deficient mice do not develop autoimmunity (Kaplan et al., 2005). Transgenic mice with a constitutive and selective deficiency in pDCs would be very useful to address this still open question. The differential depletion of $CD11c^+$ DC subsets in ΔDC and CD11c: DTA mice is likely attributed to different DTA expression levels. Since the same BAC-transgenic CD11c-Cre line was utilized in both cases, these differences could relate to the two different DTA strains used (Birnberg et al., 2008; Ohnmacht et al., 2009).

Furthermore, side effects induced by the ablation of DCs can be problematic. It was recently shown that CD11c-DTR and CD11c-DOG mice develop neutrophilia 6–24 h after DC depletion by injection of DT (Tittel et al., 2012). This is mechanistically distinct from myeloproliferation and is guided by CXCL1/2-dependent recruitment of neutrophils from the bone marrow. According to the timing of DC depletion, this can result in opposing phenotypes in a pyelonephritis model using uropathogenic *Escherichia coli* (Tittel et al., 2012). The reason underlying this early neutrophila is still unclear. One could speculate that the massive apoptosis induced by DT can have side effects on the immune system. Additionally, DT can itself have non-specific toxic effects in wildtype mice, especially when combined with protocols causing activation of the immune system (Meyer Zu Horste et al., 2010). Different DT batches can vary substantially in their toxicity, underlining the requirement for careful titration (Lahl and Sparwasser, 2011). Nevertheless, early neutrophila is not observed when treating wildtype mice with DT or when using CD11c-LuciDTR mice for DC depletion (Tittel et al., 2012). The basis for these findings requires further elucidation.

Additionally, it has been recently shown that DCs interact with high endothelial venules and thereby guide the entry of naïve T cells into lymph nodes (Moussion and Girard, 2011). Consequently, the depletion of DCs may have previously unappreciated side effects on T cell responses by altering the lymph node structure. Whether this relies on particular DC subsets has to be investigated. Taken together, both the depletion efficiency and side effects associated with DC depletion may influence the interpretation of data.

Specificity

The promoters and transgene targeting strategies used to drive the ablation of DCs or DC subsets can also strongly influence the outcome of experiments. One classical example is LCs. Three independent models allowing for the inducible or constitutive depletion of LCs based on the C-type lectin Langerin (CD207) generated apparently contradictory results (reviewed in Kaplan et al., 2008). Using Langerin-IRES-DTR-eGFP knock-in mice, no role of LCs for contact hypersensitivity (CHS) reactions was noted (Kissenpfennig et al., 2005). However, Langerin-DTR-eGFP knock-in mice revealed a contribution of LCs to CHS reactions using a similar experimental protocol (Bennett et al., 2005). In contrast, BAC-transgenic Langerin-DTA mice with constitutive LC deficiency develop enhanced CHS responses suggesting a regulatory role of LCs (Kaplan et al., 2005). One important factor contributing to these diverse findings is the specificity of Langerin expression. Langerin is not only expressed by LCs, but also at lower levels by $CD8\alpha/CD103^+$ cDCs, as well as on a subset of $CD103^-CD11b^{low}$ dermal DCs (Kaplan et al., 2008; Henri et al., 2010). Thus, the differential depletion of other $Langerin^+$ DC subsets may explain the different outcomes of the CHS reactions. Indeed, only Langerin-DTA mice specifically target LCs. This was explained by the differential regulation of the human *langerin* gene locus that was used to generate Langerin-DTA mice (Kaplan et al., 2008). Therefore, although depletion models have proven to be useful to answer several questions, these models still have limitations and the specificity of cell ablation in a particular model needs to be considered. Of course, also additional factors such as the depletion- and repopulation kinetics, transient *versus* permanent depletion approaches and the microbial environment can have a strong impact on experiments assessing DC functions and autoimmunity. The same applies to both the antigen dose or the administration route used for immunizations. These factors can for example determine whether additional and anatomically distant DC populations have access to antigen.

SUMMARY AND PROPOSED MODEL

In summary, components of the maturation model (**Figure 1A**), the functional dichotomy model (**Figure 1B**) and the subset specialization model (**Figure 1C**) can be combined to describe DC-mediated tolerance. The strict adherence to only one model can explain paradoxical experimental findings like the abrogation of particular tolerance programs during inflammation (outlined in section "Tolerogenic DCs"). At present, we favor the idea that multiple layers of tolerance (**Figure 2**) are distributed among one or multiple DC subsets. Therefore, any DC can formally be considered "tolerogenic" by executing at least one layer of tolerance. The different layers of tolerance influence each other and can be differentially regulated in order to allow powerful immune responses against pathogens while at the same time restraining autoimmunity.

In the steady state, recessive peripheral tolerance (anergy, deletion) is a default programme of DC-mediated tolerance, given that antigen and antigen-specific T cells are available. On the contrary, only few specialized DCs are capable of *de novo* generation of $Foxp3^+$ Tregs, whereas homeostasis and activation of existing $Foxp3^+$ Tregs is a global function of DCs. $Foxp3^+$ Tregs negatively control both DC numbers and maturation and thereby enforce steady-state tolerance. Inflammatory signals cause functional DC maturation that antagonises recessive peripheral tolerance. Similarly, Treg-inducing properties can be abrogated, whereas the activation and homeostasis of existing $Foxp3^+$ Tregs continue or are even enforced. $Foxp3^+$ Tregs disrupt DC activity and thereby counter-regulate immunity at different levels. Additional mechanisms of feedback regulation can be promoted, e.g., via production of IL-10. As DCs continue to execute tolerogenic programmes during inflammation, tolerance and immunity may represent parallel events tentatively executed by the same DC. Yet certain subsets can be specialized in particular layers of tolerance and some layers may be more resistant toward inflammatory

signals than others. This, in the sum, allows the initiation of immunity, whereas at the same time self tolerance is actively maintained.

Past research using DC depletion models has provided important insights into DC-mediated tolerance, yet the ablation of DC subsets so far failed to induce autoimmunity. If one excludes technical aspects as an explanation, one could postulate that a non-redundant tolerogenic DC subset simply doesn't exist. Moreover, it should be considered that DCs are required to induce (auto)immunity. Thus, the global breakdown of steady-state tolerance can be largely masked in the absence of DCs (**Figure 3**). This would explain why fatal autoimmunity occurs when disrupting non-redundant layers of tolerance (such as Foxp3$^+$ Tregs) while DCs remain present (**Figure 3C**). Unraveling the complex mechanisms of DC- and Foxp3$^+$ Treg-mediated immune regulation across all layers might be the key to the selective therapeutic manipulation of the immune system. This could allow for the enhancement of immunity against pathogens and tumors while suppressing unwanted self-specific responses, and *vice versa*.

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Functional crosstalk between dendritic cells and Foxp3$^+$ regulatory T cells in the maintenance of immune tolerance

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Peripheral immune tolerance requires a controlled balance between the maintenance of self-tolerance and the capacity to engage protective immune responses against pathogens. Dendritic cells (DCs) serve as sentinels of the immune system by sensing environmental and inflammatory signals, and play an essential role in the maintenance of immune tolerance. To achieve this, DC play a key role in dictating the outcome of immune responses by influencing the balance between inflammatory or Foxp3$^+$ regulatory T (T_{reg}) cell responses. At the heart of this immunological balance is a finely regulated DC and T_{reg} cell crosstalk whereby T_{reg} cells modulate DC phenotype and function, and DC drive the differentiation of Foxp3$^+$ T_{reg} cells in order to control immune responses. This review will focus on recent advances, which highlight the importance of this bidirectional DC and T_{reg} cell crosstalk during the induction of tolerance and organ-specific autoimmunity. More specifically, we will discuss how T_{reg} cells modulate DC function for the suppression of inflammatory responses and how DC subsets employ diverse mechanisms to drive differentiation of T_{reg} cells. Finally, we will discuss the therapeutic potential of tolerogenic DCs for the induction of tolerance in autoimmune diseases.

Keywords: Foxp3, immunity, suppression, tolerance, tolerogenic DC

INTRODUCTION

Immune tolerance consists of two main processes, namely central and peripheral tolerance. Central tolerance takes place in the thymus where most of the self-reactive T cells are deleted at an immature stage of their development (Bluestone, 2011). Despite negative selection, self-reactive T cells can escape thymic clonal deletion, and subsequently provoke autoimmune diseases such as type 1 diabetes (T1D), multiple sclerosis (MS), and inflammatory bowel disease (IBD) unless they are controlled by one of many peripheral mechanisms (Sakaguchi et al., 1995).

REGULATORY T CELLS ARE MAJOR MEDIATORS OF PERIPHERAL SELF-TOLERANCE

In order to ensure tolerance induction of such auto-reactive T cells in the peripheral immune system, a number of mechanisms including a network of regulatory T (T_{reg}) cells exist to achieve this function. T_{reg} cells constitute 1–10% of thymic and peripheral CD4$^+$ T cells in humans and mice, and arise during a thymic selection (Sakaguchi, 2000). They are characterized by the constitutive expression of the IL-2Rα chain (CD25) and expression of the forkhead winged helix transcriptional regulator Foxp3 (Hori et al., 2003). The importance of Foxp3 has been demonstrated by natural mutations of the *foxp3* gene that result in a loss of T_{reg} cell function and the development of severe autoimmune diseases, including T1D, in scurfy mice and IPEX patients (Bennett et al., 2001; Chang et al., 2006; d'Hennezel et al., 2009). T_{reg} cell population can be divided into the naturally occurring Foxp3 T_{reg} population (here defined as T_{reg}), generated in the thymus and anyone of many inducible T_{reg} cell populations (here defined as iT_{reg}), that are derived in the periphery from CD4$^+$Foxp3$^-$ precursors upon activation in presence of differentiating signals like TGF-β and IL-10 (e.g. Th3, Tr1 cells) (Haribhai et al., 2011; Pot et al., 2011).

DENDRITIC CELLS DICTATE THE BALANCE BETWEEN TOLERANCE AND IMMUNITY

Numerous studies show that different cell populations of the innate immune system such as dendritic cells (DCs), macrophages, natural killer cells, and γδ T cells can regulate tolerance induction (Lehuen et al., 2010). DCs represent a heterogeneous population of bone marrow-derived cells and are the most potent antigen presenting cells (APCs) (Banchereau and Steinman, 1998; Shortman and Naik, 2007). DCs are derived from multiple lineages, have a distinct stage of development, activation, and maturation state (Banchereau and Steinman, 1998; Steinman et al., 2003). DCs exist as a distinct subset and differ in their ontogeny, surface molecule expression, and biological functions (Banchereau and Steinman, 1998; Steinman and Nussenzweig, 2002). These factors seems to determine the T cells polarizing signals and type T cells responses induced by DCs namely Th1, Th2, Th17, or T_{reg} cells. Although all DCs are able to prime T cells, they differ in their *in vivo* niches, migration, function, and requirements

from the environment for their generation and activation (Shortman and Naik, 2007). DCs are divided into conventional, myeloid, or plasmacytoid DC (pDC) subsets. In mice, three main subsets of conventional $CD11c^{hi}$ DCs have been identified in the spleen and lymph nodes, namely the $CD8^+$, $CD4^+CD8^-$, and $CD4^-CD8^-$DCs (Vremec et al., 2000). Lymph nodes contain two additional DCs subsets; skin-derived Langerhans cells and tissue interstitial DCs that arrive from the periphery through the lymphatic circulation (Vremec et al., 2000).

Dendritic cells receive the maturation signals through the pathogen associated molecular patterns (PAMPs) and damage associated molecular patterns (DAMPs) receptors that detect certain microbial and tissue damage signals *via* activation of nuclear factor-κB (NF-κB) and interferon regulatory factors (IRF) families (Maldonado and von Andrian, 2010). Upon activation, DCs up-regulate a wide variety of gene products involved in antigen presentation and co-stimulation such as MHC II, CD86, CD80, OX40-L, inducible co-stimulator (ICOS) ligand as well cytokines involved in the modulation of effector function such as IL-1β, IL-2, IL-6, IL-8, IL-12, and IL-18 (Maldonado and von Andrian, 2010). These changes are required for DCs to initiate a three-step T cell activation process: MHC molecules displaying cognate peptide (signal 1), co-stimulatory signal expression (signal 2), and cytokine production by DCs (signal 3).

While potently capable to initiate inflammatory responses, DCs also play an important role in modulating tolerance induction. Tolerogenic DCs are characterized by high antigen uptake and processing capabilities in order to present antigen to antigen-specific T cells, but fail to deliver proper co-stimulatory signal for effector T (T_{eff}) cells activation and proliferation (Steinman et al., 2003). This results in T cell death, T cell anergy, or induction and expansion of T_{reg} cells subsets. As such, tolerogenic DCs have been shown to suppress experimental autoimmune disease and play an important role in alloimmunity (Morelli and Thomson, 2007).

SUBSETS OF TOLEROGENIC DCs: DIVISION OF LABOR IN TOLERANCE INDUCTION

Different DCs subsets are specialized in tolerance vs. inflammatory immune response decisions. Specific markers capable of discriminating tolerogenic from inflammatory DCs are still ill-defined. However, $CD8^+$ DCs expressing CD95L and DEC205 often possess tolerogenic properties (Mahnke et al., 2002; Yamazaki et al., 2008). Expression of inhibitory immunoglobulin-like transcript (ILT) receptors is also frequently observed in some subsets of tolerogenic DCs, where ILT-3 and ILT-4 mediated signals on DCs inhibits expression of co-stimulatory molecules and induce a tolerogenic state (Manicassamy and Pulendran, 2011). In humans, ILT-3 and ILT-4 expressing tolerogenic DCs can promote antigen-specific unresponsiveness in $CD4^+$ T cells and induce T_{reg} cells. Recent studies have shown that Indoleamine 2,3-dioxygenase (IDO) and IL-10 can induce the expression of ILT-3 and ILT-4 on DCs and promote tolerogenic response (Manavalan et al., 2003). Activation of ILT-3 receptor on DCs leads to the recruitment of protein phosphate SHP-1 and SHIP-1 to the immunoreceptor tyrosine-based inhibitory motif (ITIM) resulting in the inhibition of NF-κB and p38MAPK pathways that are critical for inflammatory responses (Cella et al., 1997; Svajger et al., 2008). While immature DCs appear to be good indicators for DC tolerogenicity, mature DCs might not always induce immunity. Hence, other factors such as exposure to certain differentiation signals or cues from the local environment might condition DCs beyond their expression of co-stimulatory molecules. Therefore, tolerance is not only a consequence of T cells receiving insufficient signals 2 and 3, but additional tolerance inducing factors might be in play.

GENERATION OF TOLEROGENIC DCs

T_{reg} CELLS, THROUGH VARIOUS MECHANISMS, PROMOTE THE GENERATION OF TOLEROGENIC DCs

While several environmental factors and cytokines can promote the tolerogenic phenotype of DCs, there is accumulating evidence that T_{reg} cells can also induce a tolerogenic phenotype in DCs by modulating their maturation and function. Several studies indicate that T_{reg} cells can suppress the capacity of DCs to activate T_{eff} cells by down-regulating CD80/CD86 expression on bone marrow-derived or splenic DCs *in vitro* (Cederbom et al., 2000). Depletion of T_{reg} cells from asthma susceptible mice resulted in DCs with higher expression levels of MHC II, CD80, CD86 and displayed a increased T cell stimulatory activity (Mahnke et al., 2007). Although the mechanism by which T_{reg} cells achieve this is unknown, this might be mediated through cell surface molecules or cytokines such as IL-10, CTLA-4, and TGF-β (**Figure 1**).

Role of T_{reg} cell-derived IL-10 in induction of tolerogenic DCs

The production and activity of the immunomodulatory cytokine IL-10 is often associated with tolerogenic responses. IL-10 inhibits multiple aspects of DC function including MHCI/II and CD80/CD86 co-stimulatory molecules expression and a release of pro-inflammatory cytokines such as IL-1β, IL-6, TNF-α, and IL-12 (Mahnke et al., 2002, 2007; Alexander, 2005). Interestingly, IL-10 mediated effects could be only observed when immature DCs were exposed to IL-10 (Read et al., 2000), as mature DCs were insensitive to IL-10 stimulation and displayed stable, mature phenotype (Read et al., 2000). In addition, DCs cultured in presence of T_{reg} cells has been shown to secrete of IL-10, TGF-β, and IL-27 cytokines (Awasthi et al., 2007). IL-27 cytokine plays an important role in suppressing production of Th17 polarizing cytokines, such as IL-1β, IL-6, IL-23 derived from DCs and act on naïve T cells to induce Tr1 differentiation (Pot et al., 2009). IL-10 treated DCs have also been shown to promote tumor growth, and prevent transplant rejection and ameliorate MS (Mahnke et al., 2007).

CTLA-4 is a potent inducer of tolerogenic DCs

CTLA-4 plays an important role in T_{reg} cell-mediated suppression (Salomon et al., 2000). T_{reg} cells constitutively express high levels of CTLA-4 and conditional deficiency of CTLA-4 in T_{reg} cells results in lymphoproliferation and variety of autoimmune disease, an outcome analogous to global CTLA-4 deficient mice (Wing et al., 2008). Monoclonal antibody blockade of CTLA-4 exacerbates T1D in non-obese diabetic (NOD) mice and induce IBD (Read et al., 2000; Wing and Sakaguchi, 2010). CTLA-4 mediates suppression through down regulation of CD80 and CD86 expression on DCs. T_{reg} cells from CTLA-4 deficient mice or

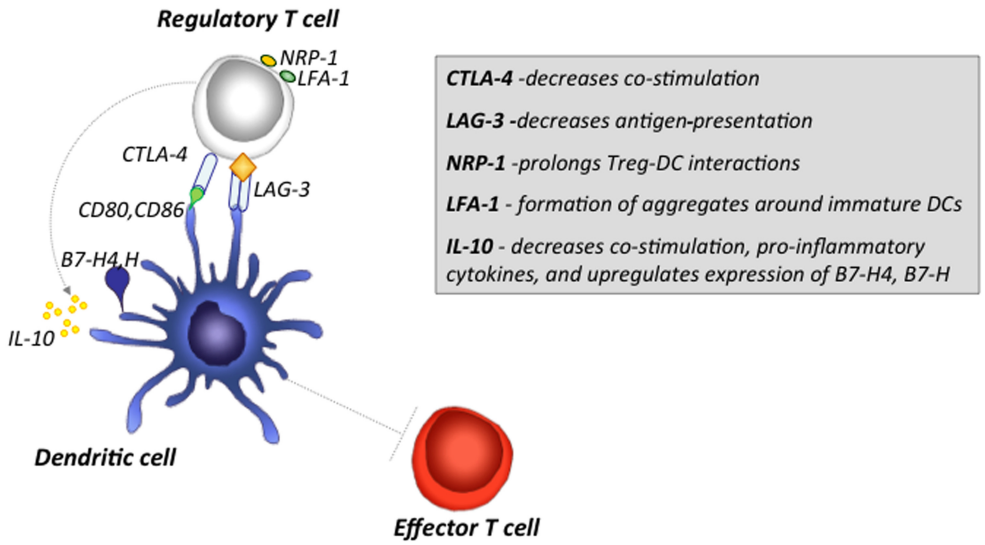

FIGURE 1 | Major mechanisms by which T_{reg} cells induce tolerogenic DCs and inhibit T_{eff} cell activation. T_{reg} cells can inhibit the function of DCs through various mechanisms. CTLA-4 expression on T_{reg} cells down-regulates up-regulation of CD80 and CD86 co-stimulatory molecules on DCs. LAG3 binding to MHCII-expressing immature DCs results in inhibitory signals that suppress DCs maturation and co-stimulatory capacity. Nrp-1 expression on T_{reg} cells promotes sustained interactions between T_{reg} cells and DCs, and limits the access of T_{eff} cells to DCs. Expression of LFA-1 on T_{reg} cells, promotes aggregate formation around DCs, and inhibit the up-regulation of CD80 and CD86 on DCs. IL-10 inhibits up-regulation of MHCI/II and B7 co-stimulatory molecule expression, suppresses release of pro-inflammatory cytokines such as IL-1β, IL-6, TNF-α, and IL-12, and up-regulates of B7-H4 and B7-H inhibitory molecules.

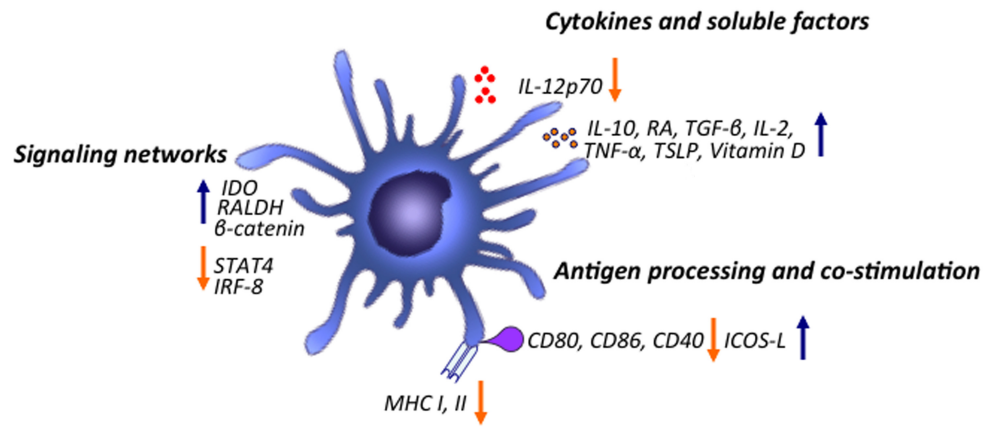

FIGURE 2 | Major mechanisms of immune modulation by tolerogenic DCs. DCs modulate the immune system by promoting T_{reg} cell generation through various mechanisms. Upon stimulation, DCs are able to increase production of various cytokines and inflammatory mediators such as IL-10, RA, vitamin D, TGF-β, IL-2, IL-10, TSLP, TNF-α and down-regulate expression of inflammatory cytokines such as IL-12p70. DCs, by down-regulating co-stimulation and antigen presentation, can favor induction of T_{reg} cells. Various signaling pathways program DCs to induce tolerogenic responses. Activation of β-catenin signaling pathway promotes induction of anti-inflammatory factors such as vitamin A and is important for promoting T_{reg} cell induction and limiting inflammatory responses. TSLPR signaling inhibits activation of STAT4 and IRF-8, factors critical for production of the Th1 polarizing factor IL-12. Triggering of DCs through TLR2-6 leads to Erk activation which mediates induction of RALDH and conversion of retinal to RA. TLR9 ligation can drive induction of IDO in DCs limiting IL-6 production and differentiation of Th17 cells while inducing differentiation of T_{reg} cells.

administration of CTLA-4 blocking antibodies results in reduced down-modulation of B7 molecules and increased T cells proliferation (Oderup et al., 2006). IDO, a potent regulatory molecule that is know to induce the production of pro-apoptotic metabolites from the catabolism of tryptophan, results in the suppression of T_{eff} cells through a mechanism dependent on interaction between CTLA-4 and CD80/CD86 (Fallarino et al., 2003; Baban et al., 2009). Baban et al. (2009) recently demonstrated that IDO is a critical molecular switch that stimulates potent T_{reg} cells suppression while simultaneously block IL-6 driven reprogramming of T_{reg} cells into Th17 cells and production pro-inflammatory cytokines such as IL-17, IFN-γ, TNF-α, and IL-2. Interestingly, it has also been demonstrated that T_{reg} cells can modulate DCs to express IDO and more specifically CTLA-4 immunoglobulin

fusion protein was found to induce IDO expression through ligation to CD80 and CD86 molecules in mouse and human DCs (Fallarino et al., 2003; Mellor and Munn, 2004; Mellor et al., 2004). In addition, IL-10 produced by T_{reg} cells is critical for sustaining IDO expression in DCs (Manicassamy and Pulendran, 2011). Thus, IDO is one of the molecular switches controlling the balance between T_{reg} and T_{eff} cell responses.

B7-H4 and B7-H: B7 family members that negatively regulate T cell immunity

A number of other mechanisms have been proposed by which T_{reg} cells can either abrogate the antigen presenting capacity of DCs or promote the secretion immunomodulatory cytokines. For example, T_{reg} cells are able to induce the expression of B7-H and B7-H4, a ligands responsible for negative regulation of cell-mediated immunity in peripheral tissues (Mahnke et al., 2007). Recently, T_{reg} cells have also been shown to trigger high levels of IL-10 production by APCs and in turn stimulate B7-H4 expression in an autocrine fashion and render these APCs immunosuppressive (Kryczek et al., 2006).

Lymphocyte activation gene 3 (LAG3)

Lymphocyte activation gene 3 is cell surface molecule expressed on T_{reg} cells that can modulate DC phenotype and function (Huang et al., 2004; Workman and Vignali, 2005; Liang et al., 2008). LAG3 is a CD4 homolog that binds MHC II molecules with very high affinity, has a negative regulatory intrinsic function and is required for maximal T_{reg} cells suppression (Huang et al., 2004; Workman and Vignali, 2005). Binding of LAG3 to MHC II molecules on immature DCs initiates a receptor tyrosine-based activation motif (ITAM) mediated inhibitor signaling pathway that suppress DC maturation (Liang et al., 2008). These findings provide a novel tolerogenic pathway that may endow T_{reg} cells to enhance tolerance by inhibiting DCs functions.

