Therapeutic potential of semi-mature dendritic cells for tolerance induction

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Dendritic cells (DCs) are major players in the control of adaptive tolerance and immunity. Therefore, their specific generation and adoptive transfer into patients or their in vivo targeting is attractive for clinical applications. While injections of mature immunogenic DCs are tested in clinical trials, tolerogenic DCs still are awaiting this step. Besides the tolerogenic potential of immature DCs, also semi-mature DCs can show tolerogenic activity but both types also bear unfavorable features. Optimal tolerogenic DCs, their molecular tool bar, and their use for specific diseases still have to be defined. Here, the usefulness of in vitro generated and adoptively transferred semi-mature DCs for tolerance induction is outlined. The in vivo targeting of semi-mature DCs as represented by steady state migratory DCs are discussed for treatment of autoimmune diseases and allergies. First clinical trials with transcutaneous allergen application may point to their therapeutic use in the future.

Keywords: dendritic cells, tolerance, epicutaneous, transcutaneous, steady state, migration

IMMATURE DCs
Tolerogenicity of dendritic cells (DCs) has been shown by many experiments in vitro and in vivo (Manicassamy and Pulendran, 2011). There has been a debate whether certain subsets or the maturation/activation state defines DC tolerogenicity. In mice, all known lymphoid organ DC subsets have been demonstrated to bear tolerogenic potential, as shown for CD4+ (Sato et al., 2003b; Chung et al., 2005), CD8αα+ (Belz et al., 2002; Ferguson et al., 2002; Yamazaki et al., 2008), and plasmacytoid DC (pDC) subsets (Martin et al., 2002; Ochando et al., 2006; Hadeiba et al., 2008) but also human monocyte-derived DCs (Sato et al., 2003a). Conditional ablation of DCs during the steady state in mice results in a loss of self-tolerance (Birnberg et al., 2007; Ohmacht et al., 2009). Experimental animal models in transplantation, autoimmunity or allergy and indications from human studies suggest a tolerogenic potential of immature DCs (Bluestone et al., 2007; Morelli and Thomson, 2007; Hilkens et al., 2010; Manicassamy and Pulendran, 2011). Human immature DCs, loaded with the influenza matrix peptide and keyhole limpet hemocyanin and then injected i.v. into healthy individuals induced tolerance (Dhodapkar et al., 2001). Together, not a defined DC subset or the presentation of foreign antigens dictates DC tolerogenicity but their maturation state.

In vivo most of tissue- and lymphoid organ-resident DCs are immature (Wilson et al., 2003) but after ex vivo isolation they lose their tolerogenic potential due to maturation induced by the preparation procedure (Maldonado-Lopez et al., 1999). Thus, immature DCs need to acquire maturation resistance to subsequent stimuli to act strictly tolerogenic. This can be achieved for in vitro generated DCs by specific conditioning to preserve their immature state (Thomson, 2010). Alternatively, targeting of immature DCs in vivo can be used to induce tolerance by targeting certain surface receptors that mediate tolerance, such as first demonstrated for the 33D1 (DCIR2) antibody binding to the CD4+ DC subset (Finkelman et al., 1996) and later for the CD8αα+ DC subset by DEC205 (CD205) antibody (Hawiger et al., 2001).

As a third possibility intravenous injection of soluble antigens reach thymic and splenic DCs, which are then presented under steady state conditions with half-lives between 3 and 22 h (Muller et al., 1993). Soluble protein injections such as myelin antigens may reach preferentially the CD4+ CD11b+ DCs and can lead to protection from autoimmunity (Li et al., 2008). Injected apoptotic cells as a source for tolerogenic antigens are captured by spleen DCs and may represent promising tolerogenic tools in allogeneic transplantation settings (Steinman et al., 2000; Morelli and Larregina, 2010).

