Dendritic Cell Immune Therapy to Break or Induce Tolerance

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Abstract Dendritic cells (DCs) represent the key regulator of the immune system. The particular milieu in which DCs encounter the antigen together with their intrinsic properties defines their ability to promote an active or a tolerogenic immune response. The development of protocols to differentiate in vitro immunogenic or tolerogenic DCs opened the possibility to employ them as cell therapy in a list of immune-mediated diseases. While a number of clinical trials with immunogenic DCs have proven the safety and the efficacy of the therapy, only few studies have been performed with tolerogenic DCs. In this review, we will discuss major obstacles encountered, and strategies applied, to improve the efficacy of DC-based immunotherapies including lentiviral vector-based approaches.

Keywords Dendritic cells · Immunotherapy · Tolerance · Lentiviral vectors

Introduction

Dendritic cells (DCs) are a heterogeneous population of cells recognized as the most powerful antigen-presenting cells (APCs). DCs are present in lymphoid and non-lymphoid tissues and continuously patrol the body as immature precursors seeking for foreign invaders (antigen (Ag)s), whether these are bacteria, viruses, or dangerous toxins. After capturing Ags, DCs travel to lymph nodes or the spleen, where they undergo maturation, up-regulate co-stimulatory molecules, and acquire the ability to activate T and B cells [1]. Since their discovery, accumulated evidences sustain the central role of DCs in immunity induction, and more recently, it has become evident that DCs play an important role in maintaining tissue homeostasis and in promoting tolerance. The latter function is mediated by DCs in steady state or by specialized subsets of DCs, named tolerogenic DCs [2–4].

A large body of reports indicates the presence of different DC subtypes characterized by specialized functions and by the expression of homing receptors, which allow them to reside in specific tissue. In humans, two major and intrinsically different subpopulations of DCs have been identified: the myeloid and the plasmacytoid DCs. Myeloid DCs are a component of the adaptive immune system and are activated upon Ag encounter in the periphery, whereas plasmacytoid DCs belong to the innate immune system and upon interaction with nucleic acids secrete type 1 interferons. Each of these subsets is defined by the expression of a set of markers and specific functions and can be activated by different cytokines [5, 6]. Myeloid and plasmacytoid DCs can be either immunogenic, able to induce an active immune response, or tolerogenic that control immune responses through several mechanisms, including inhibition of effector T cells and induction of apoptosis or of T regulatory (Treg) cells (reviewed in [7–9]).

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The prominent role of DCs in promoting T cell activation or tolerance is the rationale for DC-based immunotherapy for cancer and infectious diseases [10], or for immune-mediated diseases [11, 12], respectively. The identification of a method to differentiate a large number of DCs *in vitro* from monocyte precursors using granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4 [13] pioneered the application of DC-based therapies. While a number of clinical-grade protocols for DC differentiation for cancer immunotherapy have been established and DC-based immunotherapies have been tested in clinical settings (reviewed in [14]), culture conditions for the generation of tolerogenic DCs have only recently been defined [2–4].

In this review, we will give an overview of the state of the art, of still unsolved issues (i.e., the identification of route and time of administration), and of new strategies for a safe and effective DC-based immunotherapy.

Biology of DCs and Its Impact on Designing DC-Based Immunotherapy

Designing DC-based immunotherapy for cancer, viral infections, as well as for autoimmunity and allergy, implies comprehension of DC biology. To promote immunity and tolerance, DCs should present Ags to T cells in complex with MHC molecules (signal 1). Signal 1 *per se* does not activate T cells for a productive response, but drives T cells into anergy or cell death, two important mechanisms involved in maintaining peripheral tolerance [15, 16]. To support efficient T cell activation and clonal expansion, DCs should provide co-stimulation (signal 2). A number of different co-stimulatory pathways can be expressed by DCs: some are immunogenic (i.e., CD80-CD86/CD28, CD40/CD40L) and others are inhibitory (i.e., CD80/CTLA-4, PD-L1/PD-1) [17–20]. These arrays of DC/T cell interactions determine the level of T cell activation: when pro-immunogenic interactions prevail, T cells become effectors; on the contrary, when inhibitory interactions predominate, T cells may acquire regulatory activity, become anergic or exhausted cells, or undergo apoptosis. An additional signal is provided by the cytokine milieu present at the time of T cell priming (signal 3) and drives the specialization of the Ag-specific response toward T helper (Th) (Th1, Th2, Th17) or regulatory (FOXP3⁺ Tregs, Th3, Treg type 1 (Tr1) cells [21–25]. Moreover, DCs sense mediators of innate immunity and discriminate the nature of the encountered pathogen by expressing cytokine receptors (in particular interferon receptors), Fc receptors, CD40, lectins, and toll-like receptors (TLRs), which trigger signaling cascades able to settle DC responses toward innate or adaptive immunity [26, 27].