T_{reg}–DCs cognate interactions mechanism

It has been shown that T_{reg} cells are more motile that naïve T cells *in vitro*, in turn out-competing the latter for physical access to DCs (Tran et al., 2009). This T_{reg}–DC cell aggregation process is antigen-dependent, and the selective advantage of T_{reg} cells over T_{eff} cells for physical interactions with DCs can be attributed, at least in part, to the expression of LFA-1 (Lymphocyte function-associated antigen 1) by T_{reg} cells, as deficiency or blockade of LFA-1 abolishes this process. By forming aggregates, T_{reg} cells inhibit the up-regulation of CD80, CD86 on immature DCs and also down-regulate the expression of CD80 and CD86 by mature DCs without affecting the expression of CD40 or MHC II molecules (Onishi et al., 2008). This modification of CD80 and CD86 is CTLA-4 dependent as T_{reg} cells from CTLA-4 deficient mice fail to modify expression of CD80 and CD86 (Onishi et al., 2008). Therefore at least *in vitro*, T_{reg} cell contact dependent suppression can be dissected by two consequent steps; initial LFA-1 dependent formation of aggregates around immature DCs and subsequent LFA-1 and CTLA-4 dependent active down-regulation of CD80 and CD86 expression (Onishi et al., 2008). Overall, this results in down-regulation of T_{eff} cell activation and induction of immune suppression and tolerance. The significance of DCs and T_{reg} cells interaction has been also demonstrated in studies using NOD mice. The Bluestone group, by means of intravital microscopy, demonstrated that direct interactions between T_{reg} cells and DCs *in vivo* resulted in inhibition of T_{eff} cells activation (Tadokoro et al., 2006; Tang et al., 2006) suggesting a potential feedback loop between T_{reg} cells and DCs. In this model, T_{reg} cells up-regulate LFA-1 expression upon activation by immature DCs, adhere to DCs, and suppress the expression of CD86 and CD80, in turn inhibiting the activation and expansion of T_{eff} cells (Sakaguchi et al., 2009). While $Foxp3^+$ T_{reg} must be activated by antigen in order to exert their suppressive functions *in vitro* and *in vivo* (Takahashi et al., 1998), activated $Foxp3^+$ T_{reg} cells can mediate *in vitro* bystander suppression of T_{eff} cells with different antigen specificities. However, it still remains to be determined whether such mechanism can also apply to other cell types like DCs, and to what extent such bystander suppression occurs *in vivo*.

Recently it has been shown that neuropilin (Nrp-1) also plays a critical role in mediating T_{reg}–DC interactions (Sarris et al., 2008). Nrp-1 is a ligand binding receptor for a class of semaphorins, and is preferentially expressed by T_{reg} cells. Nrp-1 expression can be induced by ectopic expression of Foxp3 in $Foxp3^-$ T cells, and antibody blockade of Nrp-1 reduces the frequency of T_{reg}–DCs interactions. Furthermore, retroviral introduction of Nrp-1 endows T helper cells with ability to establish a long interactions with DCs (Sarris et al., 2008). Interestingly, it has been demonstrated that Nrp-1 can confer a high adhesive property of T_{reg} cells in their interaction with DCs under steady conditions; however, this potential by T_{reg} cells is lost under inflammatory conditions (Sarris et al., 2008).

The results described above demonstrate the capacity of $Foxp3^+$ T_{reg} cells, through the expression of LAG3 and Nrp-1 molecules, to influence immature but not mature DCs. Mature DCs, in contrast to immature DCs, produce pro-inflammatory cytokines such as IL-6, TNF-α, and IL-1β which can down-regulate Foxp3 expression and abolish $Foxp3^+$ T_{reg} cell-mediated suppression (Rutella et al., 2006). Although IL-6 production induced by LPS stimulation only slightly reduced $Foxp3^+$ T_{reg} cells-mediated suppression, DC-derived IL-6 may render responder T cells resistant to suppression (Pasare and Medzhitov, 2003). Moreover, IL-6 might also facilitate the reprogramming of $Foxp3^+$ T_{reg} cells into Th17 lineage (Bettelli et al., 2007).

In other instances, it has been demonstrated that T_{reg} cells, through the expression of LFA-1 and CTLA-4, can suppress TNF and LPS maturated DCs by selectively down-regulating CD80, CD86, PDL1, and PDL2, but not MHCII and CD40, expression on the DC surface. Overall, these findings demonstrate that T_{reg} cells may employ specific mechanism(s) to suppress DCs functions in different microenvironments of antigen priming.

THE GUT ENVIRONMENT PROMOTES DEVELOPMENT OF TOLEROGENIC DCs

The identification of tolerogenic DCs subsets in microenvironments such as gut, skin, and lungs suggest that local signals induce tolerogenic DCs *in situ*. Given the enormous amount of microbial stimuli in the gut, intestinal DCs represent a key regulatory mechanism to prevent excessive inflammation. Specifically, $CD11c^+$ DCs expressing CD103 have been identified in the gut-associated lymphoid tissue (GALT) and mesenteric lymph node with the

specialized function of inducing Foxp3$^+$ T_{reg} cells and maintaining gut immune homeostasis (Coombes et al., 2007; Sun et al., 2007). This CD11c$^+$ DC subset expresses high levels of CD103, MHC II, CD80, and CD86. Interestingly, absence of CD103 on DCs led to the abrogation of T_{reg} cell activity indicating a crucial role for CD103 in maintaining the balance between T_{reg} and T_{eff} cells (Annacker et al., 2005). While mesenteric lymph node derived CD103$^+$ DCs are prone to imprint expression of CCR9 on T cells, an important homing receptor enabling homing to the gut, CD103$^-$ DCs promote the differentiation of CD4$^+$ T cells producing IFN-γ (Jaensson et al., 2008). Collectively, CD103$^+$ and CD103$^-$ DCs represent functional subsets and CD103 is critical to regulate T_{eff} and T_{reg} cells in the gut (Annacker et al., 2005).

APOPTOTIC DCs FAVOR FORMATION OF TOLEROGENIC DCs

Recent studies show that immature DCs can uptake apoptotic and necrotic DCs without being recognized as an inflammatory event (Kushwah et al., 2010). This uptake results in conversion of mature DCs into tolerogenic DCs that remain resistant to LPS induced maturation and induces the production of TGF-β via the mTOR signaling pathway (Kushwah et al., 2010). TGF-β producing DCs subsequently interact with naïve T cells and drive the differentiation of iT_{reg} cells (Kushwah et al., 2010; Kushwah and Hu, 2011). Moreover, recent studies showed that α-CD3 mAb treatment transiently depletes large numbers of T cells and induce long term immune tolerance (Perruche et al., 2008; Esplugues et al., 2011). The mechanism underlying this regulatory outcome is due to increased production of TGF-β by immature DCs after engulfment of apoptotic T cells (Perruche et al., 2008). A recent study demonstrated that α-CD3 mAb treatment resulted in elimination of the inflammatory Th17 cells from intestinal lumen or resulted in acquisition Th17 cells producing a IL-10 (Esplugues et al., 2011). However, it is not known which specific subset of gut-residing DCs is responsible for production of TGF-β and induction of IL-10 producing Th17 cells with regulatory capacity.

IMMUNE MODULATION BY TOLEROGENIC DCs

DC EMPLOYS VARIOUS MECHANISMS TO DRIVE THE DIFFERENTIATION OF T_{reg} CELLS

B7 co-stimulatory molecules promote differentiation of T_{reg} cells

In the steady state, DCs are largely immature and present antigens to T cells in tolerogenic manner. Such DCs are characterized by low expression of CD80, CD86, and CD40 (Steinman and Nussenzweig, 2002; Steinman et al., 2003; Hubert et al., 2007). Moreover, targeting of antigens to immature DCs via the regulatory receptor DEC205 results in tolerance through deletion of antigen-specific T cells and preferential induction of T_{reg} cells (Hawiger et al., 2001). Interestingly, antibodies bound to DEC205 are efficiently internalized and delivered to antigen processing compartments, however internalization of the antigen by DEC205 does not induce the maturation of the DCs (Mahnke et al., 2000). Immature DCs had been shown to induce T_{reg} cells *in vitro* and anti-DEC205 targeting of antigens to immature DCs led to anergic T cells *in vivo* (Mahnke et al., 2003). These data show that that the DEC205 receptor is critical for DCs in the steady state to promote tolerance (Mahnke et al., 2003). Moreover, treatment with respective anti-DEC antigen conjugates results in significant improvement of autoimmune disorders such as T1D and MS (Hawiger et al., 2004; Bruder et al., 2005). DCs can also favor tolerance by utilizing ICOS, another CD28 family member, to specifically drive Tr1 cell differentiation (Akbari et al., 2002; Ito et al., 2007).

Despite the established role of immature DCs as inducers of T_{reg} cells, recent studies have shown that mature DCs, expressing high levels of CD86, also have the potential to preferentially expand T_{reg} cells *in vitro*, and prevent T1D development (Yamazaki et al., 2003; Tarbell et al., 2004; Sgouroudis et al., 2011). Interestingly, DCs isolated from Peyer's patches, eye, or lungs display a mature phenotype, secrete IL-10, but not IL-12 and drive the development of Tr1 cells (Akbari et al., 2001). Thus, signals derived from the local tissue environment might play a role in conditioning tolerogenic DCs and drive the differentiation of T_{reg} cells. All together, these findings suggest that the previously established paradigm whereby immature DCs leads to T_{reg} cell differentiation and mature DCs drive T_{eff} cell responses might be revisited (**Figure 2**).

DC-DERIVED CYTOKINES THAT DRIVES DIFFERENTIATION OF T_{reg} CELLS

IL-10

Dendritic cells can secrete high amounts of IL-10 upon stimulation and drive differentiation of naïve T cells into IL-10 secreting Tr1 cells (Kushwah and Hu, 2011). DCs of IL-10 transgenic mice display a particularly immature phenotype and Tr1 cells are significantly enriched in spleens of these mice (Wakkach et al., 2003). Langerhans cells in the skin also produce IL-10 and drive differentiation of Tr1 cells (Igyarto et al., 2009).

TNF-α

TNF-α can also induce tolerogenic state in DCs (Menges et al., 2002; Mahnke et al., 2007). Despite the fact that TNF-α treated DCs has a mature phenotype, they fail to secrete inflammatory cytokines such as IL-1β, IL-6, TNF-α, and IL-12 (Menges et al., 2002). Moreover, these tolerogenic DCs were able to reverse development of EAE where their suppressive effects were mediated by the induction of IL-10 producing T_{reg} cells (Mahnke et al., 2007). However, there is also a controversial data demonstrating that TNF-α blockade prevented DC maturation and T_{reg} cells were induced in the absence of TNF-α (Mahnke et al., 2007).

TGF-β

TGF-β-producing DCs might preferentially drive differentiation of T_{reg} cells rather than T_{eff} cells. Tumor-bearing mice contains a CD11c$^+$ DC subset characterized by low expression of CD80 and CD86 co-stimulatory molecules and are endowed with the capacity to secrete TGF-β which promotes T_{reg} cells differentiation and proliferation *in vivo* (Ghiringhelli et al., 2005).

IL-2

We and others have shown that a local deficiency in islets of IL-2, a critical cytokine for the homeostasis/fitness of T_{reg} cells *in vivo*, compromises T_{reg} cell function in islets, a defect readily

corrected by low dose IL-2 therapy in NOD mice. NOD mice introgressed with a protective *IL2* allelic variant from T1D-resistant C57BL6 mice (NOD.Idd3^{BL6}) are resistant to autoimmune diabetes in contrast to wild-type NOD mice which are susceptible to the disease. Moreover, T1D-protective IL2 allelic variants in NOD mice impinge T1D development by bolstering IL-2 production by CD4$^+$ T_{eff} cells in turn, driving the expansion and homeostasis of CD4$^+$Foxp3$^+$ T_{reg} cells in islets. Recently, we showed that CD11c$^+$ DCs in NOD.Idd3^{BL6} have an increased maturation status relative to wild-type NOD CD11c$^+$ DCs. We also showed that NOD.Idd3^{BL6} DCs are more potent activators of T_{reg} cells functions *in vitro* and *in vivo*, and this increased capacity of congenic DCs to prime T_{reg} cells is attributed to their ability to produce IL-2. Consistently, IL-2 blockade *in vitro* completely abolished the proliferative advantage conferred by Idd3^{BL6} DCs on Foxp3$^+$ T_{reg} cells (Sgouroudis et al., 2011). Interestingly, CD11c$^+$ MHCII$^+$ DCs isolated from pancreatic LN of NOD.Idd3^{BL6} mice promoted Foxp3$^+$$T_{reg}$ cells expansion more efficiently that WT DCs isolated from similar sites, suggesting that Idd3^{BL6} DCs display tolerogenic phenotype specifically in the pancreatic sites to enhance Foxp3$^+$ T_{reg} cells functions (Sgouroudis et al., 2011). Thus, T1D-protective *IL2* allelic variants impinge the development of β-islet autoimmunity by bolstering IL-2 mRNA expression and protein secretion by CD4$^+$ T_{eff} cells and DC and in turn, driving the functional homeostasis of CD4$^+$Foxp3$^+$ T_{reg} cells in the target organ (Sgouroudis et al., 2011).

It has been previously shown that CD40/CD40L interaction regulates T_{reg} cells homeostasis (Kumanogoh et al., 2001; Guiducci et al., 2005). Mature DCs lacking CD40L are impaired in sustaining T_{reg} cells proliferation and survival. The underlying mechanism is mediated by fact that CD40L deficient DCs are not able to produce IL-2, to as similar extent as wild-type DCs. Administration of rhIL-2 *in vivo* restored T_{reg} cells numbers in thymic and peripheral compartments of CD40L deficient mice, by increasing survival and homeostatic proliferation of T_{reg} cells (Kumanogoh et al., 2001; Guiducci et al., 2005). Therefore, CD40 triggering by T_{reg} cells contributes to induce IL-2 through CD40L on DCs needed for their maintenance. Therefore, several mechanisms have been established by DCs in order to maintain a peripheral pool of T_{reg} cells through IL-2 production (Kumanogoh et al., 2001; Guiducci et al., 2005).

IDO EXPRESSION BY DCs DRIVES T_{reg} CELLS DIFFERENTIATION

Dendritic cells populations expressing IDO play a critical role in immune tolerance by promoting iT_{reg} cells differentiation (Mellor and Munn, 2004; Matteoli et al., 2010). IDO expression by DCs appears to be dependent on Aryl hydrocarbon receptor (AHR) as DCs lacking AHR fail to up-regulate IDO and prime T cells responses rather than tolerance induction (Nguyen et al., 2010). Selective inhibition or genetic deletion of IDO affects the development of antigen-specific T_{reg} cells while promotes Th1 and Th17 development and worsens T cells mediated and dextrin sulfate sodium (DSS) colitis in mice (Matteoli et al., 2010). It has been shown that TLR9 ligation can drive induction of IDO in pDCs that subsequently suppress IL-6 production and differentiation of Th17 cells while induce differentiation of T_{reg} cells (Baban et al., 2009).

SITE SPECIFIC RESIDING DCs SUPPORTS T_{reg} CELLS DIFFERENTIATION

Thymic residential DCs play a role in induction of Foxp3$^+$ T_{reg} cells

It has been shown that DCs and medullary thymic epithelial cells (mTECs) contribute to the selection of Foxp3$^+$ T_{reg} cells in the thymus. Epithelial cells in Hassall's corpuscles in the thymus produce thymic stromal lymphopoietin (TSLP) which subsequently acts on thymic DCs by binding to TSLP receptor (TSLPR) and IL-7Rα complex and drives induction of CD80 and CD86 (Ziegler and Liu, 2006). These DCs subsequently drive differentiation of CD4$^+$CD8$^+$CD25$^-$ thymocytes into Foxp3$^+$$T_{reg}$ cells, which highly depend from IL-2 and CD28 signaling (Ziegler and Liu, 2006). These data demonstrate that TSLP activated myeloid DCs are critical for selection of self-reactive thymocytes to develop into Foxp3$^+$ T_{reg} cells. In addition to the myeloid DCs, human thymic pDCs can also induce differentiation of T_{reg} cells in a CD40L-dependant fashion (Hanabuchi et al., 2010; Martin-Gayo et al., 2010). Thymic pDCs express TSLPR along with IL-7Rα and are responsive to TSLP derived from thymic epithelial cells (Hanabuchi et al., 2010; Martin-Gayo et al., 2010). More evidence for the contribution of thymic DCs in T_{reg} cell induction comes from studies done in mouse models, where targeting of neo-self antigens to thymic DCs instructed antigen-specific T cells to develop into Foxp3$^+$ T_{reg} cells (Proietto et al., 2008). Three thymic DCs subtypes, namely conventional DCs, thymus resident DCs, and pDCs were shown to be able of instructing Foxp3$^+$ T_{reg} cells differentiation *in vitro*, supporting the model where antigen recognition on thymic DCs can directly elicit Foxp3$^+$ T_{reg} cells differentiation (Wu and Shortman, 2005).

Gut-residing DCs maintain T_{reg} cell stability via production of retinoic acid and TGF-β

Dendritic cells from the lamina propria of the small intestine preferentially promote T_{reg} cell induction relative to the DCs of lymphoid organs. This increased induction of T_{reg} cells has been shown to be dependent on TGF-β and retinoic acid (RA), a vitamin A metabolite highly expressed in small intestine (Coombes et al., 2007; Sun et al., 2007; Belkaid and Oldenhove, 2008). DCs are the major subset of immune cells able of metabolizing vitamin A to RA. RA synthesis is a highly regulated process that involves several key enzymes: vitamin A is oxidized to retinaldehyde by alcohol dehydrogenase and then to RA by retinal dehydrogenase (RALDH). Manicassamy et al. (2009) and Manicassamy and Pulendran (2011) demonstrated that TLR2 and TLR6 signaling by zymosine promote induction of RALDH1 and RALDH2 in DCs via ERK dependent mechanism. This result in conversion of retinal to RA which then exerts an autocrine effect on DCs via RA receptor to induce SOCS3, this then suppress activation of p38MAPK and pro-inflammatory cytokines. Accumulating evidence shows that RA directly influences the development and function of various immune cells. For example, RA promotes the differentiation of T_{reg} cells and suppress differentiation of Th1 and Th17 cells, and prevents IBD and EAE in mice (Kushwah and Hu, 2011). CD103$^+$ DCs in the intestine lamina propria and mesenteric lymph nodes express high levels of RALDH and have ability to induce Foxp3$^+$ T_{reg} cells. It is well documented IL-6 is an essential cytokine driving Th17 cell differentiation, while down-regulate

Foxp3 expression in T_{reg} cells. Interestingly, Zhou et al. (2010) showed that RA maintains the stability of Foxp3$^+$ T_{reg} cells and sustains their suppressive potential in the presence of IL-6, through down-regulation of IL-6 receptor expression and signaling, therefore preventing T_{reg} cells conversion into Th17 cells (Coombes and Powrie, 2008; Zhou et al., 2010). CD103$^+$ DCs from the lamina propria of the small intestine and mesenteric lymph node have been shown to be significantly better than splenic DCs at mediating the conversion of naïve T cells into Foxp3$^+$ T cells in the presence of exogenous TGF-β (Coombes et al., 2007). Moreover, expression of the $\alpha_v\beta_8$ integrin by DCs is important for TGF-β activation, accumulation of T_{reg} cells in the small intestine and prevention of colitis development (Coombes and Powrie, 2008). Overall, gut-derived signals may condition DCs to produce TGF-β for T_{reg} cell generation.

β *Catenin: a novel regulator of intestinal homeostasis*

Wnt-β-catenin signaling in intestinal DCs regulates the balance between inflammatory and regulatory responses. Recently, Manicassamy et al. (2009) performed a gene expression profile of lamina propria DCs and demonstrated that several Wnt family genes and β-catenin were constitutively expressed by intestinal DCs, but not by splenic DCs (Manicassamy et al., 2009). Furthermore, they showed that β-catenin signaling promotes the induction of T_{reg} cells while suppress Th1 and Th17 cells in the gut, indicating that β-catenin expression by intestinal DCs is important for maintaining the balance between T_{reg} and T_{eff} cells in the gut (Manicassamy et al., 2010). In addition, intestinal DCs lacking β-catenin expression produced lower levels of T_{reg} cell-promoting stimuli including RA metabolizing enzymes, IL-10, and TGF-β, but higher levels of Th17-promoting cytokines IL-23 and IL-6 (Manicassamy et al., 2010). Overall, β-catenin signaling is needed to maintain intestinal homeostasis through the induction of T_{reg} cells and the suppression of pro-inflammatory factors.

The gut microenvironment influences intestinal DC function

Many of the unique properties of intestinal DCs appear to be a result of environmental conditioning. Activation of NF-κB expression in intestinal epithelial cells, perhaps as a result of microflora signaling through PRRs, enhances TSLP production (Coombes and Powrie, 2008). Recently, important role for TSLP in dictating the quality of immune response has been suggested. TSLP and other epithelial cells factors limits the activation of STAT4 and IRF-8, essential factors for the production of Th1 polarizing cytokine IL-12-23p40 (Arima et al., 2010). In addition, TSLP signaling also induce the activation of STAT6, which programs DCs to secrete chemokines necessary for the recruitment of Th2 cells, and increase IL-10 and TGF-β production (Coombes and Powrie, 2008; Arima et al., 2010). TSLP has also been shown to confer human thymic DCs with ability to induce the differentiation of CD4$^+$CD25$^-$ thymocytes into Foxp3$^+$CD4$^+$CD25$^+$$T_{reg}$ cells (Watanabe et al., 2005). Thus, the increased ability of gut-residing CD103$^+$ DCs to induce Foxp3 may be due to TSLP.

Overall, these findings illustrate that the intestinal immune system (i.e., GALT) is a preferential site for the induction of T_{reg} cells, and provides a mechanism by which the thymus-derived T_{reg} cell pool could be complemented by *de novo* generated T_{reg} cells for the efficient control of inflammatory responses toward specific commensal bacteria and food antigens (Coombes and Powrie, 2008).

The skin and lung are naturally tolerogenic environments

Like the intestine, the skin and lungs are also constantly exposed to various microbes and environmental antigens such as allergens. The interaction between vitamin D and RANK–RANKL signaling pathway in the skin plays a role in DC-mediated iT_{reg} cells induction (Loser et al., 2006; Kushwah and Hu, 2011). This process occurs *via* the activated metabolite of vitamin D, VD3, which exerts its actions through its nuclear receptor, VDR. VDR is expressed on DCs and vitamin D treatment inhibits DCs maturation and their ability to prime alloreactive T cells responses (Loser et al., 2006). Similarly to the intestine, lung CD103$^+$ DCs promote the induction of T_{reg} cells, while the CD103$^-$ DCs represent the main producers of pro-inflammatory cytokines in response to airborne allergens and TLR ligands. In contrast to lung and mucosal sites, CD103$^-$CD11b$^+$ migratory dermal DCs, compared to CD103$^+$CD11b$^+$ DCs, are much more potent in promoting T_{reg} cells induction (Guilliams et al., 2010; Pulendran et al., 2010).

THERAPEUTIC APPLICATIONS OF TOLEROGENIC DCs

The possibility to generate or expand tolerogenic DCs *in vitro* provides significant opportunities for therapeutic interventions. DCs are generated *in vitro* from bone marrow precursors in rodents or blood monocytes in humans and can be rendered tolerogenic by modulating their culture conditions through exposure to cytokines, growth factors or pharmacologic mediators, or genetic engineering (Morelli and Thomson, 2007). *In vitro* generation of tolerogenic DC can be achieved through exposure to various anti-inflammatory agents such as vitamin D, as well as clinically approved suppressive drugs such as corticosteroids, cyclosporin, and rapamycin (Morelli and Thomson, 2007). Rapamycin acts as an inhibitor of the Akt–mTOR pathway, increases the number of Foxp3$^+$ T_{reg} cells, and promotes their resistance to apoptosis (Strauss et al., 2007; Basu et al., 2008). Moreover, rapamycin-conditioned myeloid DCs fail to produce IL-12p70 and TNF-α and are resistant to maturation induced by TLR ligands or by CD40 signaling (Turnquist et al., 2007). Advances in gene transfer technology offers the possibility to generate tolerogenic DCs by genetically inducing the expression of immunosuppressive molecules like IL-10, TGF-β, or CTLA-4, or blocking the expression of co-stimulatory molecules (Morelli and Thomson, 2007). Conversely, it is possible to generate tolerogenic DCs *in vitro* that drive T_{reg} cells differentiation in a tissue specific manner in order to inhibit inflammation in a particular site (Rescigno, 2010). For example, intestinal epithelial cells release factors that drive the development of mucosal like tolerogenic DCs (Iliev et al., 2007). Incubation of DCs with intestinal, but not mammary or epithelial cell-derived supernatants induces the expression of CD103 on DCs while inhibiting the secretion of inflammatory cytokines. These *in vitro* generated CD103$^+$ DCs can drive the induction of T_{reg} cells and expression of the gut-associated homing receptor $\alpha_4\beta_7$ (Iliev et al., 2009). Moreover, only intestinal epithelial cells conditioned DCs are able to protect against colitis development (Iliev et al., 2009). Therefore, DCs conditioned at local environment are

important for generation of T_{reg} cells that are able to suppress and home to specific sites.

CONCLUSION

Our understanding of the functional and phenotypic plasticity of DCs, as well the capacity to modulate DCs development and maturation *in vitro* and *in vivo* gives opportunity to use these cells for therapeutic purpose in autoimmunity and cancer. Tolerogenic DCs has a dual role, for example in cancer, a profound defect in DCs function are associated with accumulation of immature DCs in tumors where DCs are unable to initiate anti-tumor immune responses while contributes to the recruitment, expansion and function of T_{reg} cells. While this have negative outcome in cancer settings, similar scenario would be helpful in the case of autoimmunity. Several studies in mice suggest that DCs might be used in the treatment of autoimmunity through their ability to induce T_{reg} cells. For example, repetitive injections of semi-mature DCs results in protection from EAE and thyroiditis (Ueno et al., 2007). In the NOD mice, DC-mediated *in vitro* generation of T_{reg} cells can inhibit spontaneous T1D development in these mice (Ueno et al., 2007). In recent years, several mechanisms have been identified to explain how tolerogenic DCs mediate their function. For example, IDO expression by intestinal DCs is important for regulating intestinal immune homeostasis by keeping the balance between T_{reg} cells and Th17, Th1 cells. Deregulations of IDO activity results in increased intestinal inflammation, suggesting that IDO could be regarded as a new target for IBD. Therefore, pharmacological targeting of IDO during the chronic inflammation like IBD would be beneficial in dampening the inflammatory process and tissue damage in the gut (Matteoli et al., 2010). Moreover, as IL-6 is often present in inflammatory infiltrates in autoimmune settings, recent findings about ability of RA to stabilize T_{reg} cells in the presence of IL-6 offers the possibility that RA treated T_{reg} cells can be used in many autoimmune settings where achieve such a balance is essential.