It is of note that s.c. injection of immature DCs leads to their upregulation of costimulatory molecules and a loss of tolerogenicity (Fu et al., 1996). TNF-matured DCs that were tolerogenic when injected i.v., turn into highly immunogenic DCs when applied the s.c. route (Voigtlander et al., 2006). This may indicate that tissue injury mediated maturation by ex vivo isolation procedures or via the s.c. injection route causes danger signals strong enough to abrogate tolerogenicity of immature DCs. Recently, human autologous monocyte-derived DCs, treated with antisense oligonucleotides against CD40, CD80, and CD86 but not loaded with specific antigens were injected intradermally into type 1 diabetes patients (Giannoukakis et al., 2011). Although these DCs were not further characterized, not even on the stability of the costimulation blockade, they appeared save for the patients but also without clinical benefit. Thus, DC injections or in vivo targeting may prefer the i.v. route or require specific treatments to gain maturation resistance.
MATURE DCs
Mature DCs or, as we proposed earlier, rather fully mature DCs (Lutz and Schuler, 2002), are inducers of effector T cell responses by their costimulation, homing, and cytokine production capacities and therefore candidates for anti-microbial or tumor vaccine approaches (Steinman, 2008). Further “licensing” of DCs through CD40 signals leads to elevated cytokine secretion and resistance to Treg-mediated loss of costimulatory molecules on mature DCs (Hänig and Lutz, 2008; and references therein). However, although immature DCs are more efficient in Treg de novo induction from naive T cells, mature DCs have been demonstrated to act superior in activating the suppressor function of Tregs. Details on the role of DC costimulation for Treg generation and function has been reviewed elsewhere (Pletinckx et al., 2011a).

SEMI-MATURE DCs
Partial maturation resulting in upregulation of MHC and costimulatory molecules and lymph node homing capacity but lack of proinflammatory cytokine production was termed semi-maturation (Lutz and Schuler, 2002). An advantage of semi-mature tolerogenic DCs over immature tolerogenic DCs is their lymph node homing potential by which DCs can reach T cells at their anatomical locations. Although under debate, to create the term “semi-maturation” allowed the collection of arguments for or against it and then to keep or discard it. So far, further experimental evidences for the phenotype and tolerogenic potential of semi-mature DC stages have been obtained and reviewed (Mills and McGuirk, 2004; Morelli et al., 2005; Braun et al., 2006; Nouri-Shirazi and Thomson, 2006; Rutella et al., 2006; van Duivenvoorde et al., 2006; Young et al., 2007; Frick et al., 2010; Morel and Turner, 2011). Recently, gene-expression profiling of different semi-mature DCs (TNF, Trypanosoma antigens) was compared to fully mature DCs (LPS) and revealed mainly quantitative differences between these DC types. A common signature of only 24 proinflammatory genes characterized the semi-mature DC types with a total of 160–466 genes regulated as opposed to almost 5000 genes regulated by LPS (Pletinckx et al., 2011b). These data underline that besides the qualitative instruction of pathogen- versus self-antigen-recognition by triggering or not of pattern recognition receptors also more fine-tuned quantitative differences in gene regulation seem to determine DC tolerogenicity versus immunogenicity (Figure 1). Here, some specific aspects of semi-mature tolerogenicity will be discussed.

TOLEROGENICITY OF SEMI-MATURE DCs
Initial findings in the mouse, that TNF-matured bone-marrow-derived DCs (TNF/DCs) and intravenously injected into mice could act tolerogenic (Menges et al., 2002) were similar to findings that cross-tolerance of CD8+ T cells in vitro induced by human DCs also required TNF stimulation (Albert et al., 2001). Repetitive injections of peptide-loaded TNF/DCs into mice allowed complete protection from experimental autoimmune encephalomyelitis (EAE). The resulting T cell response was characterized by a lowered IFN-γ production, absence of IL-4, and increased IL-10 production of CD4+ T cells as detected by ELISA (Menges et al., 2002). Thus, a tolerogenic response being compatible with induction of a regulatory T cell type 1 (Tr1) (Roncarolo et al., 2006). Similar observations have been made with TNF/DCs in a murine thyroiditis model (Verginis et al., 2005), DNA-matured DCs in experimental collagen-induced arthritis (Jaen et al., 2009), MyD88-silenced, and then LPS-matured DCs in rat intestinal allograft transplantation (Yang et al., 2011). Others generated semi-mature DCs by dexamethasone and 1α,25-dihydroxyvitamin D3 (VD3) treatment alone (Unger et al., 2009) or in parallel with LPS exposure

