Role of dendritic cell maturity/costimulation for generation, homeostasis, and suppressive activity of regulatory T cells

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Tolérancy of dendritic cells (DCs) has initially been attributed exclusively to immature/resting stages, while mature/activated DCs were considered strictly immunogenic. Later, all different subsets among the myeloid/conventional DCs and plasmacytoid DCs have been shown to bear tolerogenic potential, so that tolerogenicity could not be attributed to a specific subset. Immunosuppressive treatments of immature DC subsets could prevent re-programming into mature DCs or upregulated inhibitory surface markers or cytokines. Furthermore, the different T cell tolerance mechanisms anergy, deletion, immune deviation, and suppression require different quantities and qualities of costimulation by DCs. Since expansion of regulatory T cells (Tregs) has been shown to be promoted best by fully mature DCs the role of CD80/B7-1 and CD86/B7-2 as major costimulatory molecules for Treg biology is under debate. In this review, we discuss the role of these and other costimulatory molecules on myeloid DCs and their ligands CD28 and CD152/CTLA-4 on Tregs for peripheral conversion from naive CD4+ T cells into the major subsets of Foxp3+ Tregs and Foxp3− IL-10+ regulatory type-1 T cells (Tr1) or Tr1-like cells and their role for peripheral maintenance in the steady state and after activation.

Keywords: regulatory T cells, Foxp3, IL-10, dendritic cells, costimulation

SUBSETS OF Tregs

Regulatory T cells can be generated in the thymus and are released as so-called natural regulatory T cells (nTregs) or by extrathymic induction from naive T cells (iTregs) in secondary lymphoid organs (Horwitz et al., 2008; Sakaguchi et al., 2008; Yamazaki and Steinman, 2009). Several Treg subsets or functional states, depending on their anatomical origin or mode of generation, have been described (Sakaguchi et al., 2008) but a common signature or distinct sublineages may not exist (Feuerer et al., 2010). Two major subtypes of iTregs can be distinguished on the basis of their Foxp3 expression. Many authors refer only to the Foxp3+ subtypes as iTregs, while the Foxp3− IL-10+ T cells are mostly named Tr1 (T regulatory type-1) or Tr1-like cells (Figure 1). Of note, most activated Tregs seem to release IL-10, independent of their Foxp3 expression (Figure 1). Since these Treg subsets have been in focus the last years, there is ample information about their interaction with DCs and this will be discussed here. In contrast, the less well investigated IL-35+ Tregs and TGF-β-producing Th3 or CD8+ Tregs are reviewed elsewhere (Filaci and Suciu-Foca, 2002; Wang, 2008; Bettini and Vignali, 2009; Weiner et al., 2011). Since the generation of nTregs in the thymus seems not to depend on DCs but B7 costimulation on medullary epithelial cells and other APCs, we also refer to other reviews (Kysewski and Klein, 2006; Ohkura and Sakaguchi, 2010).

The best studied costimulatory molecules are CD80 (B7-1) and CD86 (B7-2). Both B7 molecules are the ligands of CD28 and CTLA-4 receptors on T cells (Lenschow et al., 1993). Whereas signaling by B7/CD28 pathway is crucial for enhancing T cell activation and survival, signaling by B7/CTLA-4 mainly regulates inhibitory T cell responses (Sansom and Walker, 2006). Moreover, selectivity for CD28 to CD86 and CTLA-4 to CD80 interactions may have to be considered due to stoichiometric and affinity measurements in vitro (Collins et al., 2002).

ROLE OF COSTIMULATION FOR THE GENERATION OF Tr1 CELLS

MOLECULAR FACTORS DRIVING IL-10 IN T CELLS

Initial reports defined Tr1 cells as IL-10 producing T cells which developed from naive T cells and acquired suppressive activity in the presence of IL-10. They adapted a particular cytokine expression profile distinct from Th1 or Th2 effector T cells (Groux et al., 1997). However, ever since, many IL-10 producing Tregs have been described that could be induced under various experimental conditions (Table 1) and as reviewed in Hawrylucz and O’Garra (2005), Roncarolo et al. (2006). Interestingly, high production of IL-10 and acquisition of regulatory function can also occur as a result of chronic stimulation of differentiated T helper cells thereby gradually losing production of effector cytokines such as IFN-γ or IL-4 (O’Garra et al., 2004). The question remains whether all Foxp3+ IL-10+ iTregs cells that are either derived from naive T cell precursors (Tr1) or from chronically stimulated effector T cells (Tr1-like) develop via similar IL-10- and costimulation-dependent signaling mechanisms. To date this remains unclear. However, for Th1-like cells common signaling pathways have been reported.

Saravia et al. identified the mitogen-activated protein kinases Erk1 and Erk2 along with strong T cell receptor (TCR) triggering...
Whereas the role of DC maturation and costimulation for the development and activation of the respective signal transducer and activator of transcription (STAT) as a common signaling pathway for the production of IL-10 by Th1, Th2, or Th17 effector T cells (Saraiva et al., 2009; Saraiva and O’Garra, 2010). Motomura et al. (2011) further supported the concept of a common signaling cascade for the appearance of IL-10 by effector T cells by identifying the central transcription factor E4 promoter-binding protein 4 (E4BP4), also known as NFIL3, essential for the regulation of both the IL-13 and IL-10 expression in chronically stimulated Th1 cells and other innate cells. Further research is needed to clarify the signaling pathways in T cells upstream of E4BP4 and the role DC-derived costimulatory molecules in this process.

**IL-10 AND THE DEVELOPMENT OF Tr1 CELLS**

Whereas the role of DC maturation and costimulation for the generation of Tr1 cells has remained poorly investigated, the requirement of IL-10 is well documented (Groux et al., 1997; Leving et al., 2005; Roncarolo et al., 2006). Indeed, differentiation of Tr1 by means of immunosuppressive drugs such as dexamethasone and vitamin D3 is inhibited even in APC-free conditions in the presence of anti-IL-10R antibodies (Barrat et al., 2002). Other studies on Tr1 differentiation by human immature DCs suggested that DC-derived IL-10 is critical for the generation of Tr1 cells (Leving et al., 2005; Gregori et al., 2010; van der Aar et al., 2011). The use of IL-10 reporter mice indicated that mouse Tr1 cells can develop in vivo in the absence of IL-10 (Maynard et al., 2007). It has been discussed whether IL-10 may only be required to maintain T cell anergy of Tr1 cells (Roncarolo et al., 2006). In this scenario IL-10 modulates the DC or APC maturation phenotype rather than through direct activity on T cells (Wakkach et al., 2001). Indeed, IL-10 is a potent down-modulator of MHC II and costimulatory molecule expression on DCs (Moore et al., 2001; Sato et al., 2002; Gabrysova et al., 2009; Gregori et al., 2010). Immature DCs cultured in the presence of additives such as IL-10, TGF-β, glucocorticoids or vitamin D3 analogs or low doses of GM-CSF can acquire even a maturation-resistant DC phenotype, thus remaining strictly immature and generating anergic T cells in vitro (Lutz, 2006). However, not all such anergic T cells gain regulatory capacity (Berger et al., 2009). It remains open whether this can be explained by the fact that T cell anergy can result either in complete absence of CD80/CD86 signals or by CD80/CD86 engagement of CTLA-4 (Greenwald et al., 2001; Macian et al., 2004).