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Regulatory dendritic cells: there is more than just immune activation

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The immune system exists in a delicate equilibrium between inflammatory responses and tolerance. This unique feature allows the immune system to recognize and respond to potential threats in a controlled but normally limited fashion thereby preventing a destructive overreaction against healthy tissues. While the adaptive immune system was the major research focus concerning activation vs. tolerance in the immune system more recent findings suggest that cells of the innate immune system are important players in the decision between effective immunity and induction of tolerance or immune inhibition. Among immune cells of the innate immune system dendritic cells (DCs) have a special function linking innate immune functions with the induction of adaptive immunity. DCs are the primary professional antigen presenting cells (APCs) initiating adaptive immune responses. They belong to the hematopoietic system and arise from $CD34^+$ stem cells in the bone marrow. Particularly in the murine system two major subgroups of DCs, namely myeloid DCs (mDCs) and plasmacytoid DCs (pDCs) can be distinguished. DCs are important mediators of innate and adaptive immunity mostly due to their remarkable capacity to present processed antigens via major histocompatibility complexes (MHC) to T cells and B cells in secondary lymphoid organs. A large body of literature has been accumulated during the last two decades describing which role DCs play during activation of T cell responses but also during the establishment and maintenance of central tolerance (Steinman et al., 2003). While the concept of peripheral tolerance has been clearly established during the last years, the role of different sets of DCs and their particular molecular mechanisms of immune deviation has not yet fully been appreciated. In this review we summarize accumulating evidence about the role of regulatory DCs in situations where the balance between tolerance and immunogenicity has been altered leading to pathologic conditions such as chronic inflammation or malignancies.

Keywords: cancer, chronic infection, chronic inflammation, regulatory dendritic cells, IDO

MAJOR FUNCTIONAL STATES OF DCs DURING IMMUNE ACTIVATION

A major focus of research into DC biology was built on observations during immune activation. DCs are a heterogeneous cell population that can acquire diverse maturation states and functions. Intensive studies of DC development in mice lead to the conception that DCs derive under homeostatic conditions from hematopoietic $CD34^+$ stem cells in the bone marrow and potentially in the intestinal lamina propria (Bogunovic et al., 2009) while monocyte-derived DCs have been described under inflammatory conditions (Cheong et al., 2010). Also, human $CD14^+$ $CD34^+$ PBMCs were described to give rise to DCs by the influence of platelet endothelial cell adhesion molecule-1 (Ferrero et al., 1998). Myeloid precursor cells in the bone marrow give rise to common DC precursor cells with the ability to proliferate and relocate to bone marrow, spleen, and lymph nodes. The major function of DCs has been attributed to the initiation steps of immune activation leading to protection of the individual against invading pathogens and the immune surveillance against transformed cells.

At least two categories of DCs have been described for the mammalian immune system (Banchereau et al., 2000). Myeloid DCs (mDCs) also called conventional DCs have a strong capability to capture antigens which enables them to stimulate T cells. These major antigen presenting and activating cells comprise a very heterogeneous group of cells expressing high levels of MHC class II and integrin CD11c on their cell surface, but also other adhesion molecules, like LFA-1 (CD11a), LFA-3 (CD58), ICAM-1 (CD54), ICAM-2 (CD50), and ICAM-3 (CD102). The costimulatory molecules CD80 and CD86 have been established as hallmarks of DC maturation during an immune response with CD86 being expressed at early stages of maturation, while CD80 (and also CD83) become upregulated in mature DCs. The Langerhans cells of the skin are one major representative subgroup of the mDCs continuum. Murine mDCs are characterized by the expression of CD11b and CD11c and are generated *in vitro* by stimulation of bone marrow progenitor cells while in the human, DCs are often generated from peripheral blood monocytes using GM-CSF and IL-4 (Sallusto and Lanzavecchia, 1994).

A second group of DCs are plasmacytoid DCs (pDCs) that are found in circulation and in peripheral lymphoid organs. In comparison to other APC the capacity of pDCs to present antigens is rather low since immature pDCs express only low levels of MHC-II or other costimulatory molecules. Upon activation they secrete large amounts of IFNα and IFNβ (Cella et al., 1999; Siegal et al., 1999). Infection with RNA- and DNA-viruses induces IFN-related immune responses in pDCs human and mice after the recognition of viral genomes via pattern recognition receptors (PRR) such as toll-like receptors (TLRs) 7 and 9 (Lund et al., 2003; Di Domizio et al., 2009; Swiecki and Colonna, 2010). Characterization via surface receptors revealed that pDCs do not express markers commonly present on human mDCs such as CD11c, but express instead the interleukin 3 receptor (CD123) and exclusively the type II c-type lectin BDCA-2 (CD303) which is involved in the presentation of antigens to T cells (Dzionek et al., 2001). In contrast to human pDCs murine pDCs are characterized by the expression of CD11c, B220, Gr-1, CD45RA, Ly49Q, BST2, and Siglec-H (Gehrie et al., 2011). It is assumed that these cells play a major role in anti-viral immune responses since they produce high amounts of IFNα after viral infection.

A third group named follicular DCs (fDCs) can be found in the germinal centers of lymph nodes presenting antigens to B cells to maintain immune memory. fDCs extracted out of human tonsils have been found to express the surface receptors CD21, CD23 CD35, and cell cycle markers DRC-1, Ki-M4 or DR53 (Kim et al., 1994). Interestingly, in contrast to pDCs and mDCs fDCs share some common antigens such as 3C8 with fibroblasts suggesting that these cells share some molecular programs (Lindhout et al., 1999; Lee and Choe, 2003; Vinuesa et al., 2010).

Immature DCs patrol via the blood systems throughout the body and can invade peripheral tissues to take up antigens from infected or dying cells via macropinocytosis, phagocytosis, and endocytosis (Steinman et al., 1999). Migration of DCs from peripheral tissues to lymph nodes also occurs under steady state conditions in absence of infection and might contribute to tolerance induction. Receptors of the C-type lectin family like DEC205, DCIR or the mannose receptor (CD206) directly capture antigens and direct them to antigen processing antigen processing machinery in the endosomal compartment or the cytosol (Villadangos and Schnorrer, 2007). The expression of PRR including TLRs, NOD-like receptors and RIG-like helicases by DCs enables these immune cells to recognize bacterial (e.g., LPS) or viral (e.g., single-stranded RNA) compounds, so called pathogen associated molecular patterns (PAMPs) (Janeway and Medzhitov, 2002). More recently, it was shown that DCs also recognize intracellular host factors released to the extracellular space after cell damage, called damage associated molecular patterns (DAMPs) like HMGB1 or S100A/B proteins. mDCs are found to express TLR1, TRL2, TRL4, TLR5, and TLR8, while pDCs express TLR-7 and TLR-9. After activation of the TLR-signaling cascade via the adaptor molecules MYD88 and TRIF pro-inflammatory transcription factors like NFκB and several interferon regulating factors (IRFs) are activated and lead maturation of DCs and to the expression of immune activating mediators (Hemmi and Akira, 2005). A central contribution to immune activation is the presentation of processed antigens via major histocompatibility complexes (MHC) in presence of costimulatory molecules on the cell surface of DCs to T cells and B cells in secondary lymphoid organs. The transport of peptide-loaded MHC molecules to the cell surface is accompanied by an increased expression of costimulatory molecules like CD80 and CD86. Other typical maturation marker on matured human and mice DCs are elevated levels of HLA-DR, CD40, CD80, CD1a, and CD54 (Reis e Sousa, 2006). The activation of signaling cascades downstream of PRRs also induces DC migration to afferent lymph nodes to present antigens to T cells and B cells (Hemmi and Akira, 2005) which is mediated by the chemokine and homing receptor CCR7 along a gradient of the two chemokines CCL19 Epstein-Barr virus-induced molecule-1 ligand chemokine (ELC), and CCL21 Secondary lymphoid tissue chemokine (SLC) (Sanchez-Sanchez et al., 2004; Riol-Blanco et al., 2005). Mature mDCs secrete high amounts of inflammatory cytokines such as IL-12, necessary for the differentiation of naïve T cells toward certain T helper cell subsets (**Figure 1**). In principle, DCs unite at least four different signals in the immune synapse. One activation signal is established by the presentation of processed antigens via MHC molecules that interact with the T cell receptor complex. The binding of the adhesion molecule ICAM-1 with LFA-1 on the T cell surface strengthens the contact of both immune cells and the so-called signal two is established by the interaction between costimulatory molecules CD80/CD86 expressed on matured DCs with CD28 on T cells. Finally, an interaction between CD40 on DCs and CD40L on T cells is established leading to increased IL-12 production of DCs. The elevated levels of IL-12 finally lead to a T_H1 polarization and secretion of the cytokine IFNγ which is necessary for the recruitment of macrophages and cytotoxic T cells. While there is no doubt about the exceptional role of DCs during immune activation, more and more evidence has been accumulated demonstrating that these specialized cells can also exert regulatory functions.

DEFINING REGULATORY DENDRITIC CELLS

While activation and maturation of dendritic cells (DCs) and their immunostimulatory capacity are clearly linked to a distinct phenotypic change with upregulation of MHC molecules, costimulatory molecules, and the enhanced production of inflammatory cytokines, this is less clear for DCs exerting regulatory functions. Initially, regulatory, inhibitory or even tolerance-inducing capabilities were assigned to immature DCs, a differentiation state prior maturation. It has been a common view that DCs exist in an immature and a mature state. Initial experiments demonstrated that immature DCs can induce tolerance, which was explained by the finding that immature DCs process and present antigens in the absence of costimulation, leading to T cell anergy and deletion (Jonuleit et al., 2000; Lutz et al., 2000; Reis e Sousa, 2006; Manicassamy and Pulendran, 2011). However, during the last years, fully matured DCs with regulatory functions have been observed in numerous distinct settings suggesting that regulatory DCs (DC_{reg}) are a functional state rather than a unique subpopulation defined by phenotypical markers. For example, in a murine colitis model $CD103^+$ DCs acquire immune-activating functions under inflammatory conditions and express pro-inflammatory cytokines (Laffont et al.,

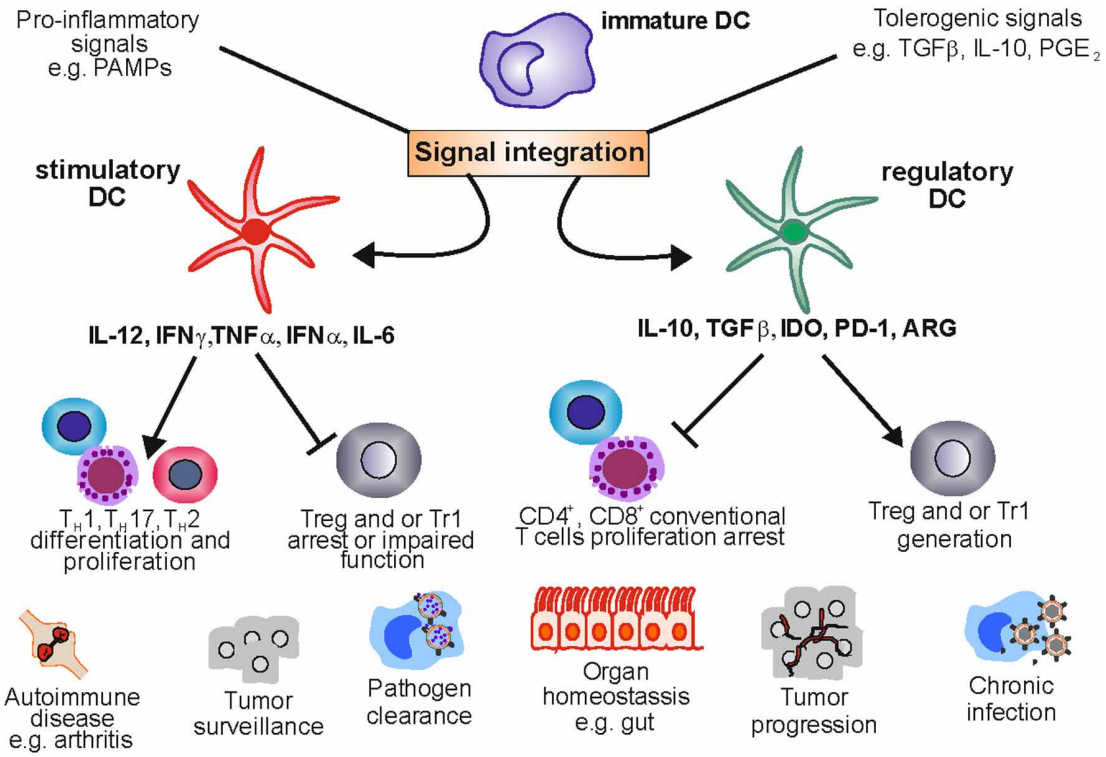

FIGURE 1 | Stimulatory and regulatory dendritic cells in health and disease. DCs are a plastic lineage able to process and integrate signals from the microenvironment. Under pro-inflammatory conditions stimulatory DCs promote an effective immune response by stimulating T cell proliferation and shaping T cell responses toward TH 1, TH 2, or TH17 phenotypes. This crucial role allows the immune system to clear pathogens and keep transformed cells in check. Nevertheless, uncontrolled DC activation can lead to tolerance ablation, fostering the development of autoimmune diseases like rheumatoid arthritis. Under a tolerogenic environment DCs acquire regulatory functions suppressing T cell activation and proliferation and providing signals that enable Treg and Tr1 differentiation and expansion. This function maintains tolerance in organs like the gut which are exposed to a variety of harmless antigens. However, DCreg function can be exploited by tumors and pathogens leading to tumor progression and chronic infection.

2010). However, under steady state conditions $CD103^+$ DCs in the gut where shown to be strong inducers of T cell tolerance, which was manifested by their capacity to induce $Foxp3^+$ T_{reg} from $CD4^+$ naïve precursors (Del Rio et al., 2010; Scott et al., 2011). A similar situation was observed in the liver where low numbers of pDCs are associated with viral persistence during chronic hepatitis C infection while elevated numbers of highly active DCs are associated with pathogen clearance (Lai et al., 2007). At the same time resident pDCs in the liver promote immune regulation through various mechanisms (Matta et al., 2012). Yet another example is the coexistence of DCs with stimulatory and regulatory functions in the tumor microenvironment. Depending on the expression of immunomodulatory factors and cytokines in the tumor, DC_{reg} together with other immunoregulatory cells can be recruited to the tumor environment (Shurin et al., 2011; Gabrilovich et al., 2012). Further evidence for the existence of fully matured DC_{reg} came from a murine asthma model, demonstrating that fully matured DCs expressing high levels of costimulatory molecules stimulated T_{reg} development via an IL-10 depending mechanism (Akbari et al., 2001). In humans, monocyte-derived DC stimulated with prostaglandin E2 (PGE_2) and TNFα exhibit a fully mature phenotype characterized by high expression of costimulatory molecules and pro-inflammatory cytokines, yet they suppress T cell activation via a combination of factors like indoleamine 2,3 deoxygenase (IDO) and IL-10 (Popov et al., 2006, 2008; Von Bergwelt-Baildon et al., 2006). Furthermore, it was shown that DCs with intermediate features between the immature and mature state expressing costimulatory molecules but only low levels of inflammatory cytokines, such as IL-12, IL-6, and TNFα, are also characterized by regulatory function (Lutz and Schuler, 2002). Moreover, the potential therapeutical application of semi-mature DCs as tolerance promoters have been recently reviewed (Lutz, 2012).

In summary, there seems to be significant heterogeneity of DC populations with regulatory function, which might be due to the plasticity of DCs capable of reacting to and integrating environmental signals from different microenvironments. Nevertheless they share the ability (1) to regulate or inhibit T cell activation, and (2) to induce and promote T_{reg} development and expansion (**Figure 1**).

ANTI-INFLAMMATORY MEDIATORS CAN DRIVE DCs TOWARD REGULATORY FUNCTION

Since regulatory function of DCs is linked to environmental cues several soluble factors such as TGFβ, IL-10, or PGE_2 known to play a role in immune inhibition have been linked to the

induction of regulatory DCs (Popov and Schultze, 2008). Even under steady state conditions, these factors play an important role for the integrity of many organ systems, particularly those with close contact to the outside world such as lung or intestine. For example the intestine is in constant contact with a large variety of antigens and it is of vital importance to discriminate between harmless nutrients, commensal flora, and potential threats (Iweala and Nagler, 2006). TGFβ is produced by intestinal epithelial cells thereby fostering the generation of DC_{reg} and subsequently T_{reg} cells. Neutralization of TGFβ directly leads to a diminished DC_{reg} capacity to induce T_{reg} cells (Iliev et al., 2009). Along the same lines, Belladonna and coworkers demonstrated that $CD8^+$ pDC rely on autocrine TGFβ stimulation but also IDO to keep tolerance under steady state conditions. Moreover, they showed that $CD8^-$ immunogenic DCs do not produce TGFβ and yet externally added TGFβ induces IDO changing immunogenic DC into DC_{reg} (Belladonna et al., 2008). TGFβ also plays an important role for immunosuppression in the brain, which at least in part is also due to regulatory functions of DCs in this compartment. In a recent report, it could be demonstrated that blockade of TGFβ receptor signaling in DCs caused severe autoimmune encephalitis indicating an important role of this factor in DCs to maintain tolerance (Laouar et al., 2008). Another well-established factor inducing immunoregulatory functions in DCs is interleukin-10 (IL-10). DCs exposed to IL-10 fail to induce immunostimulatory cytokines such as IL-12 (De Smedt et al., 1997) or TNFα. Moreover, IL-10 prevents the upregulation of immunostimulatory molecules such as MHC class II and CD86. The quintessence is an impaired ability of IL-10 primed DCs to induce allogeneic T cell responses (Jonuleit et al., 2000; Moore et al., 2001; Pletinckx et al., 2011). Since the functional outcome of DCs depends on the exogenous signals integrated by these cells, it is not surprising that some factors can have both immunostimulatory as well as regulatory functions, depending on signal strength, time of exposure, and combination with other factors. The effect of PGE_2 exemplifies such complex interaction. During acute inflammatory immune responses PGE_2 is widely expressed by epithelial cells, fibroblasts, and immune cells infiltrating the inflamed site (Kalinski, 2012). In such situations PGE_2 can serve as an enhancer of the immunostimulatory response. PGE_2 also increases CCR7 expression and is essential to promote DC migration toward the lymph node-derived chemokines CCL19 and CCL21 (Scandella et al., 2002; Legler et al., 2006). However, more recently it has been reported that the PGE_2 effect on CCR7 expression is only transient and that these PGE_2-treated DCs secret reduced levels of CCL19, the key chemokine attracting naïve and central memory T cells (Muthuswamy et al., 2010). During extended inflammatory responses and in chronic inflammation, PGE_2 might deviate DCs from a stimulatory into a regulatory phenotype. Under such conditions, PGE_2 can induce regulatory mediators such as IL-10 (Kalinski et al., 1997) or thrombospondin-1 (Doyen et al., 2003). We have previously shown that TNFα signaling in presence of PGE_2 induces regulatory DCs expressing a myriad of inhibitory molecules such as IDO, IL-10, soluble CD25 or COX-2 further increasing PGE_2 production (Von Bergwelt-Baildon et al., 2006; Driesen et al., 2008; Popov et al., 2008). Finally exposure of DCs to PGE_2 leads to increased IL-12p40 secretion which is not accompanied by production of IL-12p35 leading to an overall diminished production of the bioactive IL-12 heterodimer (Kalinski et al., 2001; Von Bergwelt-Baildon et al., 2006).

As exemplified for TGFβ, IL10 or PGE_2, DCs can integrate signals from their microenvironment in a fashion that induces regulatory rather than immunostimulatory activity by these cells. Under steady state conditions such signal integration is critical for organ homeostasis. Any changes in the balance between regulatory and immunostimulatory signals can lead to tissue pathology. While decreased regulatory capacity of DCs can be associated with enhanced inflammatory responses associated with tissue destruction, increases in DC_{reg} are linked to chronic inflammation and malignant diseases.

REGULATORY DCs IN CANCER

Although there is clear evidence that the immune system can eliminate malignant cells the generation of a clinically efficient immune response against cancer is a challenging task (Schreiber et al., 2011). Over the last 15 years, therapies based on the immunostimulatory capacities of DCs have been a major focus of tumor immunotherapy, yet, most clinical studies have not resulted in meaningful clinical responses (Palucka and Banchereau, 2012). A major hurdle for DC-based tumor immunotherapy is to overcome regulatory circuits within the tumor microenvironment. In addition to many other cell types with regulatory or suppressive function such as myeloid derived suppressor cells (Palucka and Banchereau, 2012), T_{reg} cells or deviated macrophages, DCs also seem to be altered in various ways in malignancies (**Table 1**). In some cancer types DCs are depleted from the tumor site itself but also from the circulation suggesting that these malignancies induce significant changes in DC generation (Almand et al., 2000; Gabrilovich, 2004; Satthaporn et al., 2004; Tjomsland et al., 2010). In other cancer types, DC maturation was shown to be impaired and this feature was associated with lack of T cell activation and the induction of T cell anergy thereby inducing tolerance against the tumor (Ma et al., 2012; Shurin et al., 2012). In such situations, DC maturation might not simply be blocked, but more likely DCs are deviated toward a regulatory function by integrating signals from the tumor microenvironment (Gabrilovich et al., 2012). Such tumor-associated DC_{reg} are not only able to suppress effector T cells but also induce the recruitment and expansion of T_{reg} cells.

FUNCTIONAL IMPAIRMENT OF DCs LEADS TO DEFECTIVE T CELL PROLIFERATION AND TOLERANCE

At the same time when numerous clinical trials were already conducted to test the efficacy of DCs as cellular cancer vaccines it was revealed in human tumor biopsies as well as murine and rat tumor models that DCs in many malignancies present an impaired function, manifested in poor antigen processing and presentation, impaired migration and low presence of costimulatory molecules (Gabrilovich, 2004; Yang and Carbone, 2004). Early studies revealed that tumor infiltrating DCs present poor capabilities to induce T cell proliferation in an allogeneic mixed lymphocyte reaction *in vitro* (Troy et al., 1998). Consistent with

Table 1 | Regulatory dendritic cells in immune diseases.

Disease	DC phenotype	Surface marker	Secreted immune modulators	Literature	Function
Cancer	Impaired maturation/immature	Downregulation of MHCII, CD80, CD83, CD86	Missing IL-12	Aalamian et al., 2001; Gabrilovich, 2004; Yang and Carbone, 2004; Bharadwaj et al., 2007; Michielsen et al., 2011; Ma et al., 2012; Shurin et al., 2012	Induction of: T cell anergy; T cell apoptosis
	Regulatory	CD25, PD-1, B7-H1	IL-10, TGFβ, Kynurerine, sCD25 IDO, COX-2, ARG1	Toossi et al., 1990; Williams et al., 1999; Benoit et al., 2004; Rodriguez et al., 2004; Ghiringhelli et al., 2005; Chemnitz et al., 2006; Von Bergwelt-Baildon et al., 2006; Chung et al., 2009; Dumitriu et al., 2009; Krempski et al., 2011; Gabrilovich et al., 2012; Scarlett et al., 2012	
CHRONIC INFLAMMATION					
Systemic lupus erythematosus	pDCs	HLA-DR, CD4 (CD11c reduced)	IFNα, IL-10	Blanco et al., 2001; Lee et al., 2008; Yan et al., 2008; Jin et al., 2010	Suppression of: T cell activation; T cell proliferation; T cell function
Rheumatoide arthritis	Regulatory	CD11b, CD11c, CD18	TGFβ, BAFF, IDO	Morelli et al., 2003; Zhang et al., 2005; Kavousanaki et al., 2010	
Obesity	FFA-dependent/Regulatory (USFAs)	IL10R; Downregulation of MHCII, CD80, CD83, CD86	IL-10 (missing IL-12 secretion)	Aliberti et al., 2002; Loscher et al., 2005; Miyake et al., 2010; Draper et al., 2011	
CHRONIC INFECTION					
Viral infection	Impaired maturation/function (Virus-dependent)	Downregulation of CD1a, CD1b, DC-SIGN, CD80, CD83, CD86	IFNα, IL-10, IL-1β (missing IL-12, IL-6, TNFα secretion)	Kruse et al., 2000; Sarobe et al., 2003; Smed-Sorensen et al., 2004; Martinson et al., 2007; Tilton et al., 2008; Harman et al., 2011; Chentoufi et al., 2012; Dental et al., 2012; Tu et al., 2012	
Parasitical infection	Regulatory	CD11c, CD25	TNFα, IFNγ, IL-10, TGFβ, COX2, IDO, S100	Von Bergwelt-Baildon et al., 2006; Poncini et al., 2008; Popov et al., 2008; Li et al., 2011	

these findings DCs differentiated from monocytes in presence of conditioned media derived from prostate or pancreatic cancer cell lines, showed low levels of HLA-DR, costimulatory molecules CD40, CD80, CD86, and the DC maturation marker CD83 (Aalamian et al., 2001; Bharadwaj et al., 2007). Moreover, DCs cultured in presence of human colorectal cancer explants failed to upregulate CD86 and CD80 expression in response to LPS (Michielsen et al., 2011). Other examples for immune deviation of DCs in cancer came from rat cancer models demonstrating the presence of DCs expressing low levels of costimulatory molecules that were incapable of inducing T cell activation (Chaux et al., 1997; Bonnotte et al., 2004). These and many other reports suggested that the lack of immunostimulatory function of DCs in context of malignant disease was mainly due to lack of the necessary stimulatory molecules (MHC, CD80, and CD86) which might be explained by a strong influence of this field of research by findings in T cell immunology at the same time suggesting that presence or absence of costimulation on APC is mainly

responsible for decision making between T cell immunity or tolerance. However, with the identification of regulatory cells such as T_{reg} cells and the appreciation of the complexity of immunoinhibitory signals within the tumor microenvironment, this rather simple model of lack of immune function by DCs has been gradually dismissed. In fact, it is now well appreciated that DCs in cancer can acquire a spectrum of different functional states ranging from strongly immunostimulatory to regulatory or even suppressive and inhibitory states inducing T cell tolerance or even deletion. Some of the molecular mechanisms responsible for regulatory rather than immunostimulatory functions of DCs in tumor microenvironments are described in the next section.