![Figure 1](image-url)
of cells that were protective in collagen-induced arthritis model (Stoop et al., 2010). Semi-mature DCs generated by sequential dexamethasone and LPS treatment were superior to immature DCs to prolong allograft survival in mice (Emmer et al., 2006). In a murine graft versus host model, human tolerogenic DCs were generated by TNF/DC injections to prolong allograft survival in mice (Emmer et al., 2006). Recent analyses showed that a single stimulation of T cells by TNF/DCs induced a Th2-like profile in vitro and in vivo (Pletinckx et al., 2011b), that allows immune deviation of antigen-specific T cells away from pathogenic Th1 and Th17 responses in EAE. Only repetition leads to dominant Tr1-mediated control of EAE. We also tested whether this mixed Th2/Tr1 response would influence asthma as a Th2 disease model. The data revealed that TNF/DCs could neither boost nor protect Th2-mediated asthma in mice, presumably pointing to a neutral effect of Th2 booster with Th1-suppression (Pletinckx et al., 2011b). This is different to what has been described by others with intranasally applied OVA antigen also leading to Tr1 cells without additional Th2 induction and protecting from asthma (Akbari et al., 2001). These differences in the clinical outcome may however, also be explained by IL-10 production by the endogenous lung DCs after intranasal asthma therapy, which was not observed with our adoptively transferred TNF/DCs. Alternatively, a local control of the disease in lung lymph nodes (Akbari et al., 2001) rather than systemically injected TNF/DCs, reaching the spleen, may be beneficial in the asthma model. Together, semi-mature DC-induced mixed Th2/Tr1 responses can protect from Th1/Th17-induced (Sato et al., 2003a) diseases but pure Tr1 induction will be necessary to treat also Th2-mediated diseases.

**INFLAMMATION, PATHOGENS, COMMENSALS, AND TUMORS AS INDUCERS OF SEMI-MATURATION**

There is accumulating evidence that typical Th2-inducing pathogens also induce only partial DC maturation such as shown for Leishmania amazonensis (Prina et al., 2004), Bordetella pertussis (Vojtova et al., 2006), cholera toxin (Binczok et al., 2007), Nippostrongylus brasiliensis (Balic et al., 2004), or Echinococcus multilocularis (Nono et al., 2012). As a consequence the resulting Th2 response will be dominated by Tr1 cells due to the chronicity of the infection (O’Garra et al., 2004) and was similar as observed for repetitive injections of TNF/DCs in the autoimmune model mentioned above. In addition, commensals such as Lactobacillus rhamnosus (Veckman et al., 2004) or Bacteroides vulgatus (Frick et al., 2006) but also exogenous noxes such as nicotine (Hu et al., 2012) or endogenous inflammasome triggers such as ATP (Ben Addi et al., 2008) can induce partial DC maturation.

Receptors that mediate semi-maturation include both TNFR1 and TNFR2 (Funk et al., 2000), IL-6R (Frick et al., 2010), allergen targeting to FcγRI (Hulse and Woodfolk, 2008) but also Trypanosoma brucei-derived VSG antigens with presumably low affinities for MyD88-dependent Toll-like receptors (TLR; Pletinckx et al., 2011b). Treatment of human patients with psoriasis and multiple sclerosis by fumaric acid similarly induces a Th2-inducing DC type (Ghoreschi et al., 2011). In sum, inflammatory mediators, commensal bacteria, or typical Th2-pathogens can induce DC semi-maturation. This may indicate that commensals and pathogens exploit “this is only an inflammation” signaling pathways in DCs to escape strong immunity and elimination but also immunopathology (MacDonald and Maizels, 2008).
FIGURE 2 | Two distinct DC semi-maturation pathways induce different types of regulatory T cells. Immature tissue-resident DCs or in vitro generated BM-DCs that are triggered through the Wnt/β-catenin pathway or proinflammatory cytokines become semi-mature DC with migratory potential to T cell areas. Upon induction of IL-10 production by the DCs as observed via intranasal antigen application Tr1 cell generation from naive T cells is favored. Alternatively, repetitive injections of IL-10-deficient semi-mature DC also lead to Tr1 cell generation. Different, only incompletely understood maturation pathways activate tissue-resident DC into RelB/p52+ semi-mature DCs homing to the T cell areas of peripheral lymph nodes. Transport of soluble and cell-associated antigens have been observed for ssmDCs. By using TGF-β and retinoic acid naive T cells are converted into Foxp3+ Tregs by ssmDCs.