**COSTIMULATION THROUGH CD80, CD86, CD58, AND ICOS-L**

The initial report used murine splenic APCs or human monocytes to differentiate Tr1 cells (Groux et al., 1997). Low levels of CD80/CD86 molecules are provided by both cellular sources. However, the requirement for CD28 or CTLA-4 signals for Tr1 cell generation have initially not been addressed. Several reports demonstrated that Tr1 cells typically express large amounts of CTLA-4 regardless of the effector T cell type the cells were originally derived from (Table 1). Indeed, Perez et al. (2008) demonstrated that preferential ligation of CTLA-4 by CD80 on mature DCs resulted in anergic T cells bearing suppressive activity and producing high amounts of IL-10 upon restimulation. Although the presence of anti-IL-10R antibodies abolished the differentiation of iTregs in this culture set-up, IL-10 production itself appeared largely dependent on CTLA-4 signaling (Perez et al., 2008). A role of CD58–CD2 interaction for human Tr1 has been proposed on the basis of studies using CD58-transfected artificial APCs (Leving et al., 2001; Wakkach et al., 2001). Since CD58 is not a ligand for CD2 in the mouse, this pathway may not follow a general rule for Tr1 generation. Others claimed that only immature DCs contributed to the differentiation of IL-10 producing Tr1 cells (Jonuleit et al., 2000; Leving et al., 2005). However, those human immature monocyte-derived DCs (although largely CD83 negative) express substantial CD80 and CD86 on their cell surface (Jonuleit et al., 2000; Gregori et al., 2010) and appear thereby rather semi-mature (Lutz and Schuler, 2002), whereas these markers can be hardly detected on freshly isolated Langerhans cells (McIlroy et al., 2001), splenic DCs (Berthier-Vergnes et al., 2001), or liver perfusate DCs (Bosma et al., 2006) from human donors. Only human DCs isolated from thymus or tonsils showed substantial expression of costimulatory markers (Summers et al., 2001; Vandenabeele et al., 2001). In addition, human Tr1 cells could be differentiated in the presence of exogenous IL-10 and monocyte-derived DCs, which did not produce IL-12p70 but expressed high levels of costimulatory molecules on their surface (Gregori et al., 2010). Thus, costimulation through CD80/CD86 as provided by semi-mature DCs, but not by truly immature DCs, is required to generate IL-10+ T cells from naive precursors (Figure 1).

Secretion of IL-10 for both Foxp3+ Treg or Tr1 cells has been linked to high ICOS surface expression (Hutloff et al., 1999; Akbari et al., 2002; Herman et al., 2004). Costimulation via ICOS-L (CD275) on the surface of mature myeloid DCs (Witsch et al., 2002) or mature plasmacytoid DCs (Ito et al., 2007) stimulates
Table 1 | Generation and phenotype of Tr1 and Tr1-like cells.

| Th origin | Cytokine origin | Cytokine shift toward | Antigens/DC system | Route | Anergy | CTLA-4 | In vitro/ in vivo | Human/ rodent | References |
|-----------|----------------|-----------------------|---------------------|-------|--------|--------|-----------------|--------------|------------|
| Th1       | IL-10^med      | IL-10^hi              | High dose bee venom (bee stings subcutaneously) | s.c. (skin) | +      | +      | In vivo        | Human        | Meiler et al. (2008) |
|           | IFN-γ^hi      | IFN-γ^lo              |                     |       |        |        |                 |              |            |
|           | IL-4^lo       | IL-4^hi               |                     |       |        |        |                 |              |            |
|           | IL-13^lo      | IL-13^hi              |                     |       |        |        |                 |              |            |
| ?         | ?              | IL-10^hi              | Aeroallergens       | Inhalation | +     | +      | In vivo        | Human        | Akdis et al. (2004) |
| Th2       | IL-10^hi      | IL-10^lo              | OVA protein (by pulmonary DC expressing high B7 costimulation and ICOS-L and producing IL-10) | i.n. | +      | ?      | In vivo        | Rodent       | Akbari et al. (2001, 2002) |
|           | IFN-γ^hi      | IFN-γ^lo              |                     |       |        |        |                 |              |            |
|           | IL-4^hi       | IL-4^lo               |                     |       |        |        |                 |              |            |
| Th1       | IL-10^hi      | IL-10^lo              | LPS-matured DC and anti-CD86 antibodies | (−)  | +     | +      | In vitro        | Rodent       | Perez et al. (2008) |
|           | IFN-γ^hi      | IFN-γ^lo              |                     |       |        |        |                 |              |            |
|           | IL-4^hi       | IL-4^lo               |                     |       |        |        |                 |              |            |
| Th1       | IFN-γ^hi      | IL-10^hi              | High affinity peptides | i.n. | +     | +      | In vitro        | Rodent       | Burkhart et al. (1999), Sundstedt et al. (2003), Gabrysova et al. (2009) |
|           | IL-4^lo       | IFN-γ^lo              |                     |       |        |        |                 |              |            |
|           | IL-5^lo       | IL-4^lo               |                     |       |        |        |                 |              |            |
| Th0       | Naïve (IL-4^lo only) | IL-10^hi             | Immunosuppressive drugs conditioned DC | (−)  | +     | +      | In vitro        | Both         | Barrat et al. (2002), Vieira et al. (2004) |
|           | Barrat et al., 2002 | IFN-γ^neg            |                     |       |        |        |                 |              |            |
|           | IL-5^neg      | IL-4^neg              |                     |       |        |        |                 |              |            |
|           | IL-4^lo only Bar- rat et al., 2002 | IFN-γ^neg            |                     |       |        |        |                 |              |            |
| Th0       | Naïve       | IL-10^hi              | Splenic APC, OVA peptide, and exogenous IL-10 | (−)  | +     | ?      | In vitro        | Both         | Groux et al. (1997) |
|           | IFN-γ^lo      | IFN-γ^hi              |                     |       |        |        |                 |              |            |
|           | IL-5^hi       | IL-4^lo               |                     |       |        |        |                 |              |            |