DCs EXPOSED TO THE TUMOR MICROENVIRONMENT ACQUIRE A REGULATORY FUNCTION

The tumor microenvironment provides many inhibitory signals that can be sensed by DCs leading to a change in their functional state. Soluble factors, such as IL-10, TGFβ, and PGE_2 (Popov and Schultze, 2008) secreted by the tumor have been clearly linked to the induction of DC_{reg} (**Table 1**). Furthermore, Scarllet and coworkers showed in a murine model of ovarian cancer that the switch from immunostimulatory to immunoregulatory function of mDCs at the tumor site determined the onset of aggressive malignant tumors that escaped immune surveillance (Scarlett et al., 2012). This data suggested that the interaction of DCs within the tumor microenvironment might be relevant in the control of disease progression—at least in ovarian cancer.

Among other factors, the enzymes IDO and arginase-1 as well as TGFβ have clearly been linked to the generation and enrichment of DC_{reg} in the tumor microenvironment. IDO catalyzes the first rate-limiting step in tryptophan (Trp) degradation (Yamamoto and Hayaishi, 1967). Its activity has a dual effect leading to Trp depletion and accumulation of Trp catabolites, collectively known as kynurenines (Sugimoto et al., 2006). IDO expression has been detected in biopsies of patients with different malignancies such as esophageal (Von Bergwelt-Baildon et al., 2006), squamous cell carcinoma (McGee-Lawrence et al., 2011), non-small cell lung carcinoma (Sim et al., 2012), and melanoma amongst others (Munn and Mellor, 2007). IDO activity was linked to multiple mechanisms in immune tolerance *in vitro* as well as *in vivo*. Trp starvation and accumulation of Trp catabolites lead to T cell proliferation arrest and apoptosis (Terness et al., 2002; Von Bergwelt-Baildon et al., 2006). Furthermore, DCs can induce the expansion of autologous T_{reg} via an IDO-dependent mechanism (Chung et al., 2009). More recently, it has been reported in mice that the Trp catabolite kynurenine can bind and activate the aryl hydrocarbon receptor (AHR) on T cells leading to AHR-dependent T_{reg} generation (Mezrich et al., 2010). The induction of active allograft-specific tolerance by AHR activation has been described earlier in Balb/c mice (Hauben et al., 2008). In this study DC out of VAG539-tolerized mice induced the development of $CD25^+Foxp3^+$ T cells.

Our group has shown that mDCs cultured in the presence of TNFα and PGE_2, two factors with high abundance in the microenvironment of many tumors (Williams et al., 1999; Benoit et al., 2004; Chemnitz et al., 2006), upregulated IDO expression concomitant with an increased expression of IL-10, COX-2, and the interleukin 2 receptor alpha chain (CD25) (Popov et al., 2006, 2008; Von Bergwelt-Baildon et al., 2006; Driesen et al., 2008). Soluble CD25 has been associated with poor prognosis in solid and hematological malignancies (Paietta et al., 1997) All these immunoregulatory mechanisms together equip DC_{reg} with powerful mechanisms to restrain T cell responses. IDO^+DC_{reg} can mediate apoptosis of effector T cells and at the same time promote T_{reg} cell expansion thereby shifting the balance further toward immune inhibition or suppression (Fallarino et al., 2002). Furthermore, we demonstrated that cell surface expression of CD25 and secretion of soluble CD25 act as decoy receptors for IL-2 (Toossi et al., 1990; Von Bergwelt-Baildon et al., 2006) which further inhibits T cell function. Since, IDO^+CD25^+ DC_{reg} are present in the tumor microenvironment one could postulate that these regulatory cells—similar to other myeloid cells with inhibitory functions are part of the immune deviation from activation to inhibition in the tumor microenvironment. In this context, a rather alerting observation was made during a clinical vaccine trial. Patients treated with mDC based cancer vaccines showed a recruitment of IDO^+ immune cells together with T_{reg} cells to the site of injection suggesting that under some circumstances such cellular therapies might actually enhance regulatory functions of DCs rather than potentiating immune activation (Wobser et al., 2007).

In addition to IDO^+ mDCs, the accumulation of pDCs in tumors and tumor lymph nodes is also well documented for different malignancies including melanoma (Vermi et al., 2003) or head and neck cancer (Hartmann et al., 2003). Moreover, IDO^+ pDCs present in murine and human tumor-draining lymph nodes, were reported to induce T cell anergy toward specific tumor antigens. Importantly, the interaction of CD80/CD86 receptors on IDO^+ pDCs with CTLA-4 on T_{reg} cells was an important molecular interaction leading to T_{reg} cell expansion and subsequently antigen-specific anergy in effector T cells (Baban et al., 2005). More recently, Watkins and coworkers reported the presence of IDO^+ pDCs in human and murine prostate cancer. These IDO^+ DCs suppressed T cell proliferation and induced T cell tolerance. By gene expression profiling the authors defined a regulatory gene signature of these IDO^+ pDCs, which was controlled by the transcription factor FOXO3 (Watkins et al., 2011).

Another enzyme that has been implicated as an immunoregulatory molecule in myeloid cells including DCs in the tumor context is arginase-1 (ARG1). ARG1 catalyzes arginine conversion into urea and L-ornithine, causing the depletion of the non-essential amino acid arginine (Munder, 2009). High ARG1 activity has been described in patients with various malignancies including gastric, colon, breast, and lung cancers (Rodriguez et al., 2004). In murine models it was reported that mDC upregulate ARG1 when exposed to different cancer cell lines. Moreover, $ARG1^+$ mDC suppressed T cell proliferation via arginine depletion (Liu et al., 2009; Norian et al., 2009). In T cells L-arginine deprivation leads to an arrest of the cell cycle during G0-G1 transition (Rodriguez et al., 2007) and to reduced expression of the TCR ζ-chain (Rodriguez et al., 2004). These findings suggest that upregulation of ARG1 is yet another mechanism of DC_{reg} that

impacts on the outcome of T cell activation, at least in the murine model. However, the relevance of these findings in human tumors has not yet been clarified.

A major immunosuppressive factor within the tumor microenvironment is TGFβ (Li and Flavell, 2008). It has been well documented that mDCs upregulate TGFβ expression and secrete TGFβ once exposed to tumor cell lines, e.g., non-small cell lung carcinoma cell lines (Dumitriu et al., 2009). Moreover, interaction of T cells with TGFβ producing mDCs leads to the induction of $CD4^+CD25^+Foxp3^{high}$ T cells (Dumitriu et al., 2009). Consistent with these findings Ghiringhelli and coworkers showed in mouse and rat colon cancer models that DCs exposed to tumor cells could acquire the capacity to secrete TGFβ and to stimulate naturally occurring T_{reg} cells *in vivo* (Ghiringhelli et al., 2005). Therefore, expression of TGFβ is yet another mechanisms by which DCs exert regulatory rather than immunostimulatory function in the tumor microenvironment. More recently, the expression of inhibitory cell surface molecules PD-1 and B7-H1 on a subset of DCs in a murine model of ovarian cancer was linked to suppression of T cell proliferation and this effect could be abolished by blocking anti-PD-1 antibodies (Krempski et al., 2011). These data suggest that PD-1 and B7-H1 expression on DC can also contribute to immunoregulation or suppression by DCs in the tumor microenvironment. Altogether, there is accumulating evidence that the absence of T cell immunity in cancer is not solely due to the lack of expression of MHC and costimulatory molecules on DCs. Rather the existence of multiple inhibitory pathways and effector molecules in tumor-associated DCs are major mechanisms of immune deviation in cancer and DCs showing such hallmarks are part of a larger family of regulatory DCs. Further studies are necessary to delineate whether a hierarchy of regulatory pathways exists or whether these mechanisms are co-existing. This will be particularly important when developing novel strategies to overcome immunoregulation in the tumor microenvironment as a basis for the development of effective cancer immunotherapy.

DENDRITIC CELLS IN CHRONIC INFLAMMATION

Chronic inflammation results from prolonged inflammatory immune responses that are not terminated despite the fact that the causing stimuli are often eliminated. Stimuli inducing chronic inflammatory responses vary widely ranging from numerous pathogens, self-antigens to chemical compounds. Chronic inflammatory responses are associated with diseases such as diabetes, atherosclerosis, or obesity. Another deviation from a normal immune response occurs in many autoimmune diseases and chronic exposure to allergens. Allergic responses can turn into prolonged inflammatory responses with significant changes of the inflammatory response during later time points which is similarly observed in many autoimmune diseases. In such situations, resident immune cells become continuously activated and recruit further immune cells which invade the inflamed tissue further fueling the inflammatory response. Albeit pro-inflammatory cytokines, NO, arachidonic acid metabolites and ROS are major compounds under such conditions, mechanisms exist that change the cellular programs of immune cells from immunostimulatory programs resolving inflammation to chronic inflammatory programs supporting chronification of the response. Again, myeloid cells including both macrophages and DCs play an essential role in deregulating inflammatory responses into chronic inflammation.

REGULATORY MECHANISMS IN DCs SEEM TO BE DEFECTIVE IN SYSTEMIC LUPUS ERYTHEMATOSUS

While regulatory or suppressive myeloid cells seem to play a central role for immune deviation in cancer, regulatory functions of these cells seem to be reduced or even lacking in autoimmune diseases such as systemic lupus erythematodes (SLE), rheumatoid arthritis, or psoriasis (**Table 1**). SLE is an autoimmune disease characterized by the loss of tolerance against self-antigens. Furthermore, an imbalance of T_H1 and T_H2 cells in favor of T_H2 responses has been reported to be accommodated by decreased expression of IL-18 with unchanged expression levels of IL-10 in pDCs of SLE patients (Jin et al., 2010). Despite this dysbalance in pDCs that might favor immunoregulation when using pDCs of SLE patients in allogeneic MLR assays they failed to induce the expansion of Foxp3 expressing $CD4^+CD25^+$ T_{reg} cells (Jin et al., 2010). These data follow along the lines that activated DCs in this autoimmune disease might be incapable of suppressing effector T cell activation thereby contributing to the persistence of inflammation. Despite the lack of DCs to induce T_{reg} cells, $Foxp3^+CD4^+CD25^+$ T cells have been reported to be elevated in peripheral blood of SLE patients, however their suppressive function seemed to be impaired which was related to high levels of IFNα secreted by antigen presenting cells (APCs) of SLE patients (Yan et al., 2008). In SLE patients pDCs seem to be a main source for IFNα. It is assumed that immune complexes containing DNA or RNA act as inflammatory stimuli maintaining the IFNα production in pDCs via TLR7 and TLR9 (Means et al., 2005; Savarese et al., 2006). However, other immune cells might also contribute to the interferon storm. For example, monocytes cultured in the presence of IFNα-rich sera from SLE patients differentiated into IFNα producing DCs (Blanco et al., 2001; Ueno et al., 2007) suggesting that a feed-forward loop exists that supports chronification of the autoimmune response in SLE. In a murine model of SLE chronification of the immune response inducing IFNα production in immature $Ly6C^{high}$ monocytes can also be initiated by treatment with tetramehtylpentadecane (Lee et al., 2008) further supporting the hypothesis that the lack of induction of DCs with regulatory function is a major mechanisms of prolongation of exacerbated autoimmune responses in SLE patients. As a logical consequence novel therapeutic concepts for SLE are targeting at the point of induction of regulatory DCs. For example, Zhang and coworkers recently described that treatment with immune complexes lead to elevated levels of PGE_2 secretion by FcγRIIb-overexpressing DCs which correlated with an elevated capacity to suppress T cell activation (Zhang et al., 2011). Clinically, injection of immune complex-treated FcγRIIb-overexpressing DCs into lupus-prone MRL/lpr mice was associated with prolonged survival of these mice. In another mouse model the nucleosomal histone peptide epitope H4(71-94) was used in subnanomolar doses to induce DC_{reg} which expressed TGFβ but showed

reduced levels of IL-6 supporting the generation of T_{reg} cells and suppression of pro-inflammatory T_H17 cells (Kang et al., 2007). One other therapeutical approach targeted the central pro-inflammatory transcription factor NFκB. Inhibition of this transcription factor in a murine SLE model in FcγRIIb-deficient mice with andrographolide or the anti-diabetic drug rosiglitazone lead to induction of DC_{reg} (Kalergis et al., 2009). The diureticum triamterene is another compound which might be useful for the therapy of SLE because it is able to induce IDO activity in mDCs and such IDO^+ DCs have been described to induce fully functional Foxp3 positive $CD4^+CD25^+$ T cells (Ghosh and Branch, 1975; Chung et al., 2009).

LACK OF REGULATORY DC FUNCTION IS ASSOCIATED WITH JOINT INFLAMMATION IN RHEUMATOID ARTHRITIS AND PSORIASIS

As revealed in murine model systems, DCs play an essential role in keeping immune homeostasis in the joint. Under these conditions, joint derived self-antigens from apoptotic cells can be recognized by DCs via the heterodimeric receptors CR3 (CD11c/CD18) and CR4 (CD11b/CD18) belonging to the β2-integrin family of adhesion molecules (Morelli et al., 2003). Activation of DCs by these receptors induces a regulatory DC phenotype associated with suppressed IL-12 production and elevated secretion of TGFβ. Moreover, it was shown that such DCs are capable of inducing T cell anergy (Morelli et al., 2003).

Rheumatoid arthritis (RA) is a chronic autoimmune disease associated with production of elevated levels of pro-inflammatory cytokines, including IL-12, IL-6, IL-1, IL-23, and TNFα, that are involved in activation of naïve T cells and directing their polarization into T_H1 and T_H17 cells. DCs are implicated as major players for the development and persistence of inflammation but also the resulting joint and bone destruction (**Table 1**). Albeit the number of $CD303^+$ pDCs and $CD1c^+$ mDCs is significantly reduced (Kavousanaki et al., 2010) in patients with active RA, DCs invading synovial fluids are quickly activated in response to joint-associated factors such as cartilage glycoprotein 39 but also pro-inflammatory stimuli including cytokines and chemokines and other proteins derived from dying cells within synovial fluids (Van Bilsen et al., 2004). DCs in RA patients are also involved in elevated B cell activation followed by proliferation and antibody production. As shown in a murine collagen-induced arthritis model B cell activation seems to be mainly related to the secretion of the cytokine BAFF (B-cell activating factor) which belongs to the TNF family (Zhang et al., 2005). A switch from regulatory to inflammatory functions of DCs together with an overall reduced number of DC_{reg} in RA patients suggests that a reprograming of DCs back to a regulatory phenotype might be an appealing therapeutic strategy for these patients. In fact, higher numbers of pDCs expressing IDO were found in peripheral blood of RA patients following current therapy regimens. These DC_{reg} were reported to convert naïve T cells into IL-10 secreting T_{reg} cells (Kavousanaki et al., 2010). Therefore, a more specific therapeutic attempt in comparison to currently used global immune suppressive drugs, like methotrexate and infliximab, might be to revert back highly activated DCs showing a pro-inflammatory profile into DC_{reg} capable of suppressing autoreactive T cells. Indeed, several early *ex vivo* vaccination trials, like for example the phase I clinical trial Rheumavax at the University of Queensland (Australia) or AutoDECRA at Newcastle University (UK) aim to reestablish T cell tolerance by induction of T_{reg} cells via modification of DC function. Another approach might be to disrupt costimulatory interactions between DCs and T cells. Cytotoxic T lymphocyte antigen-4 (CTLA4) fused to IgG1 interrupts the costimulatory B7/CD28 interaction between T cells and DCs preventing the activation and proliferation of T effector cells (Dall'Era and Davis, 2004). Furthermore, the binding of CTLA4 or its IgG1 fusion molecule was found to induce IDO expression in DC after binding to the B7 surface receptor (Grohmann et al., 2002). Adoptive transfer of CTLA4-Ig treated $CD11c^+$ DCs into collagen-induced arthritic mice induced the expansion of $CD4^+CD25^+Foxp3^+$ T_{reg} cells and prevented the onset of arthritis efficiently (Ko et al., 2010). Another approach to target costimulation was recently achieved by siRNA (short interfering RNAs) technology. DCs loaded with the arthritogenic Ag collagen II and treated with siRNAs targeting the costimulatory molecules CD40, CD80, and CD86 were used in an arthritis mouse model. Treatment with these re-programmed DCs resulted in downregulation of IL-2, IFNγ, TNFα, and IL-17, which in turn resulted in elevated levels of $Foxp3^+$ T_{reg} cells (Zheng et al., 2010). Another approach to induce DC_{reg} was recently published demonstrating that dual lentiviral transfer of a constitutive MEK-1 mutant with a fusion protein containing invariant chain of MHC and ovalbumin (IiOVA) leads to activation of ERK signaling thereby inducing TGFβ production by DCs (Arce et al., 2011). As a consequence ERK-activated DCs induced the expansion of $Foxp3^+$ T_{reg} cells which prevented joint destruction.

Another example for the lack of DC regulatory function in autoimmunity is psoriasis, one of the most common inflammatory diseases, which is characterized by dermatosis with hyperproliferation of keratinocytes. In this autoimmune disease autoreactive, constitutively activated T cells secrete cytokines which support the proliferation of dermal cells. Genetic, environmental and immunological factors define the severity of psoriasis, but the cause of the disease is still unknown. Psoriasis is also characterized by a reduced number of circulating pDCs while at the same time increased numbers of pDCs are recruited to psoriatic lesions and healthy skin (Skrzeczynska-Moncznik et al., 2009). IFNα itself, but also other soluble mediators like chemerin have been suggested to be responsible for this relocation of pDCs in psoriasis (Skrzeczynska-Moncznik et al., 2009). In the early phase of psoriasis pDCs themselves secrete IFNα subsequently inducing the activation and expansion of T cells (Nestle et al., 2005). Elevated expression of IFNα by DCs in psoriasis might be related to the identification of high level expression of the endogenous anti-microbial peptide LL37 in psoriatic lesions. LL37 can bind to self-DNA resulting in complex structures that activate pDCs via the pattern recognition receptor TLR9 thereby inducing IFNα secretion (Lande et al., 2007). If IFNα is essential for the pathogenesis of psoriasis, its blockade should reduce psoriatic symptoms. Indeed, using blocking anti-IFNα antibodies prevented the development of psoriasis in a xenograft model in mice (Nestle et al., 2005). In addition to IFNα blockade other attempts to reestablish DC_{reg} in psoriasis

have been suggested. An interesting strategy to induce DC_{reg} was recently reported by stimulating DCs with the neuropeptide α-melanocyte-stimulating hormone (α-MSH). α-MSH stimulated DCs downregulated the costimulatory molecules CD80 and CD86, upregulated the two coinhibitory molecules PD-L1 and PD-L2, as well as CD205 and secreted high amounts of IL-10. These DCreg subsequently induced $CD4^+CD25^+Foxp3^+$ T_{reg}. Mutation experiments of the MC-1R gene showed that the induction of DC_{reg} was mediated by the binding of α-MSH to the MC-1R and that this effect could ameliorate psoriasis *in vitro* as well as *in vivo* (Auriemma et al., 2012). Another approach to induce DC_{reg} was based on vitamin D. An activated form of vitamin D, namely 1,25-dihydroxyvitamin D3 has been described to inhibit the differentiation and maturation of DCs by its influence on RNA Polymerase II-mediated transcription of response genes (Penna and Adorini, 2000). Downregulation of activation-associated molecules such as CD1a was associated with diminished IL-12 secretion, and upregulation of IL-10 reprogramed DCs toward regulatory functions thereby gaining the capacity to induce T_{reg} cells (Piemonti et al., 2000).

MYELOID CELLS ARE INVOLVED IN CHRONIC INFLAMMATION IN OBESITY

The observation that increased adipose tissue in obesity is associated with the influx of immune cells, mainly myeloid cells has been made less than 10 years ago (Wellen and Hotamisligil, 2003; Chawla et al., 2011). Obesity is associated with Type 2 Diabetes (T2D), insulin resistance, arteriosclerosis, other cardiovascular diseases and even increased cancer risk (Lumeng and Saltiel, 2011). Elevated levels of free fatty acids (FFAs) and pro-inflammatory cytokines are hallmarks of the systemic low-grade inflammation persisting in obese individuals (Roytblat et al., 2000; Hansen et al., 2010). In mouse models massive infiltration of immune cells into adipose tissue has been described suggesting to promote high-fat died induced adipose tissue inflammation (Duffaut et al., 2009; Strissel et al., 2010). While macrophages clearly play a major role during this chronic inflammation the role of DCs in the inflammatory cross-talk of adipocytes and immune cells is not yet clear (**Table 1**). The impact of dietary fatty acids on the presence of DCs in adipose tissue adjacent to and remote from lymph nodes has been investigated under conditions of chronic mild inflammation induced by low doses of LPS in rats (Mattacks et al., 2004). Chow containing fish oil which is rich in unsaturated fatty acids (USFAs) is considered to reduce inflammatory effects while diets enriched for oils containing saturated fatty acid (SFAs) were associated with an increase of DCs in perinodal adipose tissue (Mattacks et al., 2004). So far, little is known about the role of elevated levels of FFAs on DC function. However, one could speculate that—similar to macrophages—FFAs will shape the functional program of DCs. Using fluorescently labeled palmitic acid, it was recently demonstrated that DCs can uptake fatty acids (Herber et al., 2010). Interestingly, little is known about the accumulation and storage of FFAs in DCs and what role lipid droplets might play in DCs. Increased levels of fatty acids have an impact on antigen processing and presentation. Splenic DCs derived from mice with non-alcoholic fat liver disease (NAFLD), an obesity associated disease, were found to show an impaired processing and presentation of HBsAg in the presence of the saturated fatty acid palmitic acid, but not the unsaturated fatty acid oleic acid (Miyake et al., 2010). Similarly, human blood-derived DCs challenged with antigen demonstrated reduced antigen presentation *in vitro*, lower induction of T cell proliferation and also an induction of the two pro-inflammatory cytokines IL-1β and TNFα in the presence of palmitic acid but not oleic acid (Miyake et al., 2010). Due to the complexity of FFAs concerning saturation, length and isomer structure, it is very likely that mixture of FFAs results in similarly complex immune responses due to differential signal integration. For most FFAs the impact on DC function is not yet understood. One possible receptor for fatty acids is the scavenger receptor CD36 on DCs and various other cells (Silverstein and Febbraio, 2009). But also PRR are involved in the detection of some FFAs. For example, the satured fatty acid lauric acid is recognized via TLR4, one of the major receptors for PPRs on DCs. Recognition of lauric acid via TLR4 was found to upregulate costimulatory molecules CD40, CD80, CD86, MHCII, and to enhance secretion of IL-12 subsequently triggering T cell activation (Weatherill et al., 2005). The opposite effect was shown for DCs stimulated with the omega-3 unsaturated fatty acid docosahexaenoic acid. This fatty acid inhibited LPS-induced upregulation of CD40, CD80, and CD86 as well as MHC class II molecules and prevented T cell activation. Due to these immunoinhibitory effects of unsaturated fatty acids, they might be useful as therapeutics in chronic inflammatory diseases, including obesity, cardiovascular disease, Bowel disease, Crohn's disease, or chronic obstructive pulmonary disease (Blok et al., 1996; Caughey et al., 1996; Calder, 1997; De Batlle et al., 2011; Bassaganya-Riera et al., 2012; Huang et al., 2012). A cis-9, trans-11 isomer of conjugated linoleic acid is a poly-unsaturated fatty acid naturally occurring in meat, milk, and other nutrients. It has been shown to induce the expression of the anti-inflammatory cytokine IL-10 in murine bone-marrow derived DCs in an ERK dependent fashion (Loscher et al., 2005). As negative feedback loop IL-10 inhibited NFκB leading to suppression of its downstream target IL-12 which might contribute to its immunosuppressive properties on T_H1 cell activation (Loscher et al., 2005). Besides TLR4 also peroxisome proliferation-activator receptor-gamma (PPARγ) can affect the maturation status of DCs (Chinetti et al., 2000; Nencioni et al., 2002). Upregulation of PPARγ was observed in docosahexaenoic acid or eicosapentaenoic acid stimulated bone marrow derived DCs. These cells showed reduced IL-12 secretion, reduced expression of costimulatory molecules, and reduced NFκB activity, but enhanced expression of IL-10R and its ligand IL-10 (Draper et al., 2011). Despite an increased physical interaction and cellular colocalization of NFκB and PPARγ the same study showed that the anti-inflammatory effect of the two tested omega-3 USFAs was not abolished by the inhibition of PPARγ with a chemical inhibitor compound. These results lead to the conclusion, that PPARγ might be an intracellular receptor for unsaturated fatty acids but cannot be singularly made responsible for the inflammatory effects of polyunsaturated fatty acids. Finally, it should be mentioned that resolvins and lipoxins, metabolites of docosahexaenoic acid or eicosapentaenoic acid, have anti-inflammatory

functions and are involved in downregulation of IL-12 secretion in DCs via downregulation of the chemokine receptor 5 (CCR5) (Aliberti et al., 2002).

Beneficial usage of omega-3 unsaturated fatty acids has been shown in clinical trials to improve plasma triglyceride levels and insulin activity in T2D patients (Hendrich, 2010). T2D is often associated with obesity and characterized by elevated plasma concentrations of the pro-inflammatory cytokines TNFα and granulocyte-macrophage colony-stimulating factor driving chronic low grade inflammation systemically (Surendar et al., 2012). These two pro-inflammatory mediators contribute to the activation of circulating $CD85^+CD123^+$ pDCs and $CD85^+$ $CD33^+$ $CD123^{dim}$ mDCs yet the overall number of DCs is reduced in T2D making diabetic patients prone to infections (Seifarth et al., 2008; Blank et al., 2010). Interestingly, DCs seem to be reprogrammed once concomitant health problems such as arteriosclerosis are acquired. In T2D patients with atherosclerotic complications circulating monocytes and DCs show reduced production of pro-inflammatory mediators like TNFα which is associated with impaired T cell activation (Corrales et al., 2007). Taken together, DCs like macrophages are involved in chronic low-grade inflammation observed in obesity. Elevated levels of free fatty acids, a hallmark of obesity, have substantial influences on DCs with saturated fatty acids inducing pro-inflammatory immune mediators. On the other hand, unsaturated fatty acids seem to suppress inflammation and are currently investigated as an attractive therapeutic option in obesity. Therefore, unsaturated fatty acids constitute yet another mechanism capable of inducing regulatory programs in DCs.