The downstream effects of the integration of all the abovementioned signals converge to few intracellular signaling pathways: (i) nuclear factor- κ B (NF- κ B), (ii) mitogen-

activated protein kinases (MAPK) [28], and (iii) interferon regulatory factors (IRF) [29]. The NF- κ B family (homo- or heterodimeric complexes formed by combinations of five subunits: p65/RelA, RelB, c-Rel, p50, and p52) plays a crucial role in immunity and inflammation by activating genes encoding for pro-inflammatory cytokines and chemokines (IL-6, IL-12, TNF α , MIP-1 α , MCP1) [30]. The NF- κ B pathway is regulated by negative feedback mechanisms involving several inhibitory molecules (I κ B, A20, TRIM), which prevent excessive inflammation and autoimmunity [31–35]. MAPKs are serine/threonine kinases that regulate multiple cellular processes, including immune responses [36]. MAPKs comprise the extracellular signal-regulated protein kinases (ERK) [37, 38], the c-Jun N-terminal kinases (JNK), a component of AP-1, a transcription factor that transactivates many pro-inflammatory genes [39, 40], and the p38 stress-activated protein kinases (p38) [41, 42]. ERK is involved in DC survival [43], in regulation of inflammation, and in immune suppression associated with IL-10 release [44]. p38 plays an important part in DC maturation, being involved in the up-regulation of co-stimulatory molecules and activation markers, and in IL-1 β , TNF α , IL-6, and IL-12 secretion [45–47]. Moreover, during the last decade, it has become clear that microRNAs (miR), a class of non-coding 20–22 nucleotides long RNAs, are involved in controlling DC activation. DCs derived from miR-155^{-/-} mice showed a reduced ability to stimulate Ag-specific proliferation [48], and miR-155 and miR-146a expression is induced in human and murine macrophages and DCs upon lipopolysaccharide (LPS) stimulation, demonstrating that these miRNAs are involved in the regulation of TLR signaling [49–52]. Overall, these evidences suggest that enhancement or inhibition of individual signaling pathways could generate either immunogenic or immunosuppressive DCs.

DC Immunotherapy in Cancer

The efficacy of DC-based immunotherapy in cancer relies on three major goals: (i) induction of tumor-specific effector T cell responses, (ii) inhibition of Treg cell expansion and/or function, and (iii) generation of a pool of tumor-specific memory T cells to control cancer relapse. The definition of protocols to *in vitro* generate powerful DCs is therefore of primary importance. Several methods to differentiate DCs starting from CD14⁺ monocytes or CD34⁺ cells have been established. The vast majority of clinical trials have been performed using autologous CD14⁺ monocytes cultured *in vitro* with GM-CSF and IL-4, matured with different pro-inflammatory cytokine cocktails, and loaded with tumor peptides or lysates (reviewed in [53]). To obtain high numbers of cells, CD34⁺ cells from granulocyte (G)-CSF-mobilized peripheral blood of patients have been used as alternative

sources of DC precursors [54, 55]. Repetitive administrations of CD34⁺-derived autologous DCs in cancer patients were able to induce systemic tumor-specific immune responses in the absence of major side effects [54, 55]. More recently, the use of allogeneic cord blood CD34⁺ cells has been considered as alternative to autologous CD34⁺ cells. Despite the encouraging results in obtaining high numbers of functional DCs that contain few contaminants, the need of long-term culture to generate CD34-derived DCs represents a limitation for their clinical use [56–58].