(Continued)
| Th origin | Cytokine origin | Cytokine shift toward | Antigens/DC system | Route | Anergy | CTLA-4 | In vitro/in vivo | Human/rodent | References |
|-----------|----------------|----------------------|--------------------|-------|--------|--------|-----------------|-------------|------------|
| Th0 (Naive) Cord versus peripheral blood CD4+ T cells | IL-10hi | Repetitive stimulation with allogeneic monocyte-derived DC (CD80 and CD86 high; CD83 neg) | (-) | + | + (Jonuleit et al., 2000) | In vitro | Human | Jonuleit et al. (2000), Levings et al. (2005) |
| Th0 (Naive) | IL-10hi | Artificial APC as L cell line transduced with CD58 and CD80 in addition of exogenous IL-10 and IFN-α | (-) | + | ? | In vitro | Human | Levings et al. (2001) |
| Th0 (Naive) IL-10 hi | IL-10 hi | Splenic DC with high dose OVA peptide and exogenous IL-12 | (-) s.c. | ? | ? | In vitro/ in vivo | Rodent | Saraiva et al. (2009) |
| Th1 IFN-γ hi IL-4 neg IL-5 neg IL-13 neg | IL-10 hi | Splenic APC and Th1 skewing conditions | (-) | ? | ? | In vitro | Rodent | Motomura et al. (2011) |
| Th0 (Naive) | IL-10 hi | Long-term culture of mature DC and “diffDC” or stroma conditioned DC | (-) | + | – | In vitro | Rodent | Xu et al., 2011 |
IL-10 release by Tr1 or Tr1-like cells, respectively. Enhanced expression of ICOS on in vitro generated Tr1 cells has also been attributed to the cytokine IL-27 (Pot et al., 2009). IL-27 can be produced by DCs upon interaction with Foxp3+ Tregs, which acquire a particular plasmacytoid-like DC phenotype expressing costimulatory molecules able to generate Tr1 cells (Awasthi et al., 2007). Human immature DCs specifically upregulate PD-L1 in the presence of exogenous IL-27 (Karakhanova et al., 2011). Further research is needed to define the role of a DC maturation phenotype in differentiation of Tr1 cells driven by exogenous IL-27 as most studies are performed under APC-free culture conditions.

Thus, some extent of costimulation by DCs seems necessary for the generation of Tr1 cells despite the fact that a variety of approaches have used “immature” DCs to generate Tr1 cells in vitro (Table 1).

**COSTIMULATION FOR Tr1-LIKE CELLS BY CHRONIC STIMULATION OF EFFECTOR T CELLS OR DURING INFECTION PLASTICITY OF T CELLS FOR PRODUCTION OF CYTOKINES OTHER THAN IL-10**

The concept of classical lineage commitment into Th1, Th2, and others has been challenged by recent work and it is suggested now that T cells show substantial functional plasticity in their cytokine secretion (O’Shea and Paul, 2010). As an example, IL-13 and IL-10 production by Th1 cells could be demonstrated upon chronic stimulation through TCR and CD28 pathways (Miomoto et al., 2011). The Tr1 cells described by Roncarolo and colleagues require IL-10 for their generation and then produce IL-10, some IFN-γ and IL-5 but no IL-4 (Groux et al., 1997; Levings et al., 2001). Originally IL-10 (but also IL-5 and IL-13) was defined as a classical Th2 cytokine, however, the mechanisms and/or costimulatory signals initiating IL-10 production in Th2 cells remained unclear (Moore et al., 2001). Expression of IL-10 by Th2 memory cells has been attributed to repetitive TCR receptor signaling and continuous IL-4 signals (Lohning et al., 2003; Chang et al., 2007). Alternatively, it is well established that the differentiation of IL-10 and IL-4 producing Th2 cells is CD28-signaling dependent but inhibited by CTLA-4 (Bour-Jordan et al., 2003; Coquerelle et al., 2009; Hunig et al., 2010). Indeed, injection of anti-CTLA-4 antibodies in the presence of splenic DCs induced ICOS+ Tregs producing both IL-4 and IL-10 (Coquerelle et al., 2009). In contrast, triggering CTLA-4 signaling on Th1 cells by CD80 from mature DCs seems to promote IL-10 secretion and Tr1 differentiation (Perez et al., 2008). These observations not only emphasize the necessity of DC costimulation for the induction of IL-10 secretion in effector T cells but in addition suggest that CTLA-4 and CD28 might have opposing functions dependent on the initial differentiation program that shaped the original Th1 or Th2 cells. Furthermore, since DC-derived signals are essential in directing differential T helper cell phenotypes, it is tempting to speculate that the common signature regulating IL-10 expression in T cells is in fact driven by DC-derived costimulatory signals.

**Tr1-LIKE CELLS GENERATED BY TNF OR β-CATENIN STIMULATED SEMI-MATURE DCs**

Many groups, including ours, searched intensively for the DC maturation phenotype that differentiates IL-10+ Tr1-like cells from either Th1 or Th2 cells and whether this would result in tolerance or immunity (Lutz and Schuler, 2002; Lutz, 2006; Tarbell et al., 2006). Tr1-like cell generation might be initiated by triggering the β-catenin pathway in DCs, at least when induced by disrupting E-cadherin mediated homotypic interactions between DCs or after inflammatory stimulation of the DCs (Menges et al., 2002; Spörri and Reis e Sousa, 2005; Jiang et al., 2007). These DCs show a particular semi-mature phenotype characterized by high expression of MHC II and B7 costimulatory molecules but absence of cytokine secretion (Menges et al., 2002; Spörri and Reis e Sousa, 2005).

Repetitive injections of such semi-mature DCs prevented the induction of experimental autoimmune encephalomyelitis (EAE) by inducing IL-10+ Tr1-like cells (Menges et al., 2002; Jiang et al., 2007) but also IL-4+ and IL-13+ T and NKT cells (Wiethe et al., 2007). Such TNF-stimulated semi-mature DCs were also protective in models of thyroiditis (Verginis et al., 2005) but failed to prevent CD8-mediated type-1 diabetes (Kleindienst et al., 2005). Surprisingly, semi-mature DCs deficient for the coinhibitory ligand PD-L1 (CD274) showed an increased induction of IL-10 and IL-13 secreting CD4+ T cells in vivo, indicating that PD-1 signals counteract Tr1-like cell induction (Brandl et al., 2010).

It remains open at this point whether DC maturation/costimulation profiles inducing either Th2 effector cell differentiation or tolerogenic Tr1-like cells are different or identical.

**Tr1-LIKE CELLS DERIVED FROM Th2 CELLS**

Although repetitive injections of antigen-loaded semi-mature DCs were able to prevent Th1-mediated diseases by inducing increasing numbers of Th2-derived Tr1-like cells with each injection (Menges et al., 2002), the same regimen failed to suppress Th2-mediated footpad swelling in a Leishmania major infection model (Wiethe et al., 2008). However, Th1-derived Tr1-like cells effectively suppressed immunity in a Th1-polarized, chronic, non-healing Leishmania major infection model (Anderson et al., 2007). Whether the differences in protection depend on the model or on differences between Th1- or Th2-derived Tr1-like cells remains unresolved. In murine parasitic infection models such as Trypanosoma brucei, Schistosoma mansoni, and Echinococcus multilocularis a cytokine shift to anti-inflammatory IL-10, IL-4, and IL-13 has been reported which is believed to protect the host from extensive tissue damage (Perona-Wright et al., 2006; Stijlemans et al., 2007; MacDonald and Maizels, 2008; Vuitton and Gottstein, 2010). The Th2/Tr1-like profile induced by helminth-derived secretory products of S. mansoni eggs or Nipponanglyus brasiliensis excretory/secretory antigens have been shown to result in partial DC maturation (Balic et al., 2004; Perona-Wright et al., 2006). Together, parasites or their products result in immature or semi-mature DC signatures, which trigger mild TCR and costimulatory signaling cascades and result in Th2/Tr1-like polarizations. However, how DC maturation signatures regulate the master switch leading to IL-10 production in Th2 or Tr1-like cells remains to be shown.