It has been shown that a mild DC activation can occur through disrupting DC–DC contacts formed by homotypic interaction via E-cadherin and this dissociation is indeed accompanied by partial maturation of the DCs through the Wnt/β-catenin signaling pathway (Jiang et al., 2007). These disrupted DCs upregulated MHC II and costimulatory molecules but did not secrete proinflammatory cytokines. When pulsed with myelin antigen they induced IL-10 producing T cells that controlled EAE (Jiang et al., 2007) using the same protocol and reaching very similar results as demonstrated by our group with TNF/DCs before (Menges et al., 2002). In a colitis model Wnt signals activating β-catenin in DCs were required to control the disease, indicative for a tolerogenic DC activation (Manicassamy et al., 2010). However, so far it remains unclear whether DCs matured along the β-catenin pathway are resistant to further stimulation that would be demanding for therapeutic use.

Finally, in human patients suffering from pancreatic ductal adenocarcinoma or chronic pancreatitis conventional DCs and pDCs isolated from the peripheral blood appeared at a semi-mature stage with impaired stimulatory function on T cells (Tjomsland et al., 2010). Similarly, DC infiltrating tumors of non-small cell lung cancer patients appeared immature or semi-mature or remained semi-mature when exposed to maturation stimuli (Perrot et al., 2007). Further investigations have to elucidate whether such DCs are actively tolerogenic.

LIMITATIONS OF SEMI-MATURE DC TOLERGENICITY
As already mentioned above injections of semi-mature DCs protected from Th1/Th17 immunity in the EAE model but not in Th2-mediated asthma (Pletinckx et al., 2011b). In addition, TNF/DC application for EAE therapy, i.e., after EAE induction, failed (our unpublished observations). The reasons for this failure, however, are obvious. In the preventive setting a large part of the auto-antigen-specific naive CD4+ T cell repertoire is primed and polarized into Th2 and subsequently into Tr1 phenotypes. If EAE induction follows by immunization with the same auto-antigenic peptide, the frequency of the remaining auto-antigen-specific
naive CD4+ T cells is insufficient to generate enough pathogenic Th1 and Th17 cells. This is the principle of tolerance induction by immune deviation. Reversely, if most auto-antigen-specific cells are polarized into Th1 or Th17 cells by the EAE protocol, it is difficult at later time points to generate enough protective Th2 or Tr1 cells from the remaining antigen-specific T cell pool. Thus, tolerance induction by immune deviation or induced Tregs/Tr1 cells relies on sufficient numbers of naive auto-antigen-specific T cells at the time of therapy.

In a type I diabetes model TNF/DCs loaded with an auto-antigenic peptide on MHC class I molecules also failed to act tolerogenic on CD8+ T cell autoimmunity (Kleinidjest et al., 2005), indicating that the DC semi-maturation may not allow tolerization of high affinity CD8+ T cells such as the OT-I transgenic T cells used in this system. Furthermore, dose-dependent effects have been observed in collagen-induced arthritis, where semi-mature DCs injected at low amounts were protective whereas high amounts failed to do so (Lim et al., 2009).

As for immature DCs also the stability of the semi-mature phenotype is important to maintain tolerogenicity and for this the injection route may play an essential role. While three i.v. injections of TNF/DCs were completely protective in the EAE model, s.c. application of the same DCs was deleterious and all mice died from severe EAE. One reason was a remaining responsiveness of TNF/DCs to further maturation signals such as LPS in vitro, which led to IL-12 production. In vivo, TNF/DCs injected s.c. homed to the draining lymph node but appeared cytokine negative unlike endogenous DCs, which showed proinflammatory cytokine production (Voigtländer et al., 2006). This indicates that s.c. injection abrogates semi-mature DC tolerogenicity, in part by interactions with other DCs. In cancer patients, only s.c. or intralymphatic injection routes may play an essential role. While three i.v. injections of DCs matured with tumor antigens failed to do so (Lim et al., 2009), semi-mature DCs injected at low amounts were protective whereas high amounts failed to do so (Lim et al., 2009).