REGULATORY DCs ARE INDUCED DURING CHRONIC INFECTIONS

In general, the immune system is equipped to resolve most acute infections by completely removing invading pathogens. However, some pathogens have evolved strategies to escape a resolving immune response and as a consequence induce chronification of the infection. Chronic infections are also characterized by a deviated immune response often resulting in a prevalence of immunoregulatory mechanisms including regulatory cells. In fact, increasing evidence exists suggesting that DC_{reg} also play a role in chronic infections. In some situations such as the development of chronic granulomatous infections, the development of regulatory mechanisms actually might be the last resort of the host to keep the invading pathogens in check. Using a few examples, we illustrate the evidence for DC_{reg} in chronic infections.

DEVIATION OF DC FUNCTION DURING VIRAL INFECTIONS

Under normal conditions, RNA and DNA viruses activate DCs e.g., via the PPRs TLR7 or TLR9 resulting in enhancement of IFNα production by matured DCs. In some viral infections lack of viral clearance is—at least in part—due to lack of an effective DC activation and maturation (**Table 1**). For example although hepatitis C virus (HCV) is recognized by DCs via binding of the HCV envelope 2 protein to surface receptors such as SRBI, DC-SIGN, CD81 or TLR receptors other viral proteins inhibit important steps of DC maturation, prevent IL-12 induction and subsequently reduce their capacity to induce T cell activation (Sarobe et al., 2003). During monocyte differentiation HCV inhibits the expression of CD1a, CD1b, and DC-SIGN, but induces these DCs to secret IL-10 preventing T cell expansion (Tu et al., 2012). Attempts to decipher the molecular mechanism of the immune suppressive pathways activated by HCV revealed that after exposure of pDCs to HCV-infected hepatoma cells they activated IRF7 but not the central pro-inflammatory transcription factor NFκB. These finding might explain, why pDCs in HCV infection secrete IFNα but are not able to produce IL-6 or TNFα (Dental et al., 2012). In mice, the function of DCs was recovered when DCs were pulsed *ex vivo* with the viral protein NS3 and matured in the presence of CpG (Yu et al., 2006). Unlike HCV infection, the capability to secrete IL-12 is not impaired in herpes simplex virus type 1 (HSV1) infected DCs. In an initial report, DCs infected with HSV1 were reported to show many phenotypical features of matured DCs except for a loss of CD83 and reduced T cell stimulating activity (Kruse et al., 2000). A more recent study however demonstrated in a murine model that the HSV1 latency-associated transcript (LAT) is involved in the downregulation of costimulatory molecules and pro-inflammatory cytokines like IL-6, IL-12, and TNFα (Chentoufi et al., 2012). Impaired DC function was also suggested to contribute to the reduced function of HSV-specific $CD8^+$ T cells (Chentoufi et al., 2012). Moreover, LAT can function like endogenous cFLIP inhibiting caspase-8 mediated apoptosis in HSV-infected DCs thereby ensuring spreading and replication of the virus (Kather et al., 2010). Yet other mechanisms of immune deviation operative in DCs have been suggested for human immunodeficiency virus (HIV) infection. In the early phase of primary HIV infection, numbers of $CD11c^+$ mDCs as well as $CD123^+$ pDC are decreased resulting in a lack of anti-viral IFNα expression (Pacanowski et al., 2001). Infected DCs display a rather immature phenotype because they do not really upregulate typical activation markers like MHC class II, CD83, CD80, CD86, or CCR7 (Smed-Sorensen et al., 2004; Martinson et al., 2007). Their functional potential is controversially discussed. Although the number of circulating DCs is declinedin HIV patients, the remaining DCs seem to keep a certain immune activating potential as exemplified by CD86 upregulation after stimulation with CD40L. Also these DC secrete TNFα, IL-1β, and IL-10 but do not secrete IL-12 p70 or IFNα (Smed-Sorensen et al., 2004; Martinson et al., 2007). The induction of IFN regulatory factor 1 (IRF-1) by HIV infection of DCs was shown to induce IFN-stimulated genes without the expression of type I and II interferons thereby ensuring the replication of the HIV in mDCs (Harman et al., 2011). In fact, the loss of IFNα production of HIV infected pDCs was postulated to contribute to progression of the disease (Tilton et al., 2008). Furthermore, HIV infected DCs are resistant to NK cell mediated cell death. Melki and coworkers showed an upregulation of the anti-apoptotic molecules c-FLIP and c-IAP2 in these DCs rendering them resistant to TRAIL-induced cell death via interaction between TRAIL on the cell surface of NK cells and its receptor DR4 on DCs (Melki et al., 2010).

These are only few examples, demonstrating that the induction of DCs with regulatory rather than stimulatory functions is a major theme of many viruses capable of escaping an efficient immune response thereby inducing viral persistence.

DC_{reg} ARE ESSENTIAL PLAYERS IN CHRONIC GRANULOMATOUS LISTERIOSIS

Myeloid cells with regulatory function have also been implicated in the formation and maintenance of granulomatous structures that are supposed to enclose pathogens thereby preventing their dissemination (**Table 1**). While macrophages play the major role in most granulomatous diseases chronic granulomatous listeriosis is characterized by large quantities of DCs within the cellular ring wall of the granuloma (Popov et al., 2006). Listeriosis is caused by the gram positive bacteria *Listeria monocytogenes (L.m.)*. In the immunocompetent host, clinical manifestation is characterized by a self-limiting gastroenteritis. However, in immunocompromised individuals, but also elderly people and newborns *L.m.* can cause chronical meningoencephalitis and septicemia (Swaminathan and Gerner-Smidt, 2007; Allerberger and Wagner, 2010; Mook et al., 2011). Moreover, advanced stages of chronic disease are characterized by granulomas in lymph node tissue (Gray and Killinger, 1966). Granulomas are organized immune cell aggregates that form in response to persistent stimuli of infectious or non-infectious nature (Ramakrishnan, 2012). Recently, our group discovered that $CD11c^+$ $S100^+$ IDO^+ mDCs are predominant constituent elements in the outer wall of listeria granuloma (Popov et al., 2006, 2008; Von Bergwelt-Baildon et al., 2006). Transcriptome profiling of *L.m.* infected mDCs revealed that infection of mDCs leads to the upregulation of numerous effector molecules including TNFα, IFNγ, IL-10, COX-2, IDO, and CD25 that act together in a regulatory fashion (Popov et al., 2008). Supernatants derived from mDCs infected by *L.m.* suppressed T cell proliferation via a mechanism that involves IDO activity, (Popov et al., 2008 and Schultze, unpublished data). Furthermore, IDO^+ mDCs were also capable to keep *L.m.* in check, suggesting that IDO-competent mDC possess microbicidal activity (Popov et al., 2006, 2008; Von Bergwelt-Baildon et al., 2006) and Nino-Castro, unpublished results). These regulatory DC seem to have at least three critical roles during chronic listeriosis. First, they are involved in the formation and maintenance of the granulomatous structure, second, they keep the pathogen in check, and third, they suppress T cell effector function that could destroy the granulomatous structure. Therefore, these DC_{reg} are essential for the formation and maintenance of this immune priviledged site (Mellor and Munn, 2004) at the same time acting as a barrier that controls bacterial growth and dissemination via an IDO mediated mechanism.

DC_{reg} ARE IMPORTANT CELLS IN NUMEROUS PARASITICAL INFECTIONS

Despite their phylogenetic diversity, parasitic protozoans and helminthes have the ability in common to produce long lasting chronic infections (Peters and Sacks, 2006). Understanding how parasitical infections can progress toward chronicification poses a challenge due to the complex host pathogen interactions that depend on the immunological status of the host, but also on the genetic background of both pathogen and host. Some evidence suggests a role for DCs with regulatory function during infection with parasites.

Species of the genus *Leishmania* are the causing agents of leishmaniases, a group of illnesses of the oral and respiratory mucosae, the skin, and the reticuloendothelium (Reithinger et al., 2007). The cutaneous form is the most common one and is also known to evolve into a chronic disease. Recently, it has been reported in a murine model, that a subset of $Langerin^+$ Langerhans cells might favor the recruitment of T_{reg} cells to the site of infection resulting in aggravated disease. This DC subset was able to promote T_{reg} expansion *in vitro* (Kautz-Neu et al., 2011). Moreover, it has been reported that mice infected with *Leishmania major* upregulate IDO expression in lymph node pDCs. IDO^+ pDCs suppressed T cell activation and proliferation *in vitro*. Furthermore, the inhibition of the IDO enzymatic activity resulted in an improvement of the immune response toward *L. major* manifested by a reduction in lesion size and parasitical burden (Makala et al., 2011). Although IDO has a well-known microbicidal role by limiting pathogen spread via Trp depletion, the evidence in *Leishmania* infection suggest that in this context IDO^+ pDCs are deleterious to the host, due to their capacity to attenuate adaptive immune responses. Finally, Nguyen and coworkers reported that infection of murine bone marrow stromal cells by *Leishmania donovani* enhanced their capacity to attract hematopoietic progenitor cells. *L. donovani* infected stromal cells were able to support the development DCs from hematopoietic progenitor cells. Moreover, this DC subset acquired regulatory properties being able to suppress $CD4^+$ T cell proliferation *in vitro* (Nguyen Hoang et al., 2010). In summary, there is sufficient evidence that different DC populations can acquire regulatory functions in response to *Leishmania* infection, however their exact role during chronification of the disease is not yet fully understood.

Another example for the involvement of DC_{reg} in parasite infections is Chagas disease caused by the parasite *Trypanosoma cruzi*. In most patients with Chagas disease an immune response develops, the parasitemia wanes, and signs and symptoms resolve completely within a few months. However, around 30% of the patients will progress toward a chronic disease (Hemmige et al., 2012). The mechanisms that control the progression of the disease into a chronic phase are widely unknown (Sathler-Avelar et al., 2009). Recently, it has been reported that coculture of DCs with *T. cruzi trypommastigotes in vitro* leads to production of IL-10 and TGFβ by DCs. Furthermore, these cells suppressed T cell proliferation *in vitro* (Poncini et al., 2008), which indicates that *T. cruzi* might induce DCs with regulatory functions to evade the host immune system. However, to our knowledge there is no evidence yet supporting the role of DC_{reg} as promoters of tolerance toward *T. cruzi* in infected hosts *in vivo*. Therefore, the potential role of DC_{reg} in the overall immune response against this pathogen remains unclear. Nematode infections are known to promote local immunosuppression in the host, allowing the parasite to achieve a long term survival, which is usually associated with a local chronic infection (Taylor et al., 2005). Recently, Li and coworkers described a naturally occurring DC subset with regulatory activity in the murine infection model of *Heligmosoides poligyrus*. These $CD11c^{low}$ $CD45^{mid}$ DC_{reg} expanded rapidly after *H. poligyrus* infection and promoted T_{reg} differentiation *in vitro* (Li et al., 2011).

Taken together, chronification of infectious diseases is very often accompanied by the induction of DCs with regulatory functions. Reprogramming these APC away from immunostimulatory

functions seems to be a central theme during the chronification process. However, more work is required to really understand the mechanisms that lead to the observed reprogramming of DCs.

SUMMARY AND CONCLUSIONS

During the last two decades immunostimulatory functions of DCs were the major focus of DC research (Steinman, 2007) consolidating that these extraordinary cells are the main contributors for immune activation. However, during the last years accumulating evidence strongly suggests that DCs are much more versatile in their functions allowing them to regulate (DC_{reg}) or even halt immune responses e.g., by inducing tolerance (tolerogenic DC). DC_{reg} are implicated in terminating inflammatory responses under physiological conditions yet regulatory DC functions also seem to be hijacked by pathologic conditions as different as cancer or chronic infection. While several of the molecules associated with regulatory DC function (IL-10, TGFβ, IDO, PGE_2, and CD25) have been elucidated and observed in numerous cancer entities and chronic infections, the molecular mechanisms reprogramming DC to become DC_{reg} are still poorly understood. Many previous studies are rather descriptive and many findings in murine and rodent models often could not be translated to the human, leaving researchers confronted with the problem to investigate molecular mechanisms of DC_{reg} involvement in human specimens. However, the availability of sequencing-based genomic technologies assessing networks of epigenetic, transcriptional, and translational regulation on a genome-wide scale and in an unprecedented resolution makes research into human DC_{reg} biology a very promising endeavor. We envision that the integration of such data will allow us in the future to better understand how exogenous signals are integrated in DCs to foster a regulatory rather than an immunostimulatory program. Moreover, signal integration is not only restricted to classical pro- and anti-inflammatory mediators but is further altered by a myriad of soluble and cell-contact mediated signals as exemplified here for metabolites like kynurenines or unsaturated fatty acids. Moreover, understanding DC_{reg} biology will also guide the development of better DC-based vaccines, e.g., as cancer immunotherapy. Unraveling differential and context-dependent signal integration by DCs during activation will also lead to the discovery of genes that could be targeted for immunomodulation.

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Regulatory multitasking of tolerogenic dendritic cells – lessons taken from vitamin D3-treated tolerogenic dendritic cells

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Tolerogenic dendritic cells (DCs) work through silencing of differentiated antigen-specific T cells, activation and expansion of naturally occurring T regulatory cells (Tregs), transfer of regulatory properties to T cells, and the differentiation of naïve T cells into Tregs. Due to an operational definition based on T cell activation assays, the identity of tolerogenic DCs has been a matter of debate and it need not represent a specialized DC subset. Human tolerogenic DCs generated *in vitro* using inhibitory cytokines, growth factors, natural immunomodulators, or genetic manipulation have been effective and several of these tolerogenic DCs are currently being tested for clinical use. *Ex vivo* generated tolerogenic DCs reduce activation of naïve T cells using various means, promote a variety of regulatory T cells and most importantly, frequently show stable inhibitory phenotypes upon repetitive maturation with inflammatory factors. Yet, tolerogenic DCs differ with respect to the phenotype or the number of regulatory mechanisms they employ to modulate the immune system. In our experience, tolerogenic DCs generated using the biologically active form of vitamin D (VD3-DCs), alone, or combined with dexamethasone are proficient in their immunoregulatory functions. These tolerogenic DCs show a stable maturation-resistant semi-mature phenotype with low expression of activating co-stimulatory molecules, no production of the IL-12 family of cytokines and high expression of inhibitory molecules and IL-10. VD3-DCs induce increased apoptosis of effector T cells and induce antigen-specific regulatory T cells, which work through linked suppression ensuring infectious tolerance. Lessons learned on VD3-DCs help understanding the contribution of different pattern-recognition receptors (PRRs) and secondary signals to the tolerogenic function and how a cross-talk between DCs and T cells translates into immune regulation.

Keywords: tolerogenic dendritic cells, vitamin D, regulatory T cells, autoimmune diseases, costimulatory molecules

INTRODUCTION

Dendritic cells (DCs) comprise a network of professional antigen-presenting cells (APC) throughout the body that orchestrate responses to foreign antigens, while keeping in check autoreactive T cells (Steinman et al., 2003a). DCs may initiate (inflammatory DCs) or inhibit (tolerogenic DCs) the antigen-specific response. Apart from preventing pro-inflammatory responses to antigens, tolerance comprises active inhibition of existing effector immune responses against a particular antigen. Depending on the origin of this antigen, we may differentiate tolerance for self-antigens, for antigens that originate in the environment or for alloantigens after transplantation. Breaking tolerance may lead to pathology in all cases: reaction of the adaptive immunity against alloantigen may cause graft rejection; intolerance to foreign antigen predisposes to allergy while immune responses against self-antigen may lead to development of autoimmune disease.

There are different mechanisms by which immune tolerance can be achieved: for instance, removal of pathogenic T cells, inducing, or restoring a balance between pro- and anti-inflammatory immunity or by action of regulatory T cells (Treg). A superior ability of DCs to interact with naïve and experienced T cells and contribute to all mechanisms of immune tolerance makes these attractive candidates for tolerance induction strategies. Tolerogenic DCs prevent the destructive action of self-reactive T cells that had escaped the selection in the thymus, by mechanisms of peripheral tolerance (Steinman et al., 2003a). Stimulation of Th1 and/or Th2-type of the immune response, which sometimes balances the destructive immunity, is also critically dependent on action of DCs (Singh et al., 1999; Kidd, 2003; Amsen et al., 2004). Finally, DCs and Treg are engaged in active dialog and critically depend on each other's function. Induction and maintenance of Treg depends on DCs while Treg employ their regulatory action through direct interaction with DCs, in addition to affecting effector T cells (Vendetti et al., 2000; Salcido-Ochoa and Lechler, 2002; Pasare and Medzhitov, 2003; Steinman et al., 2003b).

Tolerogenic mechanisms essentially employ the antigen-presenting function of DCs, in which co-stimulatory molecules play an important role. Activation of T cells requires that at least two signals are transferred by the APC: (Steinman et al., 2003a) antigen in the form of peptide bound to MHC molecules (Amsen

et al., 2004), co-stimulation, especially provided by molecules of the B7 and TNF-receptor families. In addition, cellular adhesion molecules and cytokines support activation of T cells that have made a productive interaction with antigen-presenting- and co-stimulatory molecules. For instance, inflammatory DCs produce and present IL-2 to naïve T cells to promote their activation (Granucci et al., 2002; Wuest et al., 2011), cytokines of the IL-12 family and IL-4 skewing T cells into a T helper-1 (Th1) or T helper-2 (Th2) polarized phenotype (Kalinski et al., 1999), and DCs secrete IL-10, TGFβ, IL-35, or TNF, which promote generation of regulatory T cells (Kushwah and Hu, 2011). Finally, stimulated T cells may be endowed with tissue-specific homing capacities as a "fourth" signal delivered by APC to T cells (Campbell and Butcher, 2002; Villablanca et al., 2008). The quantity and quality of the distinct signals delivered by APC determine the outcome of the ensuing T cell response and the ability of DCs to activate a strong effector cell response has been vastly appreciated. However, DCs are also crucial in downregulation and tolerization of the adaptive immune system and apart from being able to sense various danger signals in inflammation, DCs integrate tolerogenic signals originating from peripheral or lymphoid tissues, supporting the maintenance of immune homeostasis and avoidance of T cell autoreactivities (Steinman et al., 2003a; Yogev et al., 2012). The primary function of DCs thus might be induction and maintenance of immune tolerance rather than activation of effector responses.

Approaches to harness the immunoregulatory function of DCs encompass the search for tolerogenic DCs *in vivo* and the *in vitro* manipulation of monocyte-derived DCs to confer and secure their tolerogenic function. The emerging concept implies that *in vivo* tolerogenic DCs might not represent a specialized DC subset but might embody a particular state of DC differentiation influenced by a collection of tissue and environmental factors, making it difficult to manipulate these cells in real time. The interplay between resident DCs and naturally occurring Tregs (tTreg) from the thymus likely reinforces the default tolerogenic state of DCs. *In vitro* manipulation of monocyte-derived DCs has proven useful and modulated DCs are being currently tested for clinical use. In this review, we summarize our lessons learned using Vitamin D3-treated DCs on the role of secondary signals in the generation of tolerogenic DCs and how these may induce and maintain peripheral tolerance.

HETEROGENEITY OF TOLERANCE-INDUCING DENDRITIC CELLS: DO TOLEROGENIC DENDRITIC CELLS REPRESENT A DISTINCT SUBSET?

The highly increased awareness of the role of DCs in steering the immune system and their possible clinical applications has resulted in a wealth of information about the phenotypic heterogeneity of DCs. However, unequivocal interpretation of these findings in developmental and functional terms has proven to be difficult, especially because of the high turn-over of DCs, their significant mobility between peripheral and immune organs, the phenotypic volatility of these cells in response to environmental conditions, and the differences between strategies to endorse tolerogenic capacity. The application of phenotypic labeling to define DCs created confusion since many "new" DC types in different organs were defined, without a clear view on the possible developmental relationships between these and already known DC types. To illustrate this: in human thymus, three distinct DC populations have been found up to five populations in lymph nodes and tonsils and at least three populations in blood (Lewis and Reizis, 2012). The situation is similarly complex in mice, since up to six different DC types were found in mouse lymph nodes and spleen (Dominguez and Ardavin, 2010).

Understanding the *in vivo* function of these different DCs and establishing the identity of tolerogenic DCs has consequently taken a while. In general, two schools of thinking have arisen; one that proposes tolerogenicity to be a functional property of DC at a particular (immature) stage in development and another that hypothesizes that a specific tolerogenic DC lineage exists. In favor of the first proposal, immature DC induce anergic T cells, T cells with regulatory properties as well as T cells that secrete immunomodulatory cytokines. However, the immature phenotype of DCs is replaced by mature/inflammatory upon triggering in the periphery by danger/inflammatory signals and migration to the lymph nodes, making the tolerogeneicity of immature DCs a transient phase that can hardly be controlled for therapeutic purposes. Establishment of DCs with a "semi-mature" phenotype, representing cells that have been "matured" in the absence of inflammation, has enabled definition of functional regulatory DCs with a stable phenotype (Lutz and Schuler, 2002). The phenotype of these DCs may vary depending on the "tolerizing" signals they receive but in general, semi-mature DCs are able to present antigens like mature DCs but lack high level pro-inflammatory cytokine production and their repetitive injection prevents autoimmunity in experimental models.

The hypothesis that a specific lineage of DC induces tolerance has been widely accepted as well (Coquerelle and Moser, 2010). Studies demonstrating two types of DCs in the peripheral blood, so called myeloid or conventional (cDCs) and plasmacytoid DCs (pDCs), which had different origins and functions, supported the notion that inflammatory and tolerogenic DCs are unrelated cells with separate precursors. In mice, CD8 – cDCs and tissue-derived cDCs are predominantly immunogenic. Most notably implicated in tolerance induction are CD8+ cDCs (Yamazaki et al., 2008) and pDCs (Nikolic et al., 2009), so these were logical candidates for a special tolerogenic DC lineage in mice. However, CD8+ cDCs are not only tolerogenic but also cross-present antigens and prime CD8 T cells (Crozat et al., 2011). Similarly, the tolerogenic potential of pDCs has been demonstrated in allergic inflammation *in vivo* (Kool et al., 2009), but pDCs can also stimulate Th1 or Th2 immunity and CD8 T cell responses. This indicates that both CD8+ cDCs and pDCs can perform distinct functions depending on the sum of factors that had influenced their maturation and activation (Swiecki and Colonna, 2010; Takagi et al., 2011). Together with the identification of a common precursor for cDC and pDC in mice (Auffray et al., 2009), the notion of a separate tolerogenic DC lineage became less plausible.

Since both cDC and pDC as found in blood, bone marrow, or peripheral organs represent the immature stage of the cell, the notion is that "immature" or migratory DCs (plasmacytoid or conventional) are able to down-regulate immunity until they encounter an activation stimulus and become immunostimulatory

APC. This again strengthens the view that all DC are in principle tolerogenic in steady state, unless triggered by a "danger signal" in the inflammatory context. Therefore, a unifying platform allowing both scenarios under particular conditions would be that by conditioning during differentiation determines the functional plasticity of DCs and their diversity in origin/phenotype has evolved to enable the immune system to "sense" various danger signals with different intensity leading to different functional outcomes depending on the context (**Figure 1**).

HARNESSING TOLEROGENIC DCs FOR THERAPEUTIC PURPOSES

Dendritic cells that mediate tolerance induction *in vivo* exist without doubt. However, it is still debatable whether a particular function demonstrated *in vitro* may also be exerted *in vivo*. Immune tolerance can be achieved through different mechanisms and the experimental data from an *in vivo* system do not discern whether a particular DC subtype employs one or more mechanisms, or what other cells in the system might contribute. For example, thymic DCs have been associated with selection of developing T cells, but a recently established mouse model lacking the majority of DCs (DC-less mice) suggested that DCs are dispensable in thymic negative selection (Yogev et al., 2012). Yet, DCs in the periphery remain essential in induction and maintenance of peripheral tolerance, since DC-less mice are able to mount an immune response but fail to regulate it and develop enhanced EAE and progressive myeloproliferative disorders, resulting in spontaneous fatal autoimmunity (Birnberg et al., 2008; Ohnmacht et al., 2009; Yogev et al., 2012). The expression of the MOG auto antigen in steady state DCs in the brain induced strong tolerance, correlating with the increased expression of PD-1 on adaptive regulatory T cells (Yogev et al., 2012). Taken together, these studies show: (i) that autoimmunity may be activated by other cells than DCs; (ii) that DCs are essential for the maintenance of tolerance; (iii) that DCs may act by enhancing adaptive regulatory T cells.