**Tr1-LIKE CELLS DERIVED FROM Th1 CELLS**

The ability of Th1 differentiated T cells to produce IL-10 upon chronic stimulation is very well documented (Table 1). DCs direct Th1 differentiation as fully mature DCs by providing high TCR and costimulatory signaling in the presence of IL-12p70 cytokine
secretion (Kapsenberg, 2003) or via CD70 signals (Soares et al., 2007). As a difference between Th1- or Th2-derived Tr1-like cells, chronic Th1 stimulation seems to be required for Tr1-like cell generation, while IL-10 release by Th2 effector T cells may occur immediately (Lohning et al., 2003; Motomura et al., 2011). Several reports indicated that the presence of IL-12 released by DCs is essential for IL-10 production in Th1 cells mediated by sustained STAT-4 signaling (Chang et al., 2007; Rutz et al., 2008; Saraiva et al., 2009). Also a high antigen dose favors their IL-10 production (Saraiva et al., 2009). IL-10 induction in Th1 cells was abrogated when DC were deficient in IL-12p40 production suggesting that IFN-γ produced by Th1 cells induced continuous IL-12 production in DCs by a feedback loop (Saraiva et al., 2009).

Alternatively, complement factors, which could be produced locally as a result of cognate DC-T cell interactions, regulated the expression of MHC II, costimulatory molecules and IL-12p70 production (Heeger and Kemper, 2011). Repetitive engagement of the complement regulator proteins CD46 or CD55 on human T cells induced IL-10 production from Th1 cells although addition of exogenous IL-2 was required (Capasso et al., 2006; Cardone et al., 2010), similar as for the generation of human Tr1 cells in vitro (Groux et al., 1997; Jonuleit et al., 2000; Levings et al., 2005). It remains open how the CD55 receptor CD97 is regulated on the DC surface. Of note, Th1 cells, which acquire the ability to produce IL-10, gradually lose their expression of IL-2 (Gabrysova et al., 2009; Saraiva et al., 2009). Sustained IL-12 driven STAT-4 signaling is also required for the IL-10 production in IFN-γ-secreting Th1 cells induced by Notch signaling (Rutz et al., 2008). Hence, it is tempting to speculate that chronic stimulations by DCs shift the intrinsic T cell polarization profile from IFN-γ to IL-10 production. Radbruch and colleagues showed that Th1 cells up-regulate the transcription factor Twist1, which requires IL-12 induced STAT-4 signaling for suppression of the Th1-effector cytokines IFN-γ, IL-2, and TNF (Niesner et al., 2008). It is worth to speculate whether this shift might be supported through CTLA-4 signals since gradual CTLA-4 upregulation by Tr1-like cells differentiated from Th1 cells has been observed regularly (Table 1). In a rejection model for organ transplantation, Th1 cells adapt to persistent antigen stimulation by upregulation of inhibitory surface molecules and cytokines such as CTLA-4, Twist1, and IL-10 (Noval Rivas et al., 2009). Earlier studies demonstrated that T cells exposed to chronic antigen stimulation down-modulate effector cytokine secretion during the process of so-called adaptive tolerance (Simpson and Schwartz, 2003).

Together, IL-12 production by DCs seems to be critical for the induction of IL-10 in Th1 cells by triggering sustained STAT-4 signaling. Whether the low expression of CD80/CD86 on immature DCs is sufficient or rather semi-mature or mature DC stages are required remains open.

| COSTIMULATION FOR DIFFERENT Foxp3<sup>+</sup> IL-10<sup>+</sup> iTregs TYPES IN VIVO | LUNG |

The generation of Tr1-like cells has been reported also in the absence of overt infections (Table 1). However, Tr1-like cell induction may require some commensal stimulation during steady state conditions through TLRs for both Th1 and Th2 responses. The mucosal application route may favor a Th2 outcome as supported by experiments where exogenous antigens were administered intranasally. The antigens could enter the respiratory but also gastrointestinal tracts and migratory DC transported the intranasally applied antigens to the draining lymph nodes to induce Th2 responses (Constant et al., 2000; Piggott et al., 2005; Derbyshire et al., 2011). Studies by Umetsu and colleagues showed that high-dose antigen exposure through intranasal routes caused pulmonary DCs to mature and migrate into the draining lymph nodes where they induced IL-10 and IL-4 secreting Tr1-like cells protecting mice from airway hyper-reactivity (Akbari et al., 2001, 2002). Moreover, Akdis et al. (2004) demonstrated that a fine-tuned balance between aeroallergen-specific Tr1-like and Th2 cells exists in allergic or healthy patients, which may regulate allergy to common environmental proteins. How DCs or lung epithelium contribute to the generation of Th2 effector cells in lung-draining lymph nodes has been studied intensively (Hammad and Lambrecht, 2008). A mature DC signature has been revealed only by one study (Akbari et al., 2001), possibly resulting from minute amounts of endotoxin to allow DC maturation and indicating that costimulation would be required to induce Tr1-like cells.

**INTESTINE**

In contrast, the oral tolerance phenomenon seems to be dependent largely on DCs present in the lamina propria and mesenteric lymph nodes as shown upon administration of sugar-modified antigens, which trigger differentiation of IL-10 and IFN-γ producing Tr1-like cells (Worbs et al., 2006; Zhou et al., 2010). It has been suggested that the presence of gut microbiota and hence, the particular IL-10<sup>+</sup> semi-mature DC signature present under these conditions, contributes to the IL-10 production also of the T cells (Hrnčíř et al., 2008; Monteleone et al., 2008). TRL-2 has been previously reported to promote IL-10 production by DCs and respective Treg cell induction (Manicassamy et al., 2009). In addition, the β-catenin signaling pathway, although independent of commensal triggering, appeared critical for the tolerogenic phenotype of lamina propria DCs by promoting IL-10 production (Manicassamy et al., 2010). Okamura et al. (2009) identified lymphocyte activation gene-3 (LAG-3)-expressing Tr1-like cells in the intestine, which developed on environmental microbiota. Induction of IL-10 production by Foxp3<sup>+</sup> iTregs resulted in response to polysaccharide A from the human commensal *Bacteroides fragilis* and was largely dependent on TLR-2-signaling (Round and Mazmanian, 2010). Thus, the generation of IL-10<sup>+</sup> Tr1-like cells but also Foxp3<sup>+</sup> iTregs in the gut under steady state conditions is heavily influenced by the local microbiological stimulation leading to DC maturation and induction of costimulation via β-catenin, TLRs, or presumably other signals.