As mentioned above, it will be necessary to establish semi-mature DCs that remain stable, and this might be achieved by subsequent treatment with a maturation inhibitor followed by a maturation inducer (Sato et al., 2003a; Boks et al., 2012).

STEADY STATE MIGRATORY DCs

IN VIVO COUNTERPARTS OF IN VITRO GENERATED SEMI-MATURE DCs?

After all, the question remained whether semi-mature stages of DCs can be detected in vivo and whether they also exert tolerogenic functions. Early observations indicated that the afferent lymphatics depend on CCR7 expression for lymphocyte homing, which showed that CCR7 expression was maintained under steady state conditions, despite equivalent induction of CCR7+ DCs (van de Ven et al., 2011). Both subsets expressed more CD80, CD86 CD40, and less CD83 as compared to their resident counterparts. Despite their more mature phenotype, these DCs produced lower amounts of proinflammatory cytokines and were weaker in priming T cell responses, indicative for primarily tolerogenic functions. Supernatants of human tumor cell lines (Kuang et al., 2008) could induce partial DC maturation in vivo, similar to what has been observed in pancreatic adenocarcinomas (Tjomsland et al., 2010) and non-small cell lung cancers (Perrot et al., 2007) where such cells accumulated in the tumor tissue.

Together, ssmsDCs of the skin-draining lymph nodes in mice, pigs, and humans DCs display a semi-mature phenotype by expressing higher costimulatory molecules and having the homing capacity to lymph nodes.

TOLERGENIC FUNCTIONS OF ssmsDCs

It became evident from early studies that ssmsDCs transport self-antigens to the draining lymph nodes (Huang et al., 2000; Hemmi et al., 2001), but the consequences for T cells by the presentation of these antigens were still open, although tolerance induction was proposed. Subcutaneously implanted osmotic minipumps indicated that Foxp3+ Tregs could be de novo converted by this type of constant low dose soluble antigen delivery (Apostolou and von Boehmer, 2004). Later we could show that this low dose soluble antigen delivery by the pump system requires RelB+/p52+ CCR7+ ssmsDCs (Azukizawa et al., 2011). Since pump implantation requires a surgical intervention, this system may not fully represent steady state conditions, despite equivalent induction of Tregs. Direct comparison of soluble antigen delivery via subcutaneous minipumps with cell-associated transgenic neo-self-antigen expression of OVA in the epidermis (K5-mOVA mice), revealed the same dependency on ssmsDCs with the same kinetics and frequency of CD4+ Treg induction (Azukizawa et al., 2011; Figure 2) or CD8+ T cell depletion (Waithman et al., 2007). Recent data suggest that ssmsDCs may control the whole pool of homeostatic...
lymph node T cell circulation by producing VEGF that stimulated formation of high endothelial venules (HEVs) to enable T cell entry and stimulated fibroblastic reticular cells to secrete CCL21 that acts chemotactic for T cells (Wendland et al., 2011). Thus, ssmDCs control T cell homeostasis in peripheral lymph nodes and act tolerogenic on CD4+ and CD8+ T cells, unlike TNF/DCs.

It is however unclear to date what distinguishes these ssmDCs in the mentioned self-antigen model systems that induced IL-10− Foxp3+ Tregs from ssmDCs that captured exogenous OVA or Bordetella flagellin that was applied intranasally and induced IL-10+ Foxp3− Tr1 cells (Akbari et al., 2001; McGuirk et al., 2002). One possibility could be that tolerogenic immune evasion strategies of bacterial flagellin or low doses of endotoxin attached to OVA may lead to IL-10 release by the DCs, which is not observed under completely pathogen-free conditions.