Since the first clinical trial in B cell lymphoma [59], DC-based immunotherapy has been tested for the treatment of different malignancies, including melanoma, prostatic cancer, glioma, and renal cell cancer [10, 14•]. Overall, the limited immunogenicity of administered DCs resulted in a weak induction of tumor-specific T cells and elicited clinical responses only in a fraction of patients [60, 61]. To overcome this drawback, much effort has been made over the years, and three major approaches have been exploited and implemented: (i) the identification of the best tumor-associated Ag (TAA) to load DCs, (ii) the improvement of Ag loading to induce tumor-specific immune response, and (iii) the selection of the optimal activation stimulus and route of DC administration. By definition, tumors are not homogeneous tissues and the expression of TAAs is not uniform neither for a given type of tumor nor between primary tumors and metastases. For this reason, since the first clinical trial performed in melanoma patients, DCs were loaded with cocktails of TAAs derived from tumor lysate [55, 62]. Although tumor biopsy is not always possible, the use of tumor lysate to load DCs has been adopted in following trials [53, 63, 64]. Tumor lysate, in addition to providing a large panel of TAAs, allows Ag processing and presentation in the context of HLA class I and class II molecules, thus promoting both CD4⁺ and CD8⁺ T cell responses [65, 66]. Recently, to improve TAA loading and presentation by DCs, tumor processing in oxidative or hypoxic conditions has been implemented [67, 68]. In pre-clinical models of glioma and breast carcinoma, it has been shown that the administration of DCs loaded with lysates obtained in hypoxic conditions increased CD8⁺ T cell responses and the rate of apoptotic tumor cells [67]. Moreover, promising results have been obtained in a pilot study of five subjects with recurrent ovarian cancer, in whom immunotherapy with DC loaded with the abovementioned procedure was well tolerated and promoted a potent anti-tumor T cell response [68].

One of the critical issues of cancer immunotherapy with DCs is their relatively low ability to reach the draining lymph nodes [10]; therefore, the improvement of DC migration has been considered to enhance the efficacy of the vaccine. One interesting approach is to induce the expression of C-C chemokine receptor type 7 (CCR7) on differentiated DCs. It has been shown that addition of prostaglandin E (PGE2) to the cytokine cocktail used to mature monocyte-derived DCs

promoted CCR7 up-regulation [69] and enhanced DCs' homing capacity to tumor-draining lymph nodes [70]. Thus far, to achieve a rapid dissemination in different tissues, the majority of DC products have been administered intravenously. However, as discussed above, the limited ability of infused cells to reach tumor-draining lymph nodes, together with the fact that intravenously injected cells are primarily trapped in the liver and lungs, prompted investigators to identify different routes of DC administration including intradermal, subcutaneous, intra-nodal, and intra-tumor delivery [71]. For intradermal or subcutaneous injection, a rapid migration to the regional lymph nodes has been proven; however, this strategy requires the delivery of a high number of DCs in a small volume, which can affect cell viability [70, 72]. Alternatively, intra-nodal injection has been proposed; however, this method raises some concerns regarding the accessibility of tumor-draining lymph nodes and the potential damage of the lymph node [62, 70, 73].

In conclusion, optimizing Ag loading and activation of DCs, and the identification of the more suitable route of DC administration are warranted to generate potent immunotherapeutic DC-based vaccines.

DCs as Tolerogenic Cell Product

The discovery that DCs play an important role in modulating immune responses and in inducing tolerance introduced the use of in vitro-generated tolerogenic DCs as cell therapy to regulate immune responses in immune-mediated diseases [11, 12]. The vast majority of protocols developed to generate in vitro tolerogenic DCs for clinical purposes have in common the use of monocyte-derived DCs differentiated in the presence of GM-CSF and IL-4 in combination with cytokines, or pharmacological agents, or immunosuppressive drugs (reviewed in [2]). A general consensus for the phenotype and function of in vitro-generated tolerogenic DCs is that they should express low levels of co-stimulatory molecules and high IL-10/IL-12 production ratio. Nevertheless, it is becoming evident that, according to the differentiation protocol used, tolerogenic DCs acquired different immune modulatory properties by up-regulating a variety of inhibitory molecules [74, 75•] and the ability to promote the induction of a specific subset of Tregs [7, 11]. Despite the number of pre-clinical studies demonstrating feasibility and efficacy of tolerogenic DC immunotherapy [76–81], and the efforts dedicated to developing methods to generate clinical-grade tolerogenic DCs [75•, 82], only a limited number of clinical trials have been performed or planned [2, 12, 82].