**INTRAVENOUS ANTIGEN, CONVERSION FROM ANERGIC T CELLS**

In case of intravenous peptide-induced tolerance anergic T cells or Tregs develop from naive precursors upon TCR receptor signaling mostly in the absence of or with weak costimulation under these steady state conditions (Thorstenson and Khoruts, 2001; Leon et al., 2004; Safford et al., 2005). Intravenous antigen applications induce Foxp3<sup>+</sup> Tregs as well as Foxp3<sup>+</sup> Tr1 or Tr1-like cells both of which require high affinity TCR agonists (Gabrysova and Wraith, 2010;
Gottschalk et al., 2010). While single injections of low doses of peptides suffice to generate Foxp3+ Tregs (Gottschalk et al., 2010), their Tr1 or Tr1-like counterparts are formed after several injections of high doses of peptide (Gabrysova and Wraith, 2010). Whether antigen doses and application regimens are decisive in this process is unclear. Most likely local environmental differences such as the availability of TGF-β and all-trans retinoic acid might further contribute to Foxp3 induction (Maynard et al., 2007, 2009). In any case, these protocols follow antigen applications in the absence of inflammatory or microbial stimulation, indicating that B7 costimulation may not be involved. Whether the resulting IL-10+ cells in these assays are Tr1 or Tr1-like cells or another subtype is unclear. On one hand they may be considered as Tr1 cells because they are derived from naïve T cells under low costimulation conditions and not from polarized immunogenic Th cells. On the other hand several rounds of stimulation with strong antigenic and costimulatory conditions are more similar to Tr1-like cells. Clear genetic markers would be needed to discriminate between Tr1 cells, Tr1-like cells, and these IL-10+ T cells described here to clarify this point.

**ROLE OF COSTIMULATION FOR THE EXTRATHYMIC DE NOVO CONVERSION OF Foxp3+ iTregs**

Other major subtypes of Tregs are characterized by their expression of Foxp3. While natural Foxp3+ nTregs are directly released from the thymus independent of DCs (Kyewski and Klein, 2006; Ohkura and Sakaguchi, 2010), conversion of Foxp3+ iTregs occurs in peripheral lymphatic organs by interactions with DCs. The basal requirements for the development of Foxp3+ iTregs in vitro and in vivo are defined by TCR signaling and the presence of the cytokines IL-2 and TGF-β (Figure 1; Fantini et al., 2004; Davidson et al., 2007; Curotto de Lafaille and Lafaille, 2009). In addition, costimulatory and coinhibitory molecules on the surface of DCs importantly regulate the differentiation and functions of iTregs from naïve T cells (Bour-Jordan et al., 2011). Although more and more costimulatory and coinhibitory molecules of the B7 and TNF-families have been identified on DCs, which also affect peripheral T cell tolerance, the definite requirements for CD80/CD86 costimulation for Foxp3+ Treg biology are still under debate and therefore will be discussed here. Also PD-L1/PD-L2 heavily contribute to Treg induction and function, which is discussed in more detail elsewhere (Francisco et al., 2010) but will be briefly touched below.

**CD80/CD86 MOLECULES FOR Foxp3+ iTReg CONVERSION**

Initial evidence that these costimulatory molecules might play a role in Treg development reveal from studies using mice deficient for CD80/CD86 or CD28 or treated with the respective blocking antibodies. In both cases, mice exhibited markedly reduced numbers of Foxp3+ Tregs in the thymus and the periphery (Salomon et al., 2000; Bour-Jordan et al., 2004, 2011; Tang et al., 2004). However, these studies did not discriminate between effects of CD80/CD86 molecules on nTreg or iTreg development. First indications for peripherally induced iTregs come from investigations on the conversion of naïve CD4+ CD25− T cells into CD4+ CD25+ Tregs after adoptive transfer into CD80/CD86−/− mice (Liang et al., 2005). Under these conditions, naïve CD4+ CD25− T cells are unable to convert into Tregs, suggesting a role of CD80/CD86 molecules in iTreg induction (Liang et al., 2005). Although in this study Tregs were not defined by expression of Foxp3, these findings are in line with our in vivo conversion data using a transgenic skin-antigen model and adoptive transfer of antigen-specific naïve CD25+ Foxp3+ T cells (Azukizawa et al., 2011). In this model, elevated levels of costimulatory molecules were found on DCs migrating and transporting self-antigen under these steady state conditions, thus representing a semi-mature phenotype. These steady state migratory DCs induced peripheral conversion of naïve T cells into Foxp3+ iTregs, dependent on TGF-β/latency-associated peptide (LAP) on the DC surface (Azukizawa et al., 2011). This has been also described for specific tolerogenic DC subsets in the intestine (Coombes et al., 2007; Sun et al., 2007) and spleen (Yamazaki et al., 2008). Furthermore, mature bone marrow-derived DCs could promote iTreg differentiation in vitro, but only in the presence of exogenous TGF-β (Yamazaki et al., 2007). These reports suggest that DCs of all maturation stages are able to induce Foxp3 expressing iTregs, but the T cells prefer no or low CD28 signals. A strict requirement seems TGF-β, which can be either produced by DCs or is recruited from the environmental milieu.

**CD28 VERSUS CTLA-4 FOR iTreg CONVERSION**

Several studies indicate that the maturation of DCs and therefore a high costimulation is counteracting iTreg generation (Kretschmer et al., 2005; Yamazaki et al., 2007; Wang et al., 2008). Kretschmer et al. (2005) found that DC maturation in vivo upon injection of agonist anti-CD40 antibodies diminished their potential to promote iTreg conversion. In vitro Treg conversion assays with splenic DCs from CD80−/−/CD86−/− mice indicated that both molecules on DCs are not directly required for the differentiation of Foxp3+ iTregs (Yamazaki et al., 2007; Wang et al., 2008) but they might promote the production of IL-2 by effector T cells, which in turn enhances iTreg generation and maintenance (Yamazaki et al., 2007). On the other hand, two studies using agonistic anti-CD28 antibodies and CD80−/−/CD86−/− mice demonstrated that at least a low CD28 costimulation was required for naïve T cells to become iTregs, whereas strong CD28 interaction induces the differentiation of Teff cells (Benson et al., 2007; Semple et al., 2011). In this context it has been taken into account that both effector T cells and Tregs themselves upregulate CD80 and CD86 on their surface after activation and CTLA-4+ Tregs even suppress effector T cells by ligating their CD80/CD86 molecules (Paust et al., 2004). In this scenario also T–T cell communication through CD28/CTLA-4 and CD80/CD86 can occur in the absence of APCs and may contribute to Treg conversion. Together, CD28 costimulation appears to counter-regulate TGF-β dependent iTreg conversion and survival in vitro but also in vivo (Figure 1).