Human peripheral blood pDCs and a subtype of human blood DCs that expresses CD141 (CD141+ DCs) are believed to mediate tolerance *in vivo*. Although, there is some phenotypic and functional overlap between human and murine DCs, there are also important differences that prevent straightforward translation of findings between species. For example, while both human and mouse pDCs selectively express TLR7 and TLR9, securing the ability to produce IFN-I in response to TLR7/9 ligation, human pDCs do not produce IL-12p40, which may contribute to their tolerogenic capacities. Both human and murine pDCs express CXCR3 and CXCR4, but human pDCs lack CCR9 expression, which could be related to different maturation state (Drakes et al., 2009). CCR9 is expressed by human CD141+ DCs, which are considered to be a homolog of mouse CD8+ DCs (Jongbloed et al., 2010; Poulin et al., 2010). Yet, human CD141+ DCs do not express the same TLR receptors as the mouse counterpart, thus have different sensor functions (Kaisho, 2012). Skin resident CD141+ DCs are the

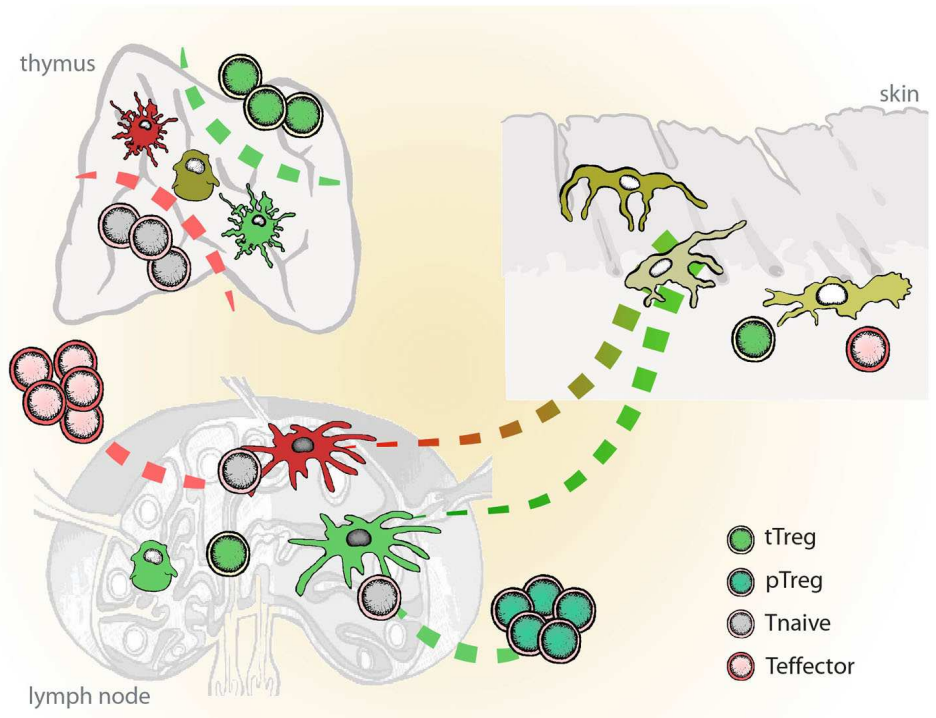

FIGURE 1 | Schematic representation of inflammatory vs. tolerogenic DCs *in vivo*. Inflammatory DCs (red) differentiate from the immature tissue-resident DCs and migrate to the lymph nodes, where they initiate immune responses and instruct generation of the effector T cells (Teff). Semi-mature DCs (green), which develop from the tissue-resident DCs in the absence of danger and inflammatory signals, induce tissue-specific adaptive regulatory T cells (pTreg). Dendritic cells in thymus play a role in the positive and negative selection of naïve lymphocytes (Tnaive) and the induction of naturally occurring regulatory T cells (tTreg). In mice, DCs seem to play dispensable role in the thymus.

major producer of IL-10, they induce T cell anergy and Tregs that suppress skin inflammation. Interestingly, treatment with vitamin D3 induces skin CD141+ DC-like phenotype on human CD141− DCs (Chu et al., 2012).

Manipulation of mouse DCs *in vivo* has shown therapeutic potential to control immune response. Selective depletion of mDCs sparing pDCs strongly attenuated autoimmune response and reduced diabetes incidence in NOD mice (Nikolic et al., 2005), and specific selective expansion of pDCs *in vivo* reduce strong allergic responses (Kool et al., 2009). Depletion of a subtype of dermal DCs prevents differentiation of effector Th cells and conferred resistance to EAE (King et al., 2010), and elimination of Langerhans cells in the skin decreased the low-dose contact hypersensitivity (Kel et al., 2010). A fine tuning between CD8+ and CD8− cDCs that differentially stimulate Th1 vs. Th2 responses can regulate immunity, but the potential of DCs to stimulate a particular type of helper T cells depends on the type of the stimulatory signal they encounter. Immunoregulatory function of tolerogenic DCs *in vivo* is usually associated with their incompletely differentiated state. It would be important to learn how to prevent DCs *in vivo* from acquiring immunizing abilities to avoid lack of control and offer safe therapeutic application in humans.

IN VITRO GENERATED TOLEROGENIC DCs

Monocytes may be manipulated *in vitro* to generate DCs with desired function and this has been investigated as immunotherapy to a great extent (Palucka et al., 2003; Figdor et al., 2004; Germain, 2010; Chia et al., 2012; Hong et al., 2012; Palucka and Banchereau, 2012). Development of DCs *in vitro* under influence of GM-CSF and IL-4, reconstructs the sequence of events established for peripheral tissue DCs although a direct approximation of the *in vitro* generated DCs to an *in vivo* existing subset is not possible. Tissue-resident DCs, like immature DCs in culture, are characterized by high endocytic capacity and low surface expression of major histocompatibility complex (MHC) and co-stimulatory molecules. Danger signals or inflammation encountered in the periphery triggers maturation of DCs, enabling these to migrate to the lymph nodes and on their way acquire all necessary tools to initiate an adequate response to phagocytosed antigens. The maturation *in vitro* is induced by stimulating DCs through pattern-recognition receptors (PRRs), CD40, or by inflammatory cytokines.

Different approaches have been tested to induce tolerogenic DCs *in vitro* and the features DCs have to fulfill are related to the semi-mature phenotype, resistance to maturation, anti-inflammatory cytokine profiles, and induction of specific T-cell profiles. A tolerogenic state of DCs can be induced using several pharmacological agents such as rapamycine, dexamethasone, Vitamin A, or Vitamin D, cytokines such as IL-10 or different growth factors such as G-CSF, VEGF, VIP, and many others (Rutella et al., 2006). Alternative approach has been tested as well using antisense nucleotides to enforce stable immature phenotype of DCs (Machen et al., 2004). These different tolerogenic DCs may share certain features, such as semi-mature phenotype or the ability to suppress allo-reactive response in the mixed leukocyte reaction (MLR), yet many have specific features not shared by others. An overview of phenotype and functional properties of different *ex vivo* generated DCs is given in **Table 1**.

VITAMIN D AND DEXAMETHASONE MODULATED DCs

The active form of the natural immunomodulator Vitamin D (i.e., $1,25(OH)_2D3$), alters the behavior of immune cells shifting T lymphocytes into Tregs and modulating differentiation of human peripheral blood monocytes (Piemonti et al., 2000; Griffin et al., 2001; Adorini et al., 2003; Unger et al., 2009; Baeke et al., 2011), or mouse bone marrow precursors (Griffin et al., 2001). Interestingly, pDCs do not respond to the modulation by $1,25(OH)_2D3$ (Penna et al., 2007). Monocyte-derived DCs generated in the presence of $1,25(OH)_2D3$ (VD3-DC) show a semi-mature phenotype with low MHC-class II expression, low activating co-stimulatory molecules, and production of interleukin IL-10 instead of IL-12 in the culture. In addition, VD3-DCs prevent priming of naïve CD4, or CD8 T cells, induce apoptosis of effector T cells and induce both allo- and auto antigen-specific Tregs from naïve CD4 T cells (van Halteren et al., 2004; Unger et al., 2009; Kleijwegt et al., 2010, 2011, 2013). Similar potential to modulate monocyte development into DCs with tolerogenic phenotype has been shown for dexamethasone (Dex) (Matasic et al., 1999; Piemonti et al., 1999; Rea et al., 2000; Chamorro et al., 2009; Unger et al., 2009). Like VD3-DCs, Dex-DCs possess durable immaturity and a sustained high IL-10 versus low IL-12 production, compared to non-treated monocyte-derived DCs. In mice, application of Dex-DC prior to allograft transplantation significantly prolonged the survival of the grafts in an antigen-dependent fashion. Similar to VD3-DCs, priming with Dex-DC leads to a decrease of IFN-γ-producing cells, while increasing the number of IL-10 producing cells, which could point to the induction of Tr1-like Tregs. However, Tregs induced using Dex-DCs suppress in a bystander fashion independent of antigen (Unger et al., 2009). $1,25(OH)_2D3$ and Dex used in combination generate a third entity of tolerogenic DCs (Combi-DCs), which share most features with VD3-DCs in terms of phenotype and function. Combined treatment with $1,25(OH)_2D3$ and Dex enhanced the modulation of DCs, compared to either compound alone with respect to surface marker expression, inhibition of pro-inflammatory cytokine production, and attenuation of T cell stimulatory capacity (Ferreira et al., 2012). Most importantly, Combi-DCs retain the capacity instilled by $1,25(OH)_2D3$ to induce antigen-specific Tregs. *In vivo*, combined-treated DCs are more potent than IL-10-treated cells to suppress colitis in a murine model (Pedersen et al., 2009).

To further investigate the effects of single vs. combined treatment with $1,25(OH)_2D3$ or Dex as DC modulators, we performed a 2D-DIGE analysis of protein profiles (Ferreira et al., 2012). At the protein level, $1,25(OH)_2D3$ induced major changes in proteins involved in iron metabolism, tricarboxylic cycle, and the purine/pyrimidine metabolism/pentose phosphate pathway. Dex alone changed proteins involved in the response to stress in addition to several proteins included in the proteolysis and the MHCII antigen presentation pathway. Combining both compounds resulted in a unique protein profile of Combi-DCs, although with a major impact of the $1,25(OH)_2D3$. Combi-DCs had a profile closer related to VD3-DCs than to Dex-DCs, which could be due to the experimental setup, since $1,25(OH)_2D3$

Table 1 | Protocols reported to date that generate tolerogenic DCs from human monocytes.

Modulator	Treatment start	HLA-DR and co-stimulatory molecules	Inhibitory molecules	Cytokine production	Inhibition of T cells*	Induction of Tregs	Additional characteristics	Reference
IL-10	Monocytes	HLA-DR low, CD80/86/40 low	PD-L1, IL-T3, mTNF	Low IL-12	Yes	Yes	Strongly polarize Th2 response	Chamorro et al. (2009)
Rapamycin	Monocytes	HLA-DR and CD86 like moDCs	Not expressed	Low cytokine production	No	No	Reduced IFNg in stimulated cells	Naranjo-Gomez et al. (2011)
Aspirine	Monocytes	Reduced CD80, CD86, CD40	ILT-3	Not reported	Yes	Yes	Induce *de novo* Tregs#	Buckland and Lombardi (2009)
Butyric acid	immDCs	CD80 low; HLA-DR and CD86 like moDCs	Not tested	Low IL-12, high IL-10	Yes	Not tested	Strong phagocytic capacity#	Downing et al. (2012)
Dexamethasone	Culture day 3	HLA-DR low, CD80/86/40 low	Low PD-L1, ILT-3	Low IL-12, high IL-10	Strong	Yes	Tregs suppress through soluble factors	Unger et al. (2009)
Vitamin D3	Monocytes	HLA-DR intermediate, CD80/86/40 low	PD-L1, IL-T3, mTNF	Low IL-12, high IL-10	Strong	Yes	Migrate to inflammation, confer infectious tolerance	Kleijwegt et al. (2010, 2011)
Aspergillus proteases	mm.DC	HLA-DR not tested,l CD86 low	ILT-4, RALDH-2, NOS, IDO	IL-8, no IL-10, no IL-12	At a high DC:T ratio	No	Induce anergic T cells#	Zimmer et al. (2012)
Semen	Monocytes	HLA-DR low, CD80/86/40 low	Not tested	Low IL-12, TNF, IL-6, high IL-10, TGFb	Not present	TGFb-producing	Maturation-resistant phenotype#	Remes et al. (2012)
Trophoblast cells	immDC	HLA-DR, CD86 and CD40 high	Not tested	low IL-12, TNF, high IL-10	Yes	Increase FoxP3 + CD25 +	Migration toward trophoblast cells	Salamone et al. (2012)
Oligonucleotides	Monocytes	Inhibited CD80 and CD86	Unknown	Unknown	Unknown	Unknown	Increase IL-10 producing B cells	Giannoukakis et al. (2011)
Wnt5a	Monocytes	HLA-DR, CD80, CD86, CD40 low	PD-L1, PD-L2 similar to moDC	Low IL-12, high IL-8, IL-10,	Yes	IL-10 producing	Wnt5a prevents normal GM-CSF/IL-4 signaling	Valencia et al. (2011)

**inhibition of proliferation of allogeneic T cells.*

#characteristics also demonstrated for VD3-DCs.

treatment started at the beginning of DC differentiation while Dex was added from day 3. Addition of Dex did not seem to interfere with the effects of $1,25(OH)_2D3$ on protein expression in DCs. The protein interaction networks and pathway analysis demonstrated that combined treatment induces drastic changes in metabolic pathways, which might affect the production of or the response to ROS generation resulting in a Combi-DCs that are less sensitive to death by nutrient starvation and have a robust antioxidative machinery possibly ensuring the survival of Combi-DCs at the inflammation site in autoimmune diseases. Since genetic polymorphisms may influence the response of immune cells to $1,25(OH)_2D3$ or Dex alone, these findings ensured us that combining the two immunomodulators to overcome potentially insufficient modulation by a single agent is the way to go with respect to clinical translation for immunomodulatory DC therapy.

MORPHOLOGY AND SURFACE PHENOTYPE OF VD3-TREATED DCs: ACTIVATING VS. INHIBITORY CO-STIMULATORY MOLECULES

Morphological features of $1,25(OH)_2D3$ and/or Dex modulated DCs are different from non-treated monocyte-derived – moDCs. Non-modulated moDCs form large clusters of non-adherent cells with small dendrites, Dex-DCs are largely non-adherent but show fewer small dendrites and form smaller clusters than moDCs. In contrast, VD3-DCs and Combi-mDCs are adherent with large spindle-shaped dendrites. Interestingly, this spindle-shaped morphology was prominent when VD3- and Combi-DCs were matured with LPS or CD40-L and less evident when DCs were matured using the inflammatory cytokine cocktail (unpublished observation). DCs treated with $1,25(OH)_2D3$ and/or Dex maintain CD14 expression and do not express CD1a, although they show high CD11c and DC-SIGN expression confirming that they are DCs with a semi-mature phenotype and not macrophages or arrested at monocyte stage (Unger et al., 2009; Ferreira et al., 2012). Upon maturation, Combi-mDCs maintain high CD14 expression and high phagocytic capacity compared to moDCs, possibly indicating a resistance to maturation. The functional consequences of residual CD14 expression and phagocytosis remain to be investigated.

Modulated DCs show lower expression of MHC-class II and co-stimulatory molecules CD80 and CD86 when compared to moDCs, upon maturation. VD3-DCs express higher levels of PD-L1 and CD86 compared to Dex-DCs and Combi-DCs. However, VD3-DCs and Combi-DCs show a similar ratio of PD-L1/CD86, being higher than that of Dex-DCs. CD86 and PD-L1 belong to the B7 family of receptors, which interact with the members of the CD28 family of molecules (CD28, CTLA-4, ICOS, and PD-1), generating potent activating or inhibitory signals in T lymphocytes. CD28/B7 interactions mediate co-stimulation and significantly enhance peripheral T-cell responses while CTLA-4 activation decreases T lymphocyte activity and limits the immune response. Similarly, PD-1 receptor interactions with its ligands PD-L1 and PD-L2 on DCs down-regulate T cell immune responses. Despite these similarities, the regulatory roles of the CTLA-4 and PD-1 pathways are different, which may be due to the differential temporal and spatial expression patterns of their ligands. CTLA-4 signaling seems to be required early in the lymph node during initiation of an immune response, while PD-1 pathway acts late in the periphery to limit T-cell activity locally (Greenwald et al., 2005). PD-L1 on VD3-DC is crucial for the acquisition of Treg function by CD4+ T cells and blockade of PD-L1 on VD3-DC during T cell priming generated Th1-like T cells incapable of suppressing allo-reactive T-cell proliferation and producing IL-10 (Unger et al., 2009). These observations suggest that PD-L1/PD-1 interactions impair the generation of effector T cells in favor of Treg formation. The underlying mechanism may involve reverse signaling by PD-L1 into DC leading to decreased expression of the positive co-stimulatory molecules CD80, CD86, and CD40 and increased IL-10 production.

Inducible T cell costimulator (ICOS) has overlapping functions with CD28 in early T-cell activation, and has emerged as an important receptor in the immune system to regulate T-cell effector functions (de la Fuente et al., 2012). The ICOS/ICOSL pathway is also involved in immune tolerance since ICOS regulates the survival of both effector memory T cells and FoxP3+ Tregs during homeostasis or antigen-specific immune response (Burmeister et al., 2008). Modulated VD3- and Combi-DCs express ICOSL at similar levels as the non-modulated moDC. The contribution of ICOSL in the "downstream" tolerogenic mechanisms installed by VD3-DCs will be discussed later in this review.

ANTI-INFLAMMATORY CYTOKINE PRODUCTION AND RELATION TO "TOLEROGENIC" PHENOTYPE

A shift in cytokine production to predominant IL-10 and reduced IL-12 production by $1,25(OH)_2D3$-treated DCs has been observed *in vitro* in multiple studies with human as well as murine DCs (Black et al., 1997; Ferreira et al., 2011). The turn-off signal for the IL-12 and related cytokines could be IL-10, which blocks IL-12 synthesis, down-regulates the expression of co-stimulatory molecules and potentiates tolerogenicity (Steinbrink et al., 1997; Rutella et al., 2006). Proteomics analysis shows that $1,25(OH)_2D3$ and Dex induce changes in DCs other than IL-10 production alone. Yet, the contribution of IL-10 to the tolerogenic phenotype might be larger than anticipated. TLR-activation induces a metabolic transition in DCs from oxidative phosphorylation to aerobic glycolysis and IL-10 blocks this transition. By switching toward oxidative phosphorylation, DCs are less reliant on glucose for survival and function, and thus less sensitive to death by nutrient starvation (Steinbrink et al., 1997; Rutella et al., 2006; Ferreira et al., 2009). IL-10 is also important for the immunomodulatory effects of VD3-DCs, attenuating Th1 driving forces and being protective in settings of autoimmunity. Together with TGF-β, IL-10 expression is a hallmark of the presence of regulatory T cells. However, IL-10 does not seem to be involved in the actual suppression by Tregs.

Tumor necrosis factor (TNF) is a pleiotropic cytokine most known for pro-inflammatory (Th1) functions in multiple autoimmune diseases (Braun et al., 2002; Feldmann and Maini, 2003). However, a beneficial effect of TNF in autoimmunity is also acknowledged (Robinson et al., 2001; Ramos-Casals et al., 2008; Ko et al., 2009; Tack et al., 2009). This dual role of TNF is seen in other pathologic conditions such as infectious diseases and cancer (Gimenez, 2003; Calzascia et al., 2007). Diverse roles of TNF in the immune response may be partly inferred by two forms of TNF: a membrane-bound TNF (mTNF), which is cleaved from

the membrane and released as a soluble cytokine (sTNF) (Black et al., 1997). We demonstrated that VD3-DC produce increased amounts of sTNF upon LPS stimulation. Yet, the expression of mTNF distinguishes VD3-DCs from moDCs (Black et al., 1997; Kleijwegt et al., 2010). We further demonstrated that mTNF is essential for the induction of allo-specific suppressive T cells by VD3-DCs through mTNF-TNFRII interaction, adding the role of mTNF in the induction of immune tolerance to the list of multiple functions of this cytokine.

Indoleamine 2,3-dioxygenase (IDO) and aryl hydrocarbon receptor (AhR) have been implicated as negative regulators of the inflammatory response by modulating the Th1/Th2 balance (Negishi et al., 2005), and regulation of Treg and Th17 cell differentiation (Quintana et al., 2008). We did not find evidence for the contribution of these molecules in modulated DCs.

PRRs AND RESISTANCE TO MATURATION

Several synthetic TLR ligands activate DC subsets and promote their adjuvant pro-inflammatory capacity, which can be a useful tool in certain types of vaccines. DC express a broad repertoire of TLRs with 10 members of the family described to date in human (Medzhitov, 2001). TLRs trigger the signals that provide DC maturation and the initiation of adaptive immune responses against pathogens. The most known ligand for TLR4 is LPS (Medzhitov, 2001), whereas TLR2 discriminates lipoproteins from Gram-positive bacteria in association with TLR1 or TLR6 (Schwandner et al., 1999; Takeuchi et al., 2002). In addition, self-proteins can be recognized by both TLR2 and TLR4 (Asea et al., 2002).

The TLR repertoire on tolerogenic DCs modulated *in vitro* with IL-10, dexamethasone, or Vitamin D appears similar to that of immunogenic DCs, although with slight differences in the expression levels of TLR2 and TLR4 (Chamorro et al., 2009). However, tolerogenic DCs responded differently to TLR mediated signals than immunogenic DCs showing partial maturation with low to intermediate levels of CD80, CD86, and CCR7, reduced ability to stimulate allogeneic T cells and secretion of the suppressive cytokine IL-10 combined with very low production of IL-12 or the related cytokines IL-23 and IL-27. The finding that partially matured DCs respond to TLR2 stimulation with an anti-inflammatory program could shed light on why patients with severe sepsis undergo long-term systemic and local immunosuppression despite their increased TLR2 expression (Armstrong et al., 2004), and how TLR2-derived signals from *Candida albicans* or *Schistosome* infections drive immunosuppression by IL-10 production and Treg induction (van der Kleij et al., 2002; Netea et al., 2004). Indeed, TLR2 participates in the induction of peripheral tolerance (Dillon et al., 2006), in the promotion of T regulatory responses leading to protection against autoimmune diseases *in vivo* (Manicassamy et al., 2009). Thus, the TLR2 up-regulation and activation on VD3- and Dex-treated DCs might enhance their tolerogenic properties.

In vivo application of DCs in patients faces possible pro-inflammatory triggering and the tolerogenic DC vaccine should not divert into pro-inflammatory DC upon injection. Preservation of tolerogenic function in the settings of an activated immune system (resistance to maturation) is therefore considered a prerequisite of tolerogenic potential for immunomodulatory vaccines. Pro-inflammatory DC maturation is induced when DCs sense microbes through PRRs, interact with T cells (through CD40) or get exposed to inflammatory cytokines. We mimicked *in vivo* DC activation by re-stimulating matured tolerogenic DCs with LPS, CD40-L, or the cocktail of cytokines (IL-6, TNF, IL-1β, and PGE2), and demonstrated that DCs modulated with Vitamin D alone or combined with dexamethasone maintained a stable regulatory phenotype, IL-10 production, and low stimulation of allogeneic T cells upon repeated maturation with either LPS, CD40-L, or cytokine mix. Similarly, a study comparing resistance to re-stimulation with LPS of the rapamycin-, dexamethasone-, and Vitamin D-treated DCs, demonstrated a stable high IL-10 and no IL-12/IL-23 production (Naranjo-Gomez et al., 2011).

MODULATED DC ARE EQUIPPED TO MIGRATE TO INFLAMED AND SECONDARY LYMPHOID TISSUES – CHEMOKINE PRODUCTION UPON TLR TRIGGERING

To be effective in silencing reactive T cells or inducing tissue-specific Tregs *in vivo*, it is of key importance that injected modulated DCs reach lymphoid tissues or the inflamed site. We therefore examined the expression of several chemokine receptors. Immature Dex-, VD3-, and moDCs expressed significant levels of CCR4 and CCR5, but no CCR7 (Unger et al., 2009). Upon maturation, CCR4 and CCR5 were down-regulated, whereas CCR7 was up-regulated by all DCs indicating the ability to migrate to secondary lymphoid tissues. CXCR3 was expressed on the surface of the modulated DCs, which may guide these cells to the inflammatory lesion in the pancreas to counteract autoreactive T cells around the distressed islets (van Halteren et al., 2005; Roep et al., 2010).

PREVENTING PRIMING OF NAÏVE T CELLS – NEGLECTION OR ACTIVE INHIBITION?

Schematic representation of molecules involved in the tolerogenic function of VD3-modulated DCs is given in **Figure 2**. VD3-DC and Combi-DC impair priming of naïve CD4 and CD8 T cells (Unger et al., 2009; Kleijwegt et al., 2013). Naïve T cells require antigen recognition, co-stimulation, and activating cytokines (mostly IL-12) to become effector T cells. VD3-DC and Combi-DCs express lower levels of co-stimulatory molecules and IL-12, providing incomplete stimulation to T cells. However, our data show that besides hampered stimulation, an active repression prevents priming of naïve T cells (Kleijwegt et al., 2013). Combi-DCs produce TNF and TGF-β (Chamorro et al., 2009; Kleijwegt et al., 2010), but neither of these cytokines contributed to the obstructed priming of CD8 T cells. Since Combi-DCs interfere with priming only when they present the cognate antigen to the T cell, mechanisms related to TCR signaling may be responsible for this effect. This could happen through failing trans-presentation of IL-15, IL-2 (Combi-DCs lack CD25 expression), or high ILT-3 expression, as demonstrated in other systems (Chamorro et al., 2009; Frasca et al., 2010; Kleijwegt et al., 2010; Huntington et al., 2011).