To define the optimal tolerogenic DCs to be used in vivo, recently studies comparing different subpopulations of in vitro-differentiated tolerogenic DCs have been performed [74, 75•]. Stability, cytokine production profile, and suppressive activity

of tolerogenic DCs have been compared, and results indicated that IL-10-modulated mature DCs are the best-suited cells for tolerogenic DC-based therapies. Our group contributed to the identification of IL-10 as key factor for promoting the differentiation of potent tolerogenic DCs. We set up a protocol to differentiate tolerogenic DCs, named DC-10, from peripheral blood monocytes cultured in the presence of GM-CSF, IL-4, and IL-10 [24]. Despite the presence of IL-10 during differentiation, DC-10 are mature myeloid cells that express CD86 and HLA-DR. DC-10 also express the tolerogenic molecules immunoglobulin-like transcript (ILT)2, ILT3, ILT4, and HLA-G [24]. The high expression levels of HLA-G and the high IL-10/IL-12 production ratio render DC-10 potent inducers of allo-specific Tr1 cells [24, 83]. The clinical use of DC-10 relies on this latter feature, and clinical-grade protocols for the induction of allo-specific Tr1 cells, suitable for cell therapy, using DC-10 have been developed [84, 85]. In addition to this clinical application, we are currently testing the potential use of DC-10 as cell therapy for tolerogenic DC-based approaches [7, 11].

Autologous monocyte-derived DCs rendered tolerogenic by the treatment with anti-sense oligonucleotides targeting CD40, CD80, and CD86 have been used in the first tolerogenic DC-based clinical trial [86]. This phase I study assessed the safety of repetitive intradermal administration of tolerogenic DCs in adult type 1 diabetes (T1D) patients. A second phase I study has been conducted in rheumatoid arthritis (RA) patients treated with a single intradermal injection of autologous DCs cultured with an inhibitor of (NF- κ B) signaling (BAY 11-7082) and pulsed with citrullinated peptides [87]. Both treatments were safe and well tolerated and paved the way to developing clinical studies to test the efficacy of tolerogenic DC immunotherapy in T1D and RA and after kidney transplantation. A phase II clinical trial to treat T1D patients at onset with autologous anti-sense oligonucleotide-treated DCs has been launched (ClinicalTrials.gov identifier: NCT02354911). Moreover, based on encouraging results obtained in pre-clinical models [88], a clinical trial with autologous DCs rendered tolerogenic by the exposure to dexamethasone and vitamin D3, activated with monophosphoryl lipid, and loaded with synovial fluids to treat RA patients has been designed (AUTODECRA, ClinicalTrials.gov identifier: NCT01352858) [12]. In this study, tolerogenic DCs will be injected intra-articularly in order to induce a local immune suppression and increase their homing to draining lymph nodes [12].

Under the umbrella of The ONE Study (The ONE Study. <http://www.onestudy.org/>), an integrated European Union-founded project, the first clinical trial testing tolerogenic DCs to prevent organ rejection has been recently started (The ONE Study ATDC Trial, ONEatDC, ClinicalTrials.gov Identifier: NCT02252055). In this study, autologous tolerogenic DCs differentiated with low doses of GM-CSF

in the absence of IL-4 (tol-DCs) will be administered to living-donor renal transplant recipients [89]. The efficacy of tol-DC treatment will be compared to that of other immunosuppressive cells (ex vivo-isolated and in vitro-expanded polyclonal or allo-specific FOXP3⁺ Tregs, Tr1 cells, and regulatory macrophages (Mregs)) [90–92].

Results from these studies will confirm the safety and define the efficacy of tolerogenic DC-based therapy in restoring tolerance in autoimmune diseases and in preventing graft rejection.

Genetically Modified DC Immune Therapy to Break or Induce Tolerance

DC-based vaccination for cancer or persistent infections is aimed at inducing or reactivating immune responses and implies the presentation of the target Ag by activated DCs. Ag presentation is a requirement also for “negative” vaccination to dampen immune reactions. As alternative to tumor lysate loading, one of the approaches used to promote immunogenic DCs for cancer immunotherapy is the delivery of TAAs by viral vectors, which are ensuring stable and prolonged Ag expression and presentation. Lentiviral vectors (LVs) efficiently transduce human DCs [93–97] and induce strong and durable anti-tumor T cell responses [98–100]. Moreover, LV-mediated DC transduction does not result in major changes in the state of DC activation [101–103], supporting the possibility to exploit LV-mediated stable and efficient Ag presentation to generate immunogenic or tolerogenic DCs. In this section, we will discuss the use of LVs as a tool to genetically modify DCs for immunogenic and tolerogenic DC-based therapies.