Unlike CD28 costimulation the interaction between CD80/CD86 molecules on DCs and CTLA-4 on T cells initially does not seem to play a role in the development of Tregs, since CTLA-4−/− mice exhibit normal numbers of Foxp3+ T cells (Tang et al., 2004; Kataoka et al., 2005; Wing et al., 2008). Two groups further analyzed the role of CTLA-4 in the differentiation of iTregs using CTLA-4 blocking antibodies and naïve CTLA-4−/− T cells in Treg conversion assays in vitro (Zeng et al., 2006; Wang et al., 2008).
Both groups found a reduced induction of Foxp3 expressing iTregs despite the presence of costimulation provided by anti-CD28 antibodies or splenic DCs (Zeng et al., 2006; Wang et al., 2008). These results cannot be explained simply by a different expression of the CTLA-4 ligands CD80/CD86 by DCs, since high costimulatory levels by anti-CD28 antibodies and low costimulatory levels by immature splenic DCs were used (Zeng et al., 2006; Wang et al., 2008). Therefore, further investigations are needed to clarify the role of CTLA-4 in iTreg development.

In this context the question remains, whether semi-mature steady state migratory DCs are tolerogenic because of or despite their costimulatory capacity. In agreement with previous results, Benson et al. (2007) could show in ex vivo studies that CD80/CD86 molecules expressed by splenic DC inhibit iTreg conversion, but administration of IL-2 and TGF-β in the absence or presence of retinoic acid can overcome the inhibition of iTreg differentiation, indicating a dominant role for IL-2 and TGF-β despite CD80/CD86 cosignaling. IL-2 production by DCs can hardly be detected under steady state conditions (Granucci et al., 2003). However, steady state migratory DCs employ endogenous TGF-β or the TGF-β binding partner LAP to induce Treg conversion (Azukizawa et al., 2011) and since these DCs are skin-derived and in close contact with TGF-β producing keratinocytes, the environmental milieu in which such migratory DCs were embeded might also affect their tolerogenic potential.

Together, these observations suggest that immature costimulatorylow DCs have the highest capacity to induce iTregs in vivo and in vitro, but costimulatoryint semi-mature and costimulatoryhigh mature DC are still able to induce iTregs. The semi-mature DC phenotype might reflect an evolutionary compromise between partial activation to upregulate CCR7 and MHC II to enable their migration to the lymph nodes and improve presentation of peripheral self-antigens with only moderate CD80/CD86 upregulation. In fact studies on human migratory DC indicated that they might maintain an immature phenotype with respect to HLA-DR and CD80/CD86 expression while CCR7 was upregulated (Geissmann et al., 2002; Verbovetski et al., 2002).

**PD-L1 AND PD-L2**

PD-L1 is constitutively expressed by most hematopoietic cells and can be further upregulated upon activation. In contrast, PD-L2 is inducible on DCs, macrophages, and B cells. PD-L1 and PD-L2 both are the ligands of PD-1, which is expressed on activated B and T cells as well as resting Tregs (Francisco et al., 2010). The role of PD-L1 and PD-L2 for iTreg generation has been first studied by Noelle and colleagues, which showed that blocking of PD-L1, but not of PD-L2, negatively affects the induction of Foxp3+ iTregs by immature splenic DCs in vitro (Wang et al., 2008). The same group also established a tumor antigen model demonstrating that DC-derived PD-L1 was required for tumor-induced iTreg conversion (Wang et al., 2008). PD-L1−/− mice used in this study show significant changes in their expression of other costimulatory molecules such as CD80, CD86, and CD40 (Wang et al., 2008). PD-L1 and TGF-β play a synergistic role in the differentiation of iTregs, since both factors alone induce less iTregs in vitro than in combination (Francisco et al., 2009). In addition, mice deficient for PD-L1, PD-L2, and Rag1 have a decreased capacity to induce iTregs in comparison with WT Rag1−/− mice (Francisco et al., 2009).

In sum, these data suggest a pivotal role for DC-derived PD-L1 on iTreg development. All studies described above were performed under steady state conditions or with isolated immature DC indicating that the constitutive level of PD-L1 expressed by DC is sufficient for iTreg generation.

**OTHER COSTIMULATORY AND COINHIBITORY MOLECULES FOR iTregs**

Despite the importance of CD80/CD86 and PD-L1/PD-L2, other regulatory molecules significantly influence iTreg conversion. Since further detailed review will be beyond the scope of this article only two examples will be mentioned. Consistent with findings from blocking CD80 and CD86 in vitro, CD40 blockage on splenic DCs increased iTreg generation (Wang et al., 2008), supporting the inhibitory effect of costimulation on Treg differentiation. In vitro conversion assays with splenic DCs treated with an agonistic GITR antibody showed a diminished induction of Foxp3+ T cells, indicating also a costimulatory role of GITR in Treg conversion (Wang et al., 2008).

**ROLE OF DC COSTIMULATION FOR Foxp3+ nTreg MAINTENANCE**

The TCR-specificities of natural Treg and effector T cell repertoires are overlapping between 10 and 40%, depending on the study (Hsieh et al., 2004; Pacholczyk et al., 2006; Wong et al., 2007). Among the Tregs some recognize self-antigens and may continuously undergo activation in those lymph nodes where steady state migratory DCs transport and present peripheral tissue antigens to specific T cells (Fisson et al., 2003), whereas others are specific for foreign antigens derived from pathogens (Belkaid and Rouse, 2005; Kretschmer et al., 2005).

**CD80/CD86**

Dendritic cells can influence the expansion and survival of Tregs by different mechanism. The interaction of CD80/CD86 molecules with CD28 on Tregs is a major requirement for the thymic development and the peripheral homeostasis of Tregs (Salomon et al., 2000; Tang et al., 2003). Previous data by Bar-On et al. (2011) support this idea since CD80/CD86 provided by DC was crucial for maintenance of the peripheral Treg pool. In this study bone marrow chimeras lacking CD80/CD86 specifically on DCs were used, which result in a significantly reduced frequency and number of Tregs in the periphery, but not in the thymus (Bar-On et al., 2011). Thus, the costimulatory molecules CD80 and CD86 on DC are required to mediate Treg homeostasis. In agreement with the above described observations costimulatoryhigh mature antigen-bearing DCs induce effectively Treg expansion in vitro and in vivo (Yamazaki et al., 2003; Fehervari and Sakaguchi, 2004b). The proliferation of Tregs in those studies was dependent on CD80/CD86 expressed by DCs and small amounts of IL-2 (Yamazaki et al., 2003, 2007; Yamazaki and Steinman, 2009), which was mainly produced by effector T cells and not DCs (Fehervari and Sakaguchi, 2004b). The proliferation of Tregs in those studies was dependent on CD80/CD86 expressed by DCs and small amounts of IL-2 (Yamazaki et al., 2003, 2007; Yamazaki and Steinman, 2009), which was mainly produced by effector T cells and not DCs (Fehervari and Sakaguchi, 2004b). Of note, costimulatorylow immature DCs are less effective than mature DCs in promoting Treg expansion in vitro (Fehervari and Sakaguchi, 2004a). However, under steady state conditions only immature and semi-mature DCs with basal to intermediate levels of costimulatory molecules are present in secondary
lymphoid organs (Azukizawa et al., 2011). Fully mature DC phenotypes appear upon infection or inflammation (Lutz and Schuler, 2002) and therefore cannot play a role in Treg homeostasis. Rather mature DCs are suggested to be required for the induction of protective effector T cell responses and its subsequent control by Treg in two ways. First, mature DCs secrete pro-inflammatory cytokines like IL-6, which inhibit the suppressive activity of Tregs (Fehervari and Sakaguchi, 2004a). Second, by interacting of CD80/CD86 with CD28 on Tregs mature DCs may induce expansion of Tregs at the peak of the immune response resulting in the suppression of effector T cells. It remains to be determined how semi-mature DCs in comparison to immature DCs affect peripheral Treg numbers.