KILLING OF T CELLS BY TOLEROGENIC DCs – THE STORY OF MR. AND MS. SMITH

Tolerogenic mechanisms of VD3-DCs include deletion of naïve T cells since the cell numbers after the co-culture with VD3-DCs are reduced compared to the cultures with non-treated moDCs. The

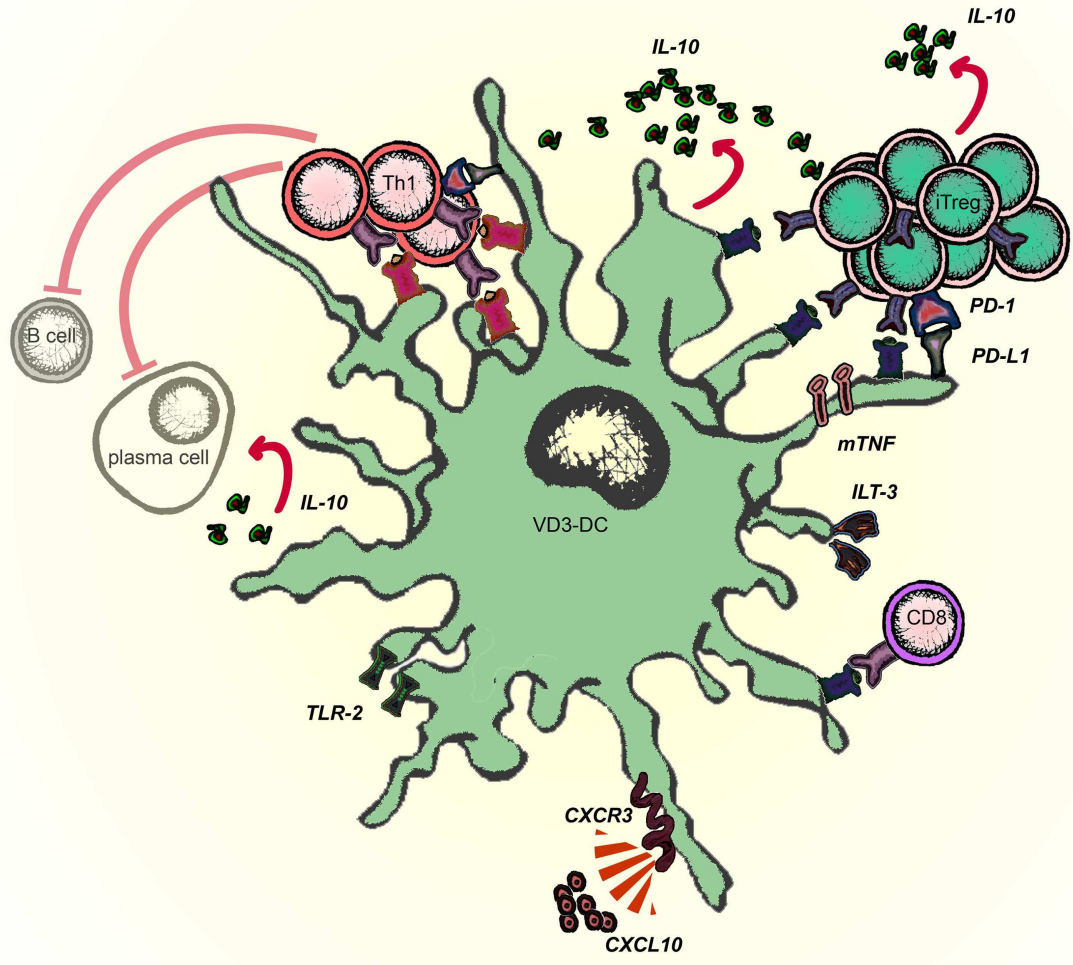

FIGURE 2 | Molecules involved in the tolerogenic function of Vitamin D-modulated DCs (VD3-DCs). VD3-DC express MHC class II in combination with low co-stimulatory molecules (CD80/86) and high expression of PD-L1, which enable antigen-dependent elimination of Th1 effector T cells and induction of antigen-specific adaptive regulatory T cells (iTregs). VD3-DCs express additional regulatory molecules: TLR2, ILT-3, and membrane-bound TNF (mTNF), and produce IL-10, which potentiate the tolerogenic function of VD3-DCs. VD3-DCs also express chemokines, which enable the migration both to lymph nodes (CCR7) and to the site of inflammation (CXCR3).

ability of DCs to kill target cells that could serve in anti-tumor immunity or T-cell killing in autoimmunity and transplantation has been described (Chauvin and Josien, 2008). A recent study in pulmonary tuberculosis patients confirms the cytotoxic activity of DCs as a mechanism of negative regulation of T lymphocytes (Sakhno et al., 2012). In case of VD3-DCs, this action is antigen-dependent but not restricted to naïve T cells since VD3-DCs also block IFNγ and IL-10 production and induce active killing of a diabetogenic Th1 clone in an antigen-specific manner (van Halteren et al., 2002). When interacting with memory CD8 T cells, Combi-DCs initially expand this cell pool, which collapses after the subsequent re-stimulation (Kleijwegt et al., 2013). This is caused by an increased cell death in the second co-culture. To test whether memory CD8 T cells undergo increased apoptosis in the second culture due to the primary interaction with tolerogenic DCs, memory CD8 T cells initially stimulated with tolerogenic DCs were subsequently re-stimulated with moDCs. This only partially rescued CD8 T cells from the collapse in the second culture, implying that during the first stimulation tolerogenic DCs endorse memory CD8 T cells with features underlying their subsequent ceased growth. The depletion of CD8 T cells by Combi-DCs could result from a combined action of the withdrawal of positive signals (co-stimulation and cytokines) and inhibition by secreted regulatory factors (such as IL-10 and TGF-β), creating a less favorable environment for cytotoxic T cell survival and expansion (Bots et al., 2007; Toda et al., 2011). This process could also involve expression of CXCR3 facilitating memory degeneration, inhibition of autocrine IL-2 production, or induction of pro-apoptotic signals as described in other models (Kurachi et al., 2011; Takata et al., 2012).

The finding that tolerogenic DCs induce elimination of effector CD8 T cells initially appeared to offer a valuable tool to tackle the

destructive forces such as autoreactive CD8 T cells in the pancreas of patients with type 1 diabetes by loading diabetogenic MHC-class I peptides onto tolerogenic DCs. However, a draw-back in this respect was our finding that antigen-loaded tolerogenic DCs were eliminated by the cytotoxic CD8 T cells through a Granzyme B-dependent mechanism (Kleijwegt et al., 2013). DCs have the ability to protect themselves from being killed using protective molecules like Serpin 9 or Cathepsin B (Bots et al., 2007) or by up-regulation of antiapoptotic proteins, such as Bcl-xL (Gutierrez et al., 2007). Indeed, tolerogenic DCs were more protected from killing than moDCs or B cells. Yet, in order to preserve the immunomodulatory action of tolerogenic vaccine on other cells types, loading of MHC-class I epitopes does not appear to add to MHC-class II epitopes on tolerogenic DCs.

VITAMIN D3-TREATED DCs INDUCE INFECTIOUS TOLERANCE THROUGH ANTIGEN-SPECIFIC TREGS

As established above, VD3- and Combi-DCs induce Tregs that suppress in an antigen-dependent manner, which is specialized feature of $1,25(OH)_2$D3-treated DCs. Rapa-DC-primed T cells exhibit reduced alloproliferation along with a concomitant expansion of naturally occurring tTregs (Kang et al., 2008; Simonetta et al., 2010), and Dex-DCs induce adaptive Tregs that suppress in an antigen-independent fashion (Unger et al., 2009). PD-L1 and mTNF play important roles in the mechanisms by which VD3-DCs induce allo-reactive or proinsulin specific Tregs (Kleijwegt et al., 2011).

Induced (adaptive) proinsulin-peptide specific Tregs (iTregs) suppress diabetogenic Th1 cells via linked suppression and do not need IL-10 or TGF-β to perform their suppressive functions (Kleijwegt et al., 2011). Tregs may exert their antigen-specific function via cytolysis of the APCs, metabolic disruption, or by modulating the immunogenic function of DCs (Vignali et al., 2008). We have demonstrated that iTregs induced by VD3-DC perform two of three described functions and the contribution of iTregs to metabolic disruption remains to be investigated. iTregs re-educate pro-inflammatory moDCs in an antigen-specific manner by changing the phenotype (@DC), which differs from VD3-DCs used to generate iTregs: VD3-DCs have low CD80 and CD86, high ILT-3 and PD-L1, low ICOSL, and no B7-H3 expression (Unger et al., 2009), whereas @DCs retain high expression of HLA-DR and co-stimulatory molecules CD80 and CD86, and low expression of inhibitory molecules PD-L1, ILT-3, and ILT-4 (Kleijwegt et al., 2011). The difference in phenotype may originate in the mode of modulation: VD3-DCs result from modulation of monocytes with vitamin D3 during differentiation, whereas iTregs alter fully differentiated mature moDCs into @DC. The specific alteration of antigen-bearing moDCs, which persisted upon removal of iTregs, was characterized by up-regulation of B7-H3 and ICOSL. Both molecules are involved in immune regulation: B7-H3 preferentially dampens Th1-mediated responses (Suh et al., 2003), whereas ICOSL promotes IL-10 secretion by T cells (Witsch et al., 2002) and induces Tr1 cells that produce IL-10 and inhibit experimental airway inflammation in mice (Akbari et al., 2002; Conrad et al., 2012). The induction of IL-10–producing T cells by human @DCs was indeed mediated through ICOSL.

Infectious tolerance is a process in which a tolerance-inducing state is transferred from one cell population to another and is supported by different mechanisms, including IL-35, TGF-β production, or essential amino acid-depleting enzymes (Jonuleit et al., 2002; Cobbold et al., 2009; Collison et al., 2010). VD3-DCs transfer tolerogenic properties to mature pro-inflammatory DC via iTregs, which operate in an antigen-dependent manner (modeled in **Figure 3**). When applied as an intervention therapy, this implies that Tregs specific for a single auto antigen

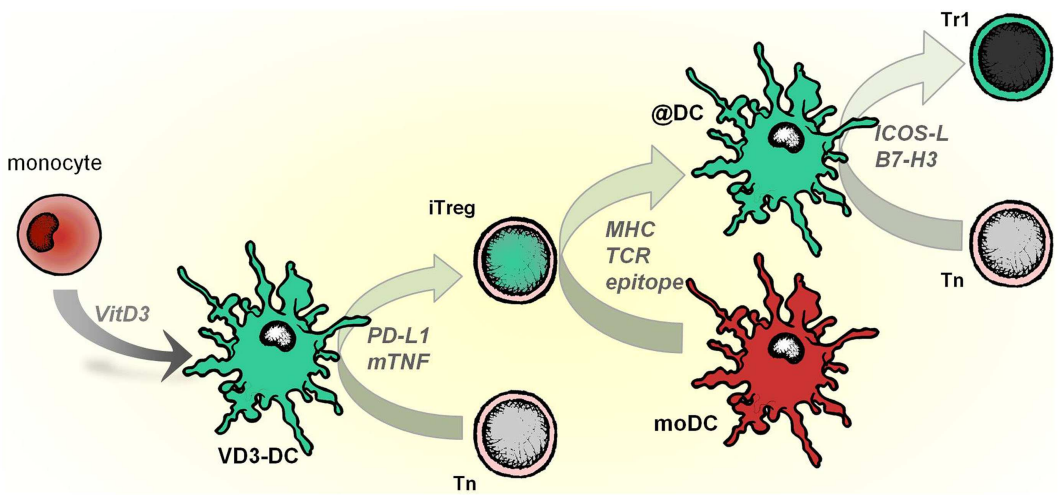

FIGURE 3 | VD3-DCs confer infectious tolerance through antigen-specific adaptive regulatory T cells (iTregs). Vitamin D modulates monocytes to differentiate into tolerogenic DCs (VD3-DCs), which induce iTregs. This process is mediated by PD-L1 and membrane-bound TNF (mTNF). iTregs, in turn, functionally modify pro-inflammatory moDCs to become anti-inflammatory DCs (@DCs), which present the cognate antigen of iTregs. As a consequence, @DCs upregulate inhibitory receptors ICOSL and B7-H3, lose the capacity to stimulate Th1 responses and instead induce IL-10–producing T cells from the naive T cell pool.

generated by tolerogenic DCs could alter other local DCs that subsequently reduce autoimmune responses to a diverse range of tissue-associated antigens.

Interplay between DCs and Tregs is essential for the immune tolerance since a direct correlation between DC numbers and Treg cells has been found as part of a feedback-control mechanism (Darrasse-Jeze et al., 2009). DCs promote and enhance the suppression by Treg cells and in turn, Tregs condition other DCs to upregulate inhibitory molecules and modulate effector responses. Instillation of VD3-DCs in this loop would support the regulatory and anti-inflammatory forces in the setting of autoimmune response that has escaped the regular control.

CONCLUDING REMARKS – TOWARD A TOLEROGENIC IMMUNOTHERAPY WITH DCs

Getting hold of a method to obtain efficient tolerogenic DCs in the clinical setting is a holy grail that will enable reshaping the immune response to particular needs. This may be accomplished using *ex vivo* modulated DCs. These cells might not be the same as tolerogenic DCs that exist *in vivo* and might need to be repetitively injected to achieve a continuous tolerogenic treatment. However, the *ex vivo* generation of tolerogenic DCs permits the propagation of sufficient amounts of cells in controlled conditions and loading with an antigen of choice thereby enabling targeted tolerizing immunotherapies.

Tolerogenic DCs modulated using the biologically active form of vitamin D ($1,25(OH)_2D3$) are a promising tool for tolerance induction in clinic. Years of investigation of these tolerogenic DCs have helped understand the contribution of different PRRs and secondary signals in facilitating tolerogenic DCs to prime antigen- and tissue-specific Tregs by mechanisms such as linked suppression and infectious tolerance. These learned lessons helped understanding how a cross-talk between DCs and T cells translates into immune regulation and may support finding therapies with small molecules that recapitulate the mode of action of cell therapy.

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Tolerogenic and activatory plasmacytoid dendritic cells in autoimmunity

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Plasmacytoid dendritic cells (pDCs) are a particular subset of DCs that link innate and adaptive immunity. They are responsible for the substantial production of type 1 interferon (IFN-I) in response to viral RNA or DNA through activation of TLR7 and 9. Furthermore, pDCs present antigens (Ag) and induce naïve T cell differentiation. It has been demonstrated that pDCs can induce immunogenic T cell responses through differentiation of cytotoxic $CD8^+$ T cells and effector $CD4^+$ T cells. Conversely, pDCs exhibit strong tolerogenic functions by inducing $CD8^+$ T cell deletion, $CD4^+$ T cell anergy, and T_{reg} differentiation. However, since IFN-I produced by pDCs efficiently activates and recruits conventional DCs, B cells, T cells, and NK cells, pDCs also indirectly affect the nature and the amplitude of adaptive immune responses. As a consequence, the precise role of Ag-presenting functions of pDCs in adaptive immunity has been difficult to dissect *in vivo*. Additionally, different experimental procedures led to conflicting results regarding the outcome of T cell responses induced by pDCs. During the development of autoimmunity, pDCs have been shown to play both immunogenic and tolerogenic functions depending on disease, disease progression, and the experimental conditions. In this review, we will discuss the relative contribution of innate and adaptive pDC functions in modulating T cell responses, particularly during the development of autoimmunity.

Keywords: plasmacytoid dendritic cells, type-I IFNs, antigen-presentation, tolerance, autoimmunity

PLASMACYTOID DENDRITIC CELL CHARACTERISTICS

Plasmacytoid dendritic cells (pDCs) were first described as either interferon (IFN) producing cells (Ronnblom et al., 1983; Chehimi et al., 1989; Fitzgerald-Bocarsly, 1993) or as plasmacytoid monocytes or plasmacytoid T cells in reference to their plasma-like morphology in secondary lymphoid organs (SLOs) (Facchetti et al., 1988). They were further defined as pre-DC2, as during activation they can differentiate into conventional DCs (cDCs)-like cells. These cells exhibit a DC morphology, with increased MHC class II (MHCII) and costimulatory molecule expression, and the ability to induce naïve $CD4^+$ T cell proliferation (Grouard et al., 1997). In 1999, the groups of M. Colonna and Y. J. Liu formally demonstrated that all these cell subtypes were actually the same entity, the pDCs (Cella et al., 1999; Siegal et al., 1999).

The development of pDCs occurs in the bone marrow (BM), after which they circulate through the blood stream, and reside in steady-state in the thymus and in the SLOs. Upon challenge via infection or inflammation, pDCs migrate and accumulate in inflamed tissues and draining lymph nodes (dLNs) (Reizis et al., 2011). The pDCs derive from the common dendritic progenitors (CDP) which express FLT3-R (CD135), CSF1-R (CD115), and low levels of c-kit (CD117) (Onai et al., 2007). Alternatively, pDCs may derive from lymphoid progenitors (Shigematsu et al., 2004; Luo and Lei, 2012; Sathe et al., 2013). Differentiation of pDCs relies essentially on Flt3-L which allows the expansion of cDC/pDC common progenitors and contributes to peripheral DC homeostasis (Waskow et al., 2008; Eidenschenk et al., 2010). The importance of E2-2 has been demonstrated for pDC differentiation (Cisse et al., 2008). It was further described that E2-2 drives the expression of other transcription factors involved in pDC fate, such as IRF-8 or Spi-B, while it inhibits other factors that are important for cDC differentiation, including Id-2 (Ghosh et al., 2010). Thus, a balance between the transcription factors E2-2 and Id-2 appears to control the differentiation toward the pDC lineage. Accordingly, in E2-2 deficient mice, pDCs exhibit an increased expression of Id-2 that correlates with a conversion into cDCs.

At the phenotypic level, pDCs are characterized by having intermediate (mouse) or no (human) expression of the DC marker CD11c. They are positive for the B cell marker CD45RA/B220, and express high levels of PDCA1, BST-2, Ly6C, and Ly49Q in mouse and BDCA-2, ILT-7, IL3Ra (CD123), and BDCA-4 in human (Reizis et al., 2011).

INNATE PLASMACYTOID DC FUNCTIONS

FEATURES OF pDC INNATE FUNCTIONS

Plasmacytoid DCs are strong sensors of non-self nucleic acids derived from bacteria or viruses through binding to Toll-like receptors (TLR). Nucleic acids come from either viruses internalized by endocytosis, cytoplasmic viral RNA by autophagy (Lee et al., 2007), or other infected cells via exosome transport (Dreux et al., 2012). The receptors TLR7 and TLR9 are selectively expressed by pDCs. Interestingly, pDCs are the only DC subset expressing TLR9 in humans (Jarrossay et al., 2001; Kadowaki et al.,

2001; Hornung et al., 2002) but not in mice (Chen et al., 2006). TLR7 senses guanosine or uridine rich single-stranded RNA from viruses or synthetic compounds such as imidazoquinoline and guanosine analogs (Diebold et al., 2004; Heil et al., 2004; Lund et al., 2004), while TLR9 recognizes single-stranded DNA containing unmethylated CpG motifs commonly found in viral and bacterial genomes (Hemmi et al., 2000; Bauer et al., 2001). The activation of TLR9 can also be through synthetic oligonucleotides (ODN) that mimic viral ssDNA responses (Kadowaki et al., 2001). TLR binding signals through MyD88, an adaptor protein that forms a signaling scaffold with IRAK-4, TRAF-6, and Btk, and induces the formation of the TRAF-3/IRAK-1/IKK-α/OPN/PI3K complex. As a consequence, IRF-7 is phosphorylated and subsequently translocated in the nucleus where it induces IFN-I gene transcription. Signaling through TRAF-6 also induces the NF-κB and MAPK pathways, leading to the secretion of inflammatory cytokines and chemokines, and the up-regulation of costimulatory molecules (Gilliet et al., 2008). Unlike other cells, where expression is dependent on IFNAR signaling, IRF-7 is constitutively expressed in pDCs (Honda et al., 2005b; Ito et al., 2006), possibly due to the low expression of the negative translational repressors 4EBPs (Colina et al., 2008).

The TLR ligands CpG-ODN have been classified as CpG-A, -B, and -C based on the different immune responses induced. In pDCs, CpG-A gives rise to robust IFN-I production, whereas CpG-B induces the production of inflammatory cytokines such as TNF or IL-6, as well as the up-regulation of MHCII and costimulatory molecules at the pDC surface (Kerkmann et al., 2005). These distinct effects of CpG-A and -B are pDC-specific and rely on different intracellular localizations after internalization. CpG-A possesses a poly-G tail, leading to the formation of a large multimeric complex that is retained in early endosomes and signals through MyD88 and IRF-7, thus inducing a strong IFN-I response. Conversely, monomeric CpG-B fails to be retained in early endosomes and rapidly travels to late endosomes/lysosomes to induce TNF and IL-6 production, and costimulatory molecule expression (Honda et al., 2005a; Guiducci et al., 2006).

Production of IFN-I by pDCs is much stronger (200–1000 times more effective) than any other cell type (Siegal et al., 1999). Multiple subtypes of IFN-I are secreted by pDCs, including IFNα, β, κ, ω, λ, and τ (Ito et al., 2006). During viral infections, pDC ablation selectively abrogates the early peak of IFN-I and leads to an increased viral burden (Swiecki et al., 2010), suggesting that IFN-I secretion is particularly crucial at the beginning of the antiviral response. By itself, IFN-I promotes the expression of IFN-stimulated genes that inhibit viral spreading through different mechanisms: (i) inhibition of viral replication by RNA degradation (Malathi et al., 2005) or by decreased protein synthesis (Barber et al., 1993); (ii) establishment of an antiviral state in uninfected cells; and (iii) induction of infected cell apoptosis (Pestka et al., 2004). Importantly, IFN-I links innate and adaptive immunity as it induces the differentiation, maturation, and activation of myeloid DCs that in turn promote antiviral T cell immunity (Paquette et al., 1998; Santini et al., 2000; Hibbert et al., 2003; Le Bon et al., 2003; Fonteneau et al., 2004; Yoneyama et al., 2005). Furthermore, IFN-I activates the antiviral functions of NK cells (Gerosa et al., 2005) and B cells (Jego et al., 2003). Using conditional targeting of the pDC-specific transcription factor E2-2, an elegant study has shown that pDC-deficient mice fail to clear chronic LCMV infection (Cervantes-Barragan et al., 2012). This defect correlates with impaired LCMV-specific $CD4^+$ and $CD8^+$ T cell numbers and functions, and relies on the lack of IFN-I production by pDCs, independently of their antigen (Ag) presenting capabilities.

INNATE pDC FUNCTIONS AND AUTOIMMUNITY

Self-DNA has been demonstrated to activate pDCs in autoimmune diseases. Under normal conditions, self-DNA is not recognized by pDCs since, when released by necrotic and apoptotic cells (Pisetsky and Fairhurst, 2007), it remains in the extracellular environment (Barton et al., 2006) and is rapidly degraded by DNAses. Following skin injury in psoriasis, self-DNA is released in the extracellular milieu (Lande et al., 2007) and sensed by pDCs (Nestle et al., 2005). In the wounded skin, the cationic anti-microbial cathelicidin LL37 is produced by keratinocytes and neutrophils (Zasloff, 2002). LL37 production is increased in psoriatic skin, binds the DNA released by dying cells, and forms aggregates which are resistant to extracellular nucleases (Lande et al., 2007). These complexes enter pDCs by endocytosis via lipid rafts and interactions with proteoglycans (Sandgren et al., 2004), and localize in early endosomes (similarly to CpG-A) to induce a strong IFN-I response. Consistently, an Imiquimod (TLR7 ligand)-containing topical cream was shown to exacerbate psoriatic lesions (Gilliet et al., 2004).

It has also been shown that IFN-I also plays a major role in the development of systemic lupus erythematosus (SLE) (Chan et al., 2012), by inducing the differentiation of both auto-antibody producing plasma cells (Jego et al., 2003; Thibault et al., 2009; Mathian et al., 2011) and cDC-driven effector T cells (Blanco et al., 2001). In SLE, apoptotic and necrotic cell derived self-DNA are complexed with (i) the peptide LL37, released by apoptotic neutrophil extracellular traps (NETs) in skin lesions (Bennett et al., 2003; Garcia-Romo et al., 2011; Lande et al., 2011), (ii) the protein HMGB1, also released in NETs, which binds aggregated nucleic acids (like CpG-A) (Tian et al., 2007), and (iii) by auto-antibodies directed against nucleic acids or nucleoproteins (Lovgren et al., 2004). These immune complexes allow the delivery of self-DNA into pDCs through the interaction of auto-Ab with FcγRII (CD32), and LC3-associated phagocytosis, a process described as a convergence between phagocytosis and non-conventional autophagy (Henault et al., 2012). In endosomes, self-DNA activates TLR signaling and subsequent IFN-I production (Lovgren et al., 2004; Barrat et al., 2005). Moreover, HMGB1 interacts with RAGE at the pDC surface, facilitating TLR9/DNA-binding in endosomes without inducing endocytosis (Tian et al., 2007). Finally, the resulting robust production of IFN-I, together with the presence of anti-LL37 auto-Abs in sera of SLE patients, induces the release of NETs and the continuous release of immune complexes (Garcia-Romo et al., 2011; Lande et al., 2011).

In multiple sclerosis (MS), many studies have suggested a protective role of pDCs and IFN-I. Indeed, relapsing patients treated with IFNβ-1a exhibit both a reduction in disease severity and a delay in relapses (Goodkin, 1996). In experimental autoimmune encephalomyelitis (EAE), a deficiency of IFNAR in central nervous system (CNS) myeloid cells exacerbates disease development (Prinz et al., 2008). In addition, pDCs have

been shown to inhibit cDC functions and, consequently, dampen the development of encephalitogenic Th17 cells (Bailey et al., 2007), whereas anti-PDCA1 mediated pDC depletion increases EAE severity (Bailey-Bucktrout et al., 2008). As discussed by the authors, and consistent with the IFN-I dependent inhibition of Th17 inflammation in the CNS (Guo et al., 2008), pDC-mediated protection may indeed depend on IFN-I. However, the molecular mechanisms accounting for the local suppression of pathogenic T cells in the CNS by pro-inflammatory IFN-I remain unclear.

Until recently, the contribution of pDCs and IFN-I to pathogenesis in type 1 diabetes (T1D) remained controversial. The role of IFN-I, first believed to be protective (Sobel and Ahvazi, 1998; Sobel et al., 1998; Tanaka-Kataoka et al., 1999; Brod, 2002) has been revisited as IFN-I expression in insulin-producing β cells exacerbates T1D progression (Stewart et al., 1993; Alba et al., 2004). Furthermore, whereas pDCs were demonstrated to secrete IFN-I in dLNs from 3-week-old Non-Obese Diabetic (NOD) mice, pDC depletion in this strain dampened IFN-I production and diabetes progression. This protective effect is seemingly IFN-I dependent, since IFNAR blockade equally delayed diabetes onset (Li et al., 2008). Finally, Diana et al. recently formally demonstrated that IFN-α-producing pDCs are required for the initiation of diabetogenic T cell responses and T1D development. The study found that spontaneous β-cell death in young NOD mice induces the recruitment of B-1a cells, neutrophils, and pDCs in the pancreas. B-1a cells secrete anti-dsDNA IgGs which activate neutrophils that in turn release DNA-binding CRAMP (cathelicidin related antimicrobial peptide) in NETs. These immune complexes activate pDCs through TLR9, leading to local IFN-α production. Using depleting antibodies, the authors further demonstrated that IFN-α producing pDCs are essential to initiate T1D in NOD mice (Diana et al., 2013).