LV-mediated transduction of DCs to achieve T cell priming with TAAs has been largely explored in vitro. Overall, results showed the potential ability of DCs transduced with LV encoding for TAAs to generate tumor-specific CD4⁺ and CD8⁺ T cells [98]. CD8⁺ T cells play a major role in cancer eradication [104]; however, CD4⁺ T cell response is crucial for sustaining CTL effector functions and for inducing a memory response in vivo [105, 106]. Priming of CD4⁺ T cells by LV-transduced DCs occurs only if the LV-encoded Ag has access to an MHC class II presentation pathway. To this aim, Rowe et al. [107] developed LVs encoding for the C-terminal of the invariant chain (Ii) fused with ovalbumin (OVA) (LV.IiOVA) that allow the presentation of OVA in the context of MHC class II. Results showed that LV-IiOVA-transduced DCs efficiently promote the induction of both OVA-specific CD4⁺ and CD8⁺ T cells and that in vivo delivery of LV.IiOVA protects mice from developing OVA-expressing EG7 tumor. Improvement in efficacy of tumor eradication may derive from the expression of multiple TAAs in order to overcome the mechanism of tumor escape due to the loss of TAA expression.

Direct in vivo LV administration to transduce DCs offers some advantages: it does not require cell manipulation, and the vector itself triggers acute inflammation providing an adjuvant effect; however, it cannot offer high specificity of cell targeting. Conversely, the in vitro LV-mediated DC transduction can significantly improve safety by minimizing off-target transduction and by the limited life span of transferred cells. Moreover, administering in vitro LV-transduced DCs allows repetitive cell administration. LV-mediated gene transfer to express modulators of activation pathways in DCs has been explored as adjuvant to design efficacious cancer immunotherapies. To this purpose, Akazawa et al. [108] over-expressed the major TLR adaptor molecules (MyD88, TRIF/TICAM-1) in mouse DCs and demonstrated that MyD88-modified DCs secrete IL-6 and IL-12p40, whereas TICAM-1-modified DCs induce IFN α production and CD86 up-regulation. These LV-modified DCs increased allogeneic responses in vitro and in vivo in a mouse model of syngeneic tumor implant and delayed tumor growth [108]. Andreaskos et al. [109] showed that the LV-mediated over-expression of NF- κ B inducing kinase (NIK) increased DC activation and their ability to promote a transgene-specific Th1 response. Analogously, Breckpot et al. [110] showed that LV-mediated expression of short hairpin (sh)RNA specific for A20, a zinc finger protein that negatively regulates TNF receptor and TLR-mediated signaling, resulted in increased and sustained production of IL-6, IL-10, and IL-12p70 by human DCs. A20 down-regulated DCs skewed naïve CD4 $^{+}$ T cells toward IFN γ -producing Th1 cells and have an enhanced capacity to prime Melan-A/MART-1-specific CD8 $^{+}$ T cells. A further example acting on the NF- κ B pathway comes from Tao et al. [111], who demonstrated that silencing of RelB expression in bone marrow-derived DCs (BMDCs), mediated by LV carrying RelB shRNA, resulted in low IL-12 secretion and co-stimulatory molecule expression. Interestingly, shRNA RelB-DCs significantly inhibited allogeneic T cell responses, down-regulated Th1 cytokine production, and promoted Th2 cytokines. Escors et al. [112] performed a comprehensive study on signaling pathways governing functional plasticity of DCs using LVs simultaneously encoding for an Ag, to stimulate CD8 $^{+}$ and CD4 $^{+}$ T cell responses, together with constitutively activated factors of the three main MAPK and IFN induction pathways, including p38 and ERK for MAPK, and JNK1 and interferon regulatory factor-3 (IRF3) for the IFN pathway. Results indicated that the activation of p38 in DCs, and to a lesser extent, of JNK1 promotes DC maturation leading to increased OVA-specific immunity, which resulted in tumor regression in vivo. Conversely, activation of ERK in DCs induced the expression TGF- β , and the expression of a constitutively activated IRF3 stimulated IL-10 secretion by DCs. The concomitant activation of ERK and IRF3 suppressed the immune response and stimulated Treg cell expansion [112]. More recently, Arce et al. [113]

successfully applied this strategy and using LV-transduced DCs suppressed experimental inflammatory arthritis.