In this context it has to be considered that under steady state conditions lymph node resident immature DCs and migratory semi-mature DCs present self-antigens of different sources (Lutz et al., 2010; Azukizawa et al., 2011). While migratory DCs take up low doses of soluble or cell-associated self-antigens in peripheral tissues, lymph node resident immature DC can only capture and present high doses of soluble antigens transported from the conduit system (Sixt et al., 2005).

**IL-2**

As mentioned above, DCs are also required for Treg homeostasis by inducing IL-2 production in effector T cells (Yamazaki et al., 2003, 2007; Fehervari and Sakaguchi, 2004a). IL-2, rather than IL-7 and IL-15, is an essential growth factor for Tregs to survive and expand in the periphery (Figure 1; Almeida et al., 2002; Malek et al., 2002; D'Cruz and Klein, 2005; Setoguchi et al., 2005). To induce IL-2 expression in effector T cells, B7/CD28 interaction between DCs and effector T cells is required (Tang et al., 2003; Yamazaki et al., 2003, 2007). However, the contribution of DC-derived IL-2 in Treg homeostasis is controversial. Whereas several *in vitro* studies have revealed that IL-2 secreted by DCs plays no role in Treg maintenance (Yamazaki et al., 2003, 2007; Fehervari and Sakaguchi, 2004a), another study indicated that DC-Treg interaction via CD40/CD40L induces IL-2, which is required for Treg expansion (Guiducci et al., 2005). However, DCs have been shown to produce IL-2 only upon microbial activation but not under steady state conditions (Granucci et al., 2003). Thus, the question remains whether immature and semi-mature DCs under steady state conditions are capable to produce IL-2 and if so, what is its relevance for Tregs.

**Fms-LIKE TYROSINE KINASE 3 LIGAND**

The control of Treg homeostasis has also been linked to DC numbers and the level of Fms-like tyrosine kinase 3 ligand (Flt3L; Liu et al., 2003; Darrasse-Jeze et al., 2009; Swee et al., 2009; Suffner et al., 2010). Flt3L is a hematopoietic growth factor supporting proliferation and differentiation of DC precursors (Karsunky et al., 2003; Liu et al., 2003). Repetitive injections of Flt3L into mice resulted not only in an increased number of DCs, but also enhanced the proliferation of peripheral nTregs (Swee et al., 2009). *In vitro* experiments by the same group indicated that Flt3L-mediated nTreg expansion was directly dependent on DC contact and IL-2, but not on TCR engagement (Swee et al., 2009). In contrast, earlier studies showed that fully mature DC generated from bone marrow by GM-CSF most efficiently induced Treg expansion (Yamazaki et al., 2003), while both immature and mature Flt3L-generated bone marrow-derived DCs had a similar capacity to promote Treg proliferation (Swee et al., 2009). Others claimed that a feedback loop exists, which regulates the homeostasis of both Tregs and DCs in a Flt3L- and MHC II-dependent manner (Darrasse-Jeze et al., 2009; Hochweller et al., 2009).

**TONIC SIGNALS FROM MHC II MOLECULES**

Under steady state conditions T cells scan self-MHC molecules to achieve basal activation state enabling a faster subsequent response upon recognition of foreign antigens (Stefanova et al., 1989). A recent study by Hochweller et al. (2010) revealed that the responsiveness of T cells toward their cognate antigen specifically depends on the recognition of self-MHC molecules on DCs but not other APC. Since Tregs express a TCR with a higher affinity for self-MHC molecules as compared to effector T cells, it was found by two-photon microscopy *in situ* that antigen-presenting DCs interact for longer periods with TCR transgenic antigen-specific Tregs than effector T cells (Tadokoro et al., 2006; Tang et al., 2006). It would be interesting to investigate if such tonic TCR signals also play a role in Treg homeostasis *in vivo*. An initial study showed that inactivation of Lck in Tregs and thereby abrogating their TCR signaling results in an impaired proliferation and homeostatic expansion of those Tregs (Kim et al., 2009). These data indicate that DC–T cell interaction via TCR and MHC II molecules affect the maintenance of Tregs.

In sum, these observations strongly support a crucial role of DCs in Treg homeostasis and demonstrate that DCs affect the proliferation of Tregs by several mechanisms such as their expression of costimulatory molecules, surface TGF-β/LAP, the induction of IL-2 production and their frequency in lymphoid organs.

**SUPPRESSIVE ACTIVITY OF Tregs ON DCs**

Despite the fact that numerous effector mechanism for Treg-mediated suppression have been described (Roncarolo et al., 2006; Sakaguchi et al., 2008; Shevach, 2009; Yamazaki and Steinman, 2009; Weiner et al., 2011), the question remains, whether Tregs can act directly on effector T cells or all regulation occurs indirectly through DCs or other APCs? Clearly, Tregs can directly suppress T cell responses *in vitro* since APC-free co-cultures of Tregs and effector T cells, stimulated by CD3 and CD28 antibodies, show suppressive activities (Dieckmann et al., 2002). On the other hand major suppression mechanisms can also act on DCs, as described below. The relative contribution of both Treg targeting strategies *in vivo* is less well understood (Miyara and Sakaguchi, 2007; Shevach, 2009). Our own findings indicated that both direct and indirect mechanisms occur simultaneously in the spleen by using adoptively transferred DCs and CD4+ CD25+ Foxp3+ Tregs. In this setting the indirect effect on DC exceeded the direct suppression of effector T cells (Hänig and Lutz, 2008).

**COMPETITION FOR DC**

Some published data suggest that Tregs interfere with the ability of DCs to activate effector T cells, either by physically obstructing effector T cells to DCs contact as shown *in vitro* (Onishi et al., 2008). However, competition could not be confirmed *in vivo* during effector T cell responses or under regulatory conditions.
even using high numbers of adoptively transferred mature DCs, Tregs and effector T cells and then following the cluster formation and sizes in spleens of mice (Hommel and Kyewski, 2003; Hänig and Lutz, 2008). Most probably, the three dimensional and highly dynamic T cell turnover in lymphatic organs cannot be reflected \textit{in vitro} where DCs highly aggregate homotypically via E-cadherin interaction (Jakob and Udey, 1998; Riedl et al., 2006; Jiang et al., 2007).