**IN VIVO TARGETING OF ssmDCs FOR TOLERANCE INDUCTION**

The existence of ssmDCs, which bear lymph node homing potential, are partially mature but still tolerogenic, offer their clinical exploitation by specific targeting. In fact earlier studies may have targeted ssmDCs for tolerance induction in an unscheduled manner. We found that DEC205 is expressed at higher levels on ssmDCs than lymph node resident or splenic DCs (own unpublished observations). Therefore antigen-targeting to this marker by i.v. injection may also or even preferentially target ssmDC in peripheral lymph nodes (Hawiger et al., 2001; Kretschmer et al., 2005).

An alternative route to reach ssmDCs is via epicutaneous antigen application. Plaster-mediated delivery of self-antigenic myelin peptide was able to prevent EAE induction (Bynoe et al., 2003; Szczepanik et al., 2005). Although not further investigated, it is likely that ssmDCs have been the vehicle to induce myelin-specific Tregs in the skin-draining lymph nodes. Surprisingly, even approaches using gene gun delivery of antigens, that has been developed for immunogenic vaccines, may be used to induce stable tolerance by induction of Foxp3+ Tregs and this may occur through ssmDCs (Ettinger et al., 2012).

This principle of targeting ssmDCs through the skin may also account for tolerogenic strategies in allergy treatment (Werfel, 2009; Senti et al., 2011). Such treatment showed therapeutic success in murine allergy models using OVA, pollen, house dust mite, or peanut as allergens (Mondoulet et al., 2010, 2011, 2012; Dioszeghy et al., 2011). First clinical studies using epicutaneous immunotherapy in childhood cow milk allergy patients demonstrated safety although the three months of treatment did not reach therapeutic success (Dupont et al., 2010). The reversal of an existing allergy may need extended periods of treatment as suggested from other studies in patients with pollen allergy that showed a moderate benefit (Senti et al., 2009, 2010). Alternatively, the intranasal application route may be superior to the skin and also employs partially mature migratory DCs and led to Tr1 induction in the pulmonary lymph nodes (Akbari et al., 2001).

Finally, the potential success of such epicutaneous or transcutaneous tolerance strategies may be encouraged by the fact that some pathogens hitchhike ssmDCs for immune evasion. A prominent example is HIV, which infects peripheral immature DCs resident in the skin or mucosa and then awaits to be transported to the draining lymph nodes for further infection of CD4+ T cells as their major targets, and even converting some of these into HIV-specific Tregs (Smed-Sorensen and Lore, 2011). Together, semi-mature DCs as represented by ssmDCs may prove valuable targets for clinical epicutaneous or transcutaneous tolerance induction protocols in the future.

**SEMI-MATURE pDCs**

So far this review concentrated on conventional/myeloid semi-mature DC or ssmDCs. However, this does not exclude the existence of semi-mature stages also for pDCs. The biology of pDCs is very different as compared to conventional DCs but certainly they have in common to present antigens to T cells in tolerogenic or immunogenic fashions.

Recent data indicate that pDCs infected in vitro with HIV may be modified by the virus to reach a semi-mature stage that facilitates Treg induction (Smed-Sorensen and Lore, 2011). Similar observations have been made with tumor-infiltrating pDCs that show impaired maturation potential but without providing T cell assays (Perrot et al., 2007; Tjomsland et al., 2010). In contrast, freshly isolated pDC from mice also appeared semi-mature, but pulsed with Leishmania antigen and reinjected into mice showed a protective effect, indicative for their immunogenic activity (Remer et al., 2007).

Together, more detailed analyses for pDCs are required to evaluate a therapeutic potential of semi-mature pDCs.

**CONCLUSION**

The initially surprising finding that partially matured DCs can still act tolerogenic has now reached a broader base by numerous reports and more mechanistic insights. Semi-mature DCs can be generated in vitro and exert a distinct spectrum of tolerogenicity after injection. The finding that semi-mature ssmDCs are continuously engaged to tolerize lymph node T cells against peripheral self-antigens opens further perspectives for therapies, especially against autoimmun diseases and allergies. Thus, tolerogenic regimens employing semi-mature DCs may in the future either be concentrated on in vivo targeting with antibodies or transcutaneous antigen application regimens.

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