The over-expression of immune regulatory molecules, such as IL-10, ILT3, and vasoactive intestinal peptide (VIP), has been proposed as an alternative approach to generate tolerogenic DCs. LV-mediated over-expression of IL-10 converted BMDCs in tolerogenic DCs that upon in vivo transfer prevent allergic contact dermatitis [114]. Human CD34 $^{+}$ cells transduced with LV encoding ILT3, and differentiated into DCs, displayed reduced allo-stimulatory capacity and the ability to promote FOXP3 $^{+}$ Tregs in vitro. Furthermore, a single inoculation of myelin oligodendrocyte glycoprotein (MOG)35-55-pulsed BMDCs engineered to express VIP in experimental autoimmune encephalomyelitis (EAE) mice resulted in protection from disease development [115]. Alternative candidates to confer tolerogenic properties to DCs via LV gene transfer are indoleamine 2,3-dioxygenase (IDO1), a tryptophan catabolizing enzyme, regulator of immunity in several physiopathologic conditions, IL-4, and TGF β [116–119].

An interesting and innovative approach to generate potent tolerogenic DCs is targeting the Ag expression and presentation to DCs at the immature state. The genetic modification of BM cells with a LV encoding for an Ag under the control of DC-STAMP promoter resulted in stable and persistent output of DCs expressing and presenting the Ag at the steady state that generate Ag-specific tolerance in RIP-OVA mice [120]. Similarly, in vivo transfer of LV.DC-STAMP.MOG transduced DCs prevented EAE progression [121]. Alternatively, Ag expression and presentation by DCs at immature steady state can be obtained by expressing the Ag under the post-transcriptional negative regulation mediated by those miRNAs, which are up-regulated in DCs upon activation. Brown et al. [122] showed that the addition of miR155-specific target sequences at the 3' untranslated region of LV expression cassette allowed a significant reduction of GFP expression as consequence of a TLR-driven activation.

Overall, the efficacy of LV-mediated gene transfer into DCs and their precursors offers several clinically applicable opportunities to exploit functional plasticity of DCs to design specific immunotherapies both for tolerance induction in autoimmunity and transplants, and for immunity to cancer and persistent infectious.

Conclusions

Significant progress has been made in understanding how DC immunotherapy can mediate immunity or promote tolerance and how DCs can be generated and manipulated in vitro in order to improve their immunogenicity or their tolerogenicity. The feasibility, the safety, and the effectiveness of immunotherapy with immunogenic DCs have been repetitively

demonstrated. Conversely, only recently the safety and the efficacy of immunotherapy with tolerogenic DCs have been demonstrated in pre-clinical models and in patients. These results supported the development of further clinical trials in cancer and infectious disease for immunogenic DCs and in autoimmunity and transplantation for tolerogenic DCs. However, several questions remain to be addressed to optimize immunotherapy: the selection of the optimal route of administration, the stability and the lifespan of the infused cells, and the identification of biomarkers of efficacy. During the last years, a number of consortia are investing efforts to identify hurdles for DC-based immunotherapies. SITC Immune Biomarkers Task is now investigating how to improve immunotherapy in cancer (<http://www.sitcancer.org/about-sitc/general-society/committees/biomarkers-task-force>) while the action BM1305: Action to Focus and Accelerate Cell-based Tolerance-inducing Therapies (www.afactt.eu), under the umbrella of the European Cooperation in the field of Scientific and Technical Research (COST) action, is working to establish minimal requirements to define a tolerogenic DCs, and to identify shared and disease-specific biomarkers of tolerance in patients undergoing tolerogenic DC-based therapies. At the same time, a deep knowledge on DC biology, together with advances in LV-mediated gene transfer, gave the possibility to improve the tolerogenic potential and the stability of DCs by inducing the constitutive expression of tolerogenic molecules.

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Compliance with Ethics Guidelines

Conflict of Interest Giada Amodio, Andrea Annoni, and Silvia Gregori declare that they have no conflict of interest.

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