**TREGS PREVENT DC MATURATION**

Tregs preclude upregulation of costimulatory molecules such as CD80, CD86, PD-L1, PD-L2, and CD40 but also the number of MHC-peptide complexes. In addition Tregs may reduce the production of inflammatory cytokines by DCs such as IL-12, IL-1β, IL-6, and IL-8 (Fallarino et al., 2003; Misra et al., 2004; Feunou et al., 2007; Larmonier et al., 2007; Hänig and Lutz, 2008; Wing et al., 2008; Andre et al., 2009). Many of these functions remained unexplained or could be attributed to soluble factors like IL-10 or TGF-β production by Tregs as discussed above for IL-10 by Tr1 and Tr1-like cells.

**Treg SURFACE MARKERS: LAG-3, CD39**

Onishi et al. (2008) showed that DC-Treg aggregation occurs as a first step where LAG-3, a CD4-related transmembrane protein, expressed by Tregs and activated effector T cells binds MHC II molecules on DCs, followed by CTLA-4-dependent prevention of CD86 upregulation. Additional function of LAG-3 has been described, proving that LAG-3 expressed on the Tregs surface leads to MHC II molecule crosslinking and consequential Erk-mediated recruitment of SHP-1 responsible for the inhibition of DC maturation (Liang et al., 2008).

Another surface molecule with immunosuppressive activity expressed by Tregs is CD39 (nucleoside triphosphate diphosphohydrolase-1), an ectoenzyme that degrades ATP to AMP. Extracellular ATP has an adjuvant feature in inflammation (Gallucci et al., 1999), whereas its degradation product AMP acts anti-inflammatory (Kumar and Sharma, 2009). CD39 expression is constitutive in Tregs and controlled by Treg master transcription factor Foxp3. After TCR ligation, the activity of membrane-bound CD39 increases (Borsellino et al., 2007).

**Treg SOLUBLE MEDIATORS: IL-10, TGF-β, IDO, GLUTATHIONE**

In the early nineties it has been demonstrated that exogenous or Th cell-produced IL-10 can inhibit costimulatory function of macrophages, Langerhans cells, and DCs (Ding and Shevach, 1992; Caux et al., 1994; Péguet-Navarro et al., 1994). In most of the studies exogenous IL-10 was used, although Ding and Shevach (1992) designated a Th2 subset as a source of this cytokine. Activated Tr1 or Tr1-like cells are the source of IL-10 and as such prevent DC maturation (see above). Similar effects were observed with TGF-β (Epstein et al., 1991; Bonham et al., 1996). Soon after Tregs were discovered, it was found that this cell population represents the source for both cytokines and can inhibit DC maturation in a similar manner as exogenous cytokines (Hara et al., 2001; Suri-Payer and Cantor, 2001; Belgith et al., 2003; Li et al., 2007).

Tregs express high level of CTLA-4 and several CTLA-4-dependent mechanisms exist through which Tregs inhibit DC function. DCs incubated with CTLA-4+ Tregs or CTLA-4–Ig fusion proteins triggering CD80/CD86 lead to indoleamine 2,3-dioxygenase (IDO) production (Fallarino et al., 2003; Feunou et al., 2007). The major effect of IDO is tryptophan degradation, causing its depletion, and the production of proapoptotic metabolites.

Another effect of the CTLA-4 interaction is Treg-mediated redox perturbation. In this case, Treg-DC contact results in CTLA-4-dependent diminished glutathione (GSH) synthesis in DCs via decreased expression of γ-glutamylcysteine synthetase, the limiting enzyme for GSH synthesis (Yan et al., 2010).

Altogether, Treg-modified DCs maintaining an immature or only semi-mature status are incapable of eliciting strong, productive immune responses, and lead to induction of tolerance.

**DOWNREGULATION OF COSTIMULATORY MOLECULES FROM THE MATURE DC SURFACE**

While prevention of DC maturation could be a significant regulatory mechanism to avoid unwanted immunity, the question remains to which extent are mature/activated DCs sensitive to Treg cell inhibitory action? This topic has been partially assessed by several groups. Some authors claim that strong TLR-stimuli completely abrogate auto-regulation (Pasare and Medzhitov, 2003; Iwasaki and Medzhitov, 2004; Kabelitz et al., 2006). Furthermore, inflammatory cytokines produced by activated DCs seem to impede Treg functions (King and Segal, 2005; Valencia et al., 2006; Wan et al., 2007). On the other hand, although not directly addressing this question in the light of time kinetics, Velldhoen et al. (2006) could show that the repertoire of cytokine production of the LPS- and CpG-matured DCs changes from inflammatory to immunosuppressive depending on the presence of Tregs in the cultures. This result partially opposes the previous claim that strong TLR-stimuli completely disqualify Treg effects. Our own group and that of Santamaria could show that mature DCs are only partially sensitive to Tregs by modulating CD80/CD86 and PD-L1/PD-L2 but no other markers, since the Treg effect could be abrogated by CD40 licensing (Serra et al., 2003; Hänig and Lutz, 2008). Although not using additional stimuli for DC maturation beside KLH and OVA, a study of Oldenhouse et al. (2003) presented convincing set of data pointing to the ability of Tregs to attenuate Th1 responses initiated by transfer of mature DCs. Remarkably, Treg depletion in the same model reflected negatively on Th2 development. Recently, a mechanism for the disappearance of CD80 and CD86 molecules on AppCs was provided by a mechanism called trans-endocytosis, meaning the removal of individual CD80/CD86 molecules but not whole membrane patches (trogocytosis) out of the APC membrane by CTLA-4 molecules (Qureshi et al., 2011). The captured molecules are then rapidly internalized, explaining the known high turnover and predominant intracellular expression of CTLA-4.

**CONCLUSION**

All available data indicate that DCs interact by many means with T cells not only for induction of effector T cells but also to generate and maintain Tregs and in turn also to be controlled by Tregs. While TCR-specific activation is required for all Treg suppressor functions so far, the induction of Tr1 or Tr1-like cells...
clearly requires also CD80/CD86 costimulation. Tr1 or Tr1-like cell generation may be further supported by IL-10. In contrast, Foxp3+ iTreg generation occurs more efficiently without CD80/CD86 signals and requires TGF-β. The maintenance and activation of nTreg is strongly supported by high levels of CD80/CD86 and IL-2. Finally a major control mechanism of mature, but not CD40-licensed, DCs occurs through CTLA-4-dependent activity on CD80/CD86 molecules by trans-endocytosis and IDO induction at the DC-Treg interface. Future research activities, which have to consider also the CD80/CD86 expression on activated Tregs and effector T cells, will lead to a more complete understanding of these molecules and how this can be exploited to develop new therapies.

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