ADAPTIVE PLASMACYTOID DC FUNCTIONS

pDCs FUNCTION AS BONA FIDE Ag PRESENTING CELLS

Accumulating evidence has revealed that pDCs can function as Ag presenting cells (APCs). In steady-state, pDCs can be easily detected in the blood, the thymus, and all SLOs (Bendriss-Vermare et al., 2001; Nakano et al., 2001; Summers et al., 2001; Asselin-Paturel et al., 2003; Seth et al., 2011). Upon inflammation, pDCs are recruited on a massive scale to infected or inflamed tissues, as well as to associated dLNs, and, importantly, in the LN T cell area, supporting a role for pDCs in activating naïve T cells (Cella et al., 1999; Krug et al., 2002; Vanbervliet et al., 2003; Irla et al., 2010). *In vitro*, pDCs exhibit the ability to capture, process, and present Ags through MHCI and MHCII molecules (Villadangos and Young, 2008; Tel et al., 2012). Steady-state pDCs were mainly described to be tolerogenic. Moreover, following TLR activation, pDCs upregulate MHCII and costimulatory molecules, which allow the direct modulation of the adaptive immune response. However, distinct mechanisms regulate Ag capture and processing in cDCs and pDCs, as well as resulting T cell outcome, suggesting complementary and, for the most part, non-overlapping functions of these two DC subsets.

Intracellularly Ag derived peptides either expressed by the cell itself (Krug et al., 2003; Young et al., 2008) or derived from intracellular-virus (Fonteneau et al., 2003; Salio et al., 2004; Schlecht et al., 2004; McGill et al., 2008; Young et al., 2008) are efficiently presented by pDCs through MHCI. Whether pDCs can phagocytose bacteria is still unclear (Villadangos and Young, 2008), while they have been shown to endocytose virions and exogenous proteins. pDCs express various endocytic receptors: Siglec-H (Zhang et al., 2006); the tetherin BST-2 (CD317) (Neil et al., 2008; Viswanathan et al., 2011); DCIR, which mediates clathrin-dependent endocytosis (Meyer-Wentrup et al., 2008); FcgRII (CD32), which allows the uptake of opsonized-Ags (Benitez-Ribas et al., 2006; Bjorck et al., 2008); and, specific to human pDCs, BDCA-2 which allows Ag delivery in the Ag processing compartment (Dzionek et al., 2001). Other receptors, such as ILT-7 (Cao et al., 2006) or NKp44 (Brown et al., 2004) may mediate endocytosis by pDCs, although this hypothesis needs further experimental confirmation (Villadangos and Young, 2008). Interestingly, ligation of these endocytic receptors potently suppresses IFN-I production by pDCs after TLR9 triggering (Dzionek et al., 2001; Blasius et al., 2004; Cao et al., 2007; Meyer-Wentrup et al., 2008). Finally, pDCs also acquire Ags from exosomes or apoptotic bodies (Hoeffel et al., 2007; Bastos-Amador et al., 2012).

Recent data suggests that mouse and human pDCs efficiently cross-present exogenous Ags to $CD8^+$ T cells. *In vitro*, TLR-activated pDCs capture, process, and cross-present exogenous proteins to $CD8^+$ T cells to induce proliferation, IFN-γ production, and cytolytic activity (Mouries et al., 2008; Kool et al., 2011). Accordingly, *in vivo* OVA delivery to CpG-activated pDCs via Siglec-H induces the generation of Ag-specific $CD8^+$ T cells (Zhang et al., 2006), although T cell effector functions were not investigated in this study. More recently, it was shown that pDC-depleted Siglec-H-DTR mice immunized with OVA protein in the presence of TLR ligands exhibit impaired OVA-specific $CD8^+$ T cell proliferation. Furthermore, the generation of MHC-I-OVA tetramer$^+$ $CD44^{hi}$ $CD8^+$ T cells, as well as OVA-specific effector CTL functions, are impaired after pDC depletion (Takagi et al., 2011). However, this study cannot exclude that decreased $CD8^+$ T cell activation and differentiation observed following pDC depletion does not reflect the abrogation of pDC cross-presenting functions, rather than simply the absence of pDC-mediated licensing of cDCs (Yoneyama et al., 2005). Accordingly, the depletion of cDCs in mice further co-immunized with OVA protein and CpG completely abrogates OVA-specific $CD8^+$ T cell responses, suggesting that pDCs do not cross-present exogenous proteins to $CD8^+$ T cells (Sapoznikov et al., 2007). Thus, the ability of murine pDCs to cross-present Ags to $CD8^+$ T cells remains controversial. In humans however, it is accepted that blood pDCs efficiently cross-present viral Ags and initiate Ag-specific antiviral $CD8^+$ T cell responses after being exposed to either influenza virus (Di Pucchio et al., 2008) or HIV-1 infected apoptotic cells (Hoeffel et al., 2007; Lui et al., 2009). The ability of pDCs to cross-present viral Ags does not seem to require IFN-I production but this function is strongly enhanced after TLR activation with synthetic compounds or influenza virus infection (Hoeffel et al., 2007).

Although some studies cited above suggested that the efficiency of Ag cross-presentation by pDCs to $CD8^+$ T cells was comparable to that of cDCs, pDCs appear to be much less potent APCs compared to cDCs in stimulating $CD4^+$ T cells. For instance, both *in vitro* and *in vivo*, CpG-activated pDCs were found not

to be as efficient as cDCs at presenting exogenous Ag through MHCII to $CD4^+$ T cells (Young et al., 2008; Kool et al., 2011). One possible explanation is that mouse cDCs and pDCs exhibit major differences in their Ag presentation machinery. Notably, MHCII molecules are differentially regulated in pDCs and cDCs. MHCII expression is regulated by the class II master transactivator gene CIITA, itself expressed under the control of distinct cell specific promoters (Reith et al., 2005). In particular, pDCs rely strictly on the B cell promoter pIII, whereas macrophages and cDCs depend on pI (LeibundGut-Landmann et al., 2004). In contrast to cDCs, activated pDCs fail to shut down MHCII synthesis (LeibundGut-Landmann et al., 2004) and turnover (Young et al., 2008), thereby allowing continued Ag presentation after activation. These features may indeed render pDCs better equipped for the continuous presentation of Ags, and allow them to present newly formed MHCII-peptide complexes even after activation (Sadaka et al., 2009), a feature with obvious advantages at sites of infection (Villadangos and Young, 2008). On the other hand, since pDCs lack the ability to accumulate long-lived MHCII-peptide complexes generated shortly after activation, it may also render these cells less efficient compared to cDCs at promoting effector $CD4^+$ T cell responses, due to the low the exposure time of the peptide on cell surface.

IMPACT ON T HELPER FUNCTIONS

As discussed above, activated pDCs present Ags to naïve $CD4^+$ T cells and thus directly contribute to T cell responses as APCs. In addition, pDCs can also indirectly impact T cells by producing inflammatory cytokines. As most studies eliminate pDCs genetically (Swiecki et al., 2010; Takagi et al., 2011) or through depleting mAbs (Bailey-Bucktrout et al., 2008; Jongbloed et al., 2009), it has been difficult so far to decipher the relative contribution of innate and adaptive pDC functions. However, accumulating evidence, mainly supported by *in vitro* studies, *in vivo* Ag targeting and abrogation of MHCII expression on pDCs, suggest a direct role of pDCs in impacting T cell responses. It is largely accepted that steady-state cDCs constantly present self- and non-self-Ags in a fashion that promotes T cell tolerance. Conversely, signals derived from pathogens or tissue damage generally boost cDC maturation, which promotes their capacity to induce effector T cell responses (Steinman, 2007). For pDCs however, their ability to promote either tolerance or immunity seems not to rely entirely on their activation state. Indeed, whereas immature pDCs exclusively promote tolerance, activated pDCs, depending on the anatomical localization and the cytokine milieu, may have both immunogenic and tolerogenic functions, although the exact nature of these functions remains to be established.

Immunogenic pDCs

Previous studies performed *in vitro* showed that both human and mouse activated pDCs, given an antigenic peptide together with appropriate activating signals, activate naïve $CD4^+$ T cells, and promote Th1 differentiation (Cella et al., 2000; Krug et al., 2001; Boonstra et al., 2003). In mice devoid of cDCs, CpG-treated LN pDCs promote Th1 development and memory differentiation *in vivo* (Sapoznikov et al., 2007). Similarly, Ag-specific delivery to TLR-activated pDCs via BST-2 induces Ag-specific Th1 development *in vivo*, as demonstrated by the production of IFN-γ by $CD4^+$ T cells and subsequent immunoglobulin production (Loschko et al., 2011b) (**Figure 1**). pDCs have also been shown to induce Th17 responses in different experimental models. Human TLR7-triggered pDCs promote Th17 differentiation from either naïve or memory $CD4^+$ T cells (Yu et al., 2010). In patients with GVHD, increased pDCs and Th17 cell numbers in the intestinal mucosa correlate with disease severity, suggesting a role of pDCs in driving Th17 responses during disease (Bossard et al., 2012). Furthermore, in tumor bearing mice, pDCs activation through CpG correlates with an increase of tumor-specific Th17 cells and an inhibition of the tumor growth (Xu et al., 2012) (**Figure 1**). The ability of pDCs to promote Th17 differentiation seems to be enhanced in the presence of TGF-β. Indeed, the transfer of TGF-β treated pDCs to collagen-induced arthritic mice leads to increased Th17 responses in LNs leading to increased disease severity (Bonnefoy et al., 2011). In EAE, anti-PDCA1 mediated pDC depletion resulted in impaired encephalitogenic Th17 responses significantly reducing early clinical scores (Isaksson et al., 2009). Moreover, pDCs may be able to convert T_{regs} into Th17 cells. Indeed, in rats, $Foxp3^+$ T cells start to produce IL-17 when activated by mature pDCs (Gautreau et al., 2011). Using genetically modified mice selectively lacking MHCII expression by pDCs (LeibundGut-Landmann et al., 2004), we have found that CpG-activated pDCs function as APCs to induce Th17 responses *in vivo* (Guery et al., manuscript in preparation) (**Figure 1**). Our unpublished data further suggest that the ability of activated pDCs to promote Th17 cells may be used as a vaccination strategy against tumors.

Tolerogenic pDCs

Plasmacytoid DCs have also been demonstrated to be involved in the induction of central and peripheral tolerance. It has been suggested that the function of pDCs within the thymus, as in other tissues, might simply be to protect the tissue from viral infections (Wu and Shortman, 2005). However, it was recently suggested that recirculating pDCs might present self-Ags in the thymus and contribute to the inactivation, or deletion, of autoreactive T cells. pDCs were detected in human thymus, colocalize with $Foxp3^+$ T_{regs}, and, when activated with CD40L plus IL-3, efficiently promote the development of $Foxp3^+$ natural T_{regs} (nT_{regs}) from autologous thymocytes (Martin-Gayo et al., 2010). Similarly, human thymic pDCs activated with CpG and TSLP induce nT_{reg} generation (Hanabuchi et al., 2010). In these two studies, T_{regs} generated by pDCs produce more IL-10 and less TGF-β compared to nT_{regs} primed by cDCs under the same conditions, suggesting a complementary effect of the two DC subsets in the development of central tolerance. However, whether pDCs actually promote thymocyte differentiation into nT_{reg} *in vivo* remains to be firmly demonstrated. In contrast to human pDCs, murine thymic pDCs do not efficiently induce T_{reg} differentiation from thymocytes *in vitro* (Proietto et al., 2009). *In vivo*, a recent study illustrated the importance of CCR9 in targeting peripheral immature pDCs to the thymus. This indicates a role for pDCs in presenting extrathymically acquired Ags to further induce the deletion of Ag-specific $CD4^+$ thymocytes (Hadeiba et al., 2012). No role for pDCs in the generation of nT_{regs} was observed in this study, suggesting that

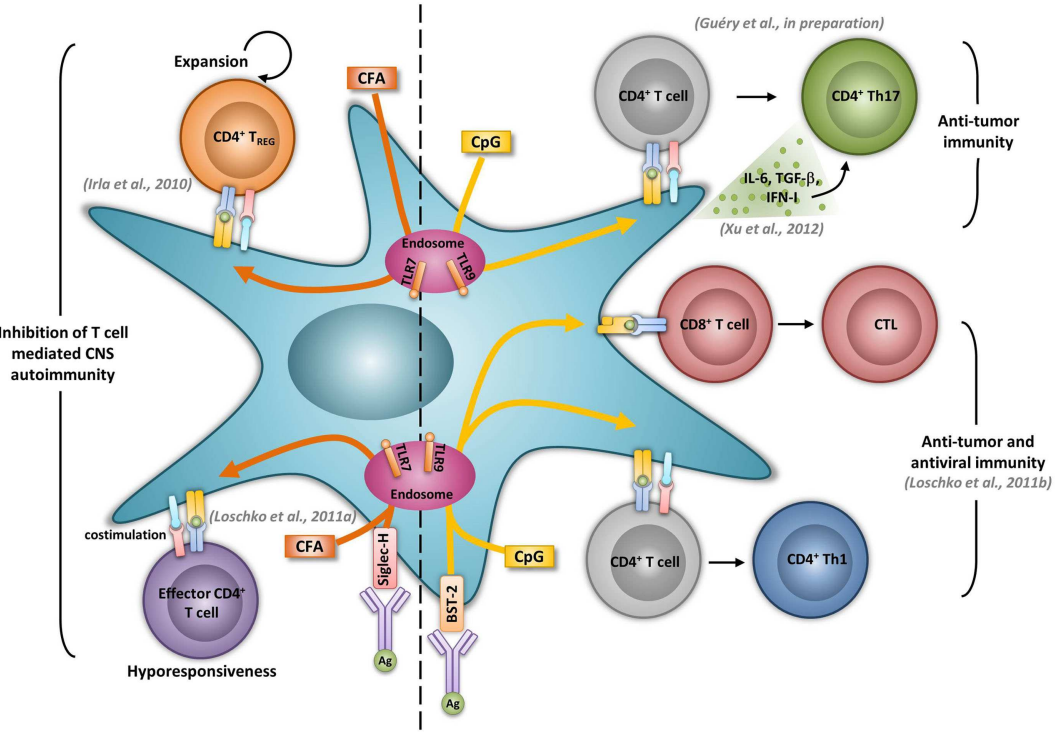

FIGURE 1 | Activated pDCs induce dual tolerogenic and immunogenic T cell responses. The most relevant *in vivo* experimental animal systems are represented in this figure.

mouse pDCs are intrinsically inefficient at inducing T_{regs} in the thymic environment *in vivo*.

Although it was suggested that pDCs are poor presenters of Ag in the absence of microbial stimulation (Colonna et al., 2004), immature pDCs promote T cell anergy, T cell deletion, as well as T_{reg} differentiation. In humans, freshly isolated pDCs induce anergy in human $CD4^+$ T cell clones, but it is reversed upon the addition of exogenous IL-2 (Kuwana, 2002). In mice, pDCs prevent oral T cell priming and are responsible for systemic tolerance to dietary Ags including proteins and haptens (Goubier et al., 2008). Indeed, pDC depletion induces hypersensitivity and $CD8^+$ T cell responses toward oral Ags. In contrast, the transfer of Ag-loaded immature pDCs in naive mice suppresses both Ag-specific $CD4^+$ and $CD8^+$ T cell responses by inducing either anergy or deletion, suggesting that oral tolerance relies on Ag presentation by pDCs (Goubier et al., 2008) (**Figure 2**). Immature pDCs expressing CCR9 were defined as inducers of $Foxp3^+$ T_{regs} that suppress Ag-specific immune response in a GVHD model. Importantly, transferred $CCR9^+$ pDCs efficiently suppressed allogenic GVDH (Hadeiba et al., 2008). pDCs were also identified as phagocytic APCs essential for tolerance to vascularized cardiac allografts (Ochando et al., 2006) (**Figure 2**). In this model, alloantigen-presenting pDCs home to the LNs under tolerogenic conditions, where they mediate alloantigen-specific T_{reg} development and allograft tolerance. In an other model of cardiac allograft transplantation, the absence of pDCs in LNs from $CCR7^{-/-}$ mice impairs T_{reg} induction, and results in graft rejection, whereas pDC transfer restores both T_{reg} frequencies and tolerance to the cardiac allograft (Liu et al., 2011) (**Figure 2**). In rats, pDCs also induce tolerance to allografts by inhibiting $CD4^+$ T cells either directly through an indoleamine 2,3-dioxygenase (IDO)-dependent mechanism, or indirectly through the induction of $CD8^+$ T_{regs} (Li et al., 2010) (**Figure 2**). Furthermore, pDC depletion in mice induces classical features of asthma after inhalation of an inert Ag, including IgE sensitization, airway eosinophilia, goblet cell hyperplasia, and Th2 cytokine production. In contrast, the adoptive transfer of pDCs before sensitization prevents asthma, possibly through the induction of T_{regs} (de Heer et al., 2004). Using Siglec-H inducible deficient mice, a recent study nicely showed that Siglec-H controls the ability of steady-state pDCs to induce the conversion of naïve $CD4^+$ T cells in inducible T_{regs} (iT_{regs}) *in vivo* (Takagi et al., 2011). In the contexts of tumors, the microenvironment maintains a resting pDC phenotype, characterized by low expression of costimulatory molecules and low IFN-I production (Hartmann et al., 2003; Conrad et al., 2012; Sisirak et al., 2012). Consequently, pDCs induce mainly tolerogenic tumor $CD4^+$ T cell responses, through IDO-dependent T_{reg} generation (Sharma et al., 2007) (**Figure 2**). Accordingly, IDO inhibition in pDCs promotes the conversion of T_{regs} into Th17 cells that efficiently inhibit tumor growth (Sharma et al., 2009). ICOS-L expression by pDCs

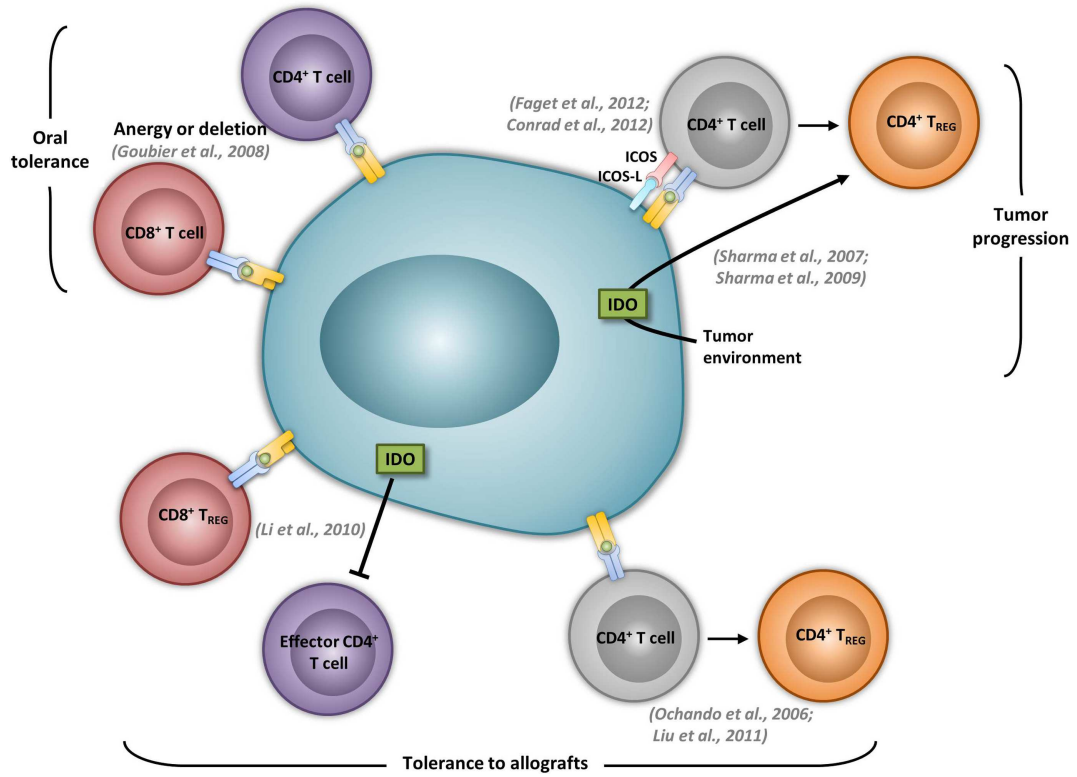

FIGURE 2 | Immature pDCs induce tolergenic T cell responses. The most relevant *in vivo* experimental animal systems are represented in this figure.

in the tumor context has also been implicated in their ability to generate T_{regs} (Conrad et al., 2012; Faget et al., 2012) (**Figure 2**).

Activated pDCs were demonstrated to favor T_{reg} development. T_{reg} induction has been correlated with low peptide-MHCII densities on APCs (Kang et al., 2007; Turner et al., 2009). Thus, the ability of activated pDCs to induce T_{regs} might be explained by the fact that they do not stabilize peptide-MHCII complexes at their cell surface, and, thus, provide a weak TCR engagement promoting T_{reg} development. In humans, TLR-activated pDCs induce the development of IL-10 producing T_{regs} in an ICOS dependent manner (Ito et al., 2007; Ogata et al., 2013). Furthermore, HIV-stimulated IDO expressing pDCs induce the differentiation of naive $CD4^+$ T cells into T_{regs} (Manches et al., 2008). CpG-activated human pDCs can also promote T_{reg} differentiation (Moseman et al., 2004), possibly in an IDO-dependent fashion (Chen et al., 2008). Finally, TLR-activated rat pDCs induce T_{reg} proliferation *in vitro* (Ouabed et al., 2008).

The severity of several autoimmune diseases has been demonstrated to be regulated by pDCs. In rheumatoid arthritis, mature pDCs from patients express high levels of IDO and are necessary for the differentiation of allogeneic naïve $CD4^+$ $CD25^-$ T cells into IL-10 producing T_{reg} (Tr1) cells (Kavousanaki et al., 2010). Moreover, in an *in vivo* mouse model of arthritis, pDC depletion correlates with enhanced articular pathology and increased T and B cell autoimmune responses to type II collagen (Jongbloed et al., 2009). In lupus, low dose Ag therapy induces the production of TGF-β by pDCs and dampens their ability to respond to TLR stimulation. The transfer of these tolerogenic pDCs promotes T_{reg} expansion while simultaneously suppressing inflammatory Th17 infiltrating the kidney of lupus-prone mice (Kang et al., 2007). Ag targeting in pDCs has also been shown to inhibit T helper cell dependent autoimmunity. In EAE, Siglec-H mediated $MOG_{35–55}$ delivery to pDCs dampens EAE, by inducing MOG-specific $CD4^+$ T cell hyporesponsiveness resulting in the impaired induction of Th1 and Th17 cells, without promoting T_{reg} differentiation (Loschko et al., 2011a) (**Figure 1**). Using a genetic mouse model in which MHCII is specifically abrogated in pDCs, we also identified a tolerogenic role for Ag presenting pDC functions during EAE. We demonstrated that pDCs, by presenting myelin Ags to naïve $CD4^+$ T cells, induce the expansion of nT_{regs}. As a consequence, mice carrying a selective abrogation of MHCII on pDCs exhibit impaired nT_{reg} expansion, increased encephalitogenic Th1 and Th17 responses and exacerbated EAE (Irla et al., 2010) (**Figure 1**).

As discussed before, pDCs contribute to the pathology of T1D through the production of IFN-I. However, pDCs are also implicated in the inhibition of diabetogenic T cells during infections. In RIP-LCMV mice, OX40-OX40L dependent pDC–iNKT cell

interactions control viral replication in pancreatic islets of LCMV infected mice by inducing IFN-I production by pDCs (Diana et al., 2009). In addition, this pDC–iNKT cooperation has been reported to promote TGF-β production by pDCs, that in turn, acquire the ability to convert naive anti-islet $CD4^+$ T cells into $Foxp3^+$ T_{regs} in pancreatic dLNs. These T_{regs} are then recruited in the pancreatic islets where they produce TGF-β that inhibits islet-specific $CD8^+$ T cells and dampens T1D severity (Diana et al., 2011). In NOD mice, pDC depletion leads to accelerated insulitis and disease onset, and correlates with a local loss of IDO, suggesting that during T1D, pDCs may exert IDO-dependent tolerogenic functions by regulating islet-specific $CD4^+$ T cell responses (Saxena et al., 2007).

In summary, pDCs in SLO were shown to contribute to T cell tolerance in several experimental systems, regardless of whether they exhibit a steady-state or activated phenotype (**Figures 1** and **2**). Interestingly, tolerogenic pDC functions were demonstrated to be dependent on IDO in several models. Furthermore, IDO contribution does not seem to rely on a pDC inflammatory environment. Why and how IDO is involved in the ability of pDCs to dampen some, but not all, Ag-specific T cell responses is still a matter of debate. This phenomenon is more complex due to recent findings demonstrating a second function for pDC-derived IDO. Together with the tryptophan catalytic activity inhibiting effector T cell function, IDO has been postulated to act as a signaling protein in response to TGF-β inducing the conversion of naïve $CD4^+$ T cells into T_{regs} (Pallotta et al., 2011). Thus, the relative contribution of the dual IDO functions needs to be addressed in the different models where an IDO-dependent inhibition of T cell responses by pDCs has been described.

CONCLUDING REMARKS

Thus, steady-state pDCs exclusively promote T cell tolerance. However, the emerging picture of pDC functions during the development of inflammatory autoimmune disorders is that they contribute to disease pathogenesis by the production of IFN-I, while promoting self-Ag-specific $CD4^+$ T cell tolerance though their ability to present auto-Ag. Altogether, studies show that innate and adaptive pDC functions may have opposite effects on T cell tolerance toward self-tissues. Interestingly, the engagement of endocytic receptors favors Ag presenting pDC functions, while it dampens their ability to produce IFN-I, suggesting that Ag targeting in pDCs would represent an attractive therapeutical strategy to control autoimmunity and graft rejection.

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