Immune Network

http://dx.doi.org/10.4110/in.2016.16.1.13

pISSN 1598-2629 eISSN 2092-6685

REVIEW ARTICLE

Induction of CD4^{+} Regulatory and Polarized Effector/helper T Cells by Dendritic Cells

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Dendritic cells (DCs) are considered to play major roles during the induction of T cell immune responses as well as the maintenance of T cell tolerance. Naive CD4^{+} T cells have been shown to respond with high plasticity to signals inducing their polarization into effector/helper or regulatory T cells. Data obtained from *in vitro* generated bone-marrow (BM)-derived DCs as well as genetic mouse models revealed an important but not exclusive role of DCs in shaping CD4^{+} T cell responses. Besides the specialization of some conventional DC subsets for the induction of polarized immunity, also the maturation stage, activation of specialized transcription factors and the cytokine production of DCs have major impact on CD4^{+} T cells. Since *in vitro* generated BM-DCs show a high diversity to shape CD4^{+} T cells and their high similarity to monocyte-derived DCs *in vivo*, this review reports data mainly on BM-DCs in this process and only touches the roles of transcription factors or of DC subsets, which have been discussed elsewhere. Here, recent findings on 1) the conversion of naive into anergic and further into Foxp3^{-} regulatory T cells (Treg) by immature DCs, 2) the role of RelB in steady state migratory DCs (ssmDCs) for conversion of naive T cells into Foxp3^{+} Treg, 3) the DC maturation signature for polarized Th2 cell induction and 4) the DC source of IL-12 for Th1 induction are discussed.
[Immune Network 2016;16(1):13-25]

Keywords: Dendritic cells, CD4^{+} T cells, Polarization, Regulatory T cells, Anergy

INTRODUCTION

Adaptive immune responses are characterized by the activation and differentiation of CD4^{+} T cells into distinct effector T helper (Th) cell subsets but also induced regulatory T cells (iTregs). The latter support pre-existing thymus-derived, natural regulatory T cells (nTregs) to allow but at the same time also control the effector cell responses against pathogens and prevent autoreactive T cells from autoimmune attack. While effector CD4^{+} T cell responses are characterized by the polarization of naive T cells into Th1, Th2, Th9 or Th17 subsets, iTregs of either Foxp3^{+} or Foxp3^{-} IL-10^{+} (Tr1) subtypes can be polarized from either naive T cells or already polarized effector Th1 or Th2 cells (1-3).

It appears that repetitive stimulation of CD4^{+} Th1 or Th2 cell leads to a loss of their effector cytokine production but an increase and finally dominance of IL-10 release as shown in mice (1) but also with cultured human T cell clones (4). The induction of Th17 effector or Foxp3^{+} iTregs from naive T cells has been described to depend on specific cytokines. While IL-6 is necessary for generation of both T cell phenotypes, TGF-β plays a pivotal

Received on November 28, 2015. Revised on January 15, 2016. Accepted on January 18, 2016.
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Abbreviations: BM, bone marrow; DCs, dendritic cells; HEV, high endothelial venules; iTregs, induced regulatory T cells; ssmDCs, steady state migratory DCs; Treg, regulatory T cells; VSGs, variant surface glycoproteins

role in generating Foxp3$^+$ iTregs while in its absence Th17 cell development is observed (5). However, it remains unclear which type of DCs and DC-derived factors would induce all of these tolerogenic (Foxp3$^+$ iTregs or Foxp3$^-$ IL-10$^+$ Tr1 cells) or immunogenic T cell polarization programs (Th1, Th2 and Th17 cells). While recent efforts concentrated on the role of conventional DC subsets and their transcription factors for CD4$^+$ T cell polarization (6-8), not much has been investigated on the DC phenotype or maturation stage such as non-migratory immature or migratory semi-mature or migratory fully mature DCs. Since quantitative aspects of T cell stimulation like peptide concentrations, TCR signal intensity or quality and quantity of costimulation play considerable roles for Th polarization (9,10), we believe that studying DC maturity may also help to contribute to the understanding of this aspect in Th cell polarization (11) and regulatory T cell induction (3). The strength of DC maturity/activation, represented by the surface expression of MHC and diverse costimulatory molecules as well as cytokines (12), will also be translated into the strength of T cell stimulation (13).

For the priming of Th cells *in vivo* DCs play a central role due to their antigen presentation capacity together with highly expressed costimulatory molecules and the production of pro-inflammatory cytokines. One additional key feature of DCs is their migratory capacity from infection sites to the draining lymph nodes. DC migration requires coordinate mechanisms of soluble and matrix-associated CCL19 and CCL21 chemokines recognized by the receptor CCR7 (14,15). Antigen capture and migration of blood DCs into lymphoid organs has been observed during immune responses but is less well understood (16). Under inflammatory conditions, monocyte-derived DCs infiltrating into atherosclerotic plaques may direct i/nTreg expansion by secretion of CCL17 chemokine (17). Since CCR7-dependent migration of DCs also occurs under steady state conditions (14), the question remained whether in mice such ssmDCs induce tolerance in naive CD4$^+$ T cells by inducing anergy, as observed *in vitro* (18), or induce deletion, as observed for CD8$^+$ T cells (19), or by converting the naive cells into iTregs.

For the latter it remained to be determined, whether i) Foxp3$^+$ iTregs would be induced as shown by using an osmotic mini-pump system (20) or whether Tr1 cells would result by employing endogenous tolerizing migratory DCs in an asthma model found by others (21) or as we observed by adoptive transfer of TNF-matured DCs in the experimental autoimmune encephalomyelitis (EAE) model (22). Finally, the question remained whether anergic T cells were stably anergic and non-suppressive or whether certain DC-derived signals may further polarize them into another phenotype such as Tregs.

Our lab addressed these topics mainly by using BM-DCs generated with GM-CSF (23) (Fig. 1) where immature and mature stages can be easily generated in contrast to *ex vivo* isolated spleen DCs or the in vivo-counterpart of BM-DCs which are inflammatory monocyte-derived DCs (24,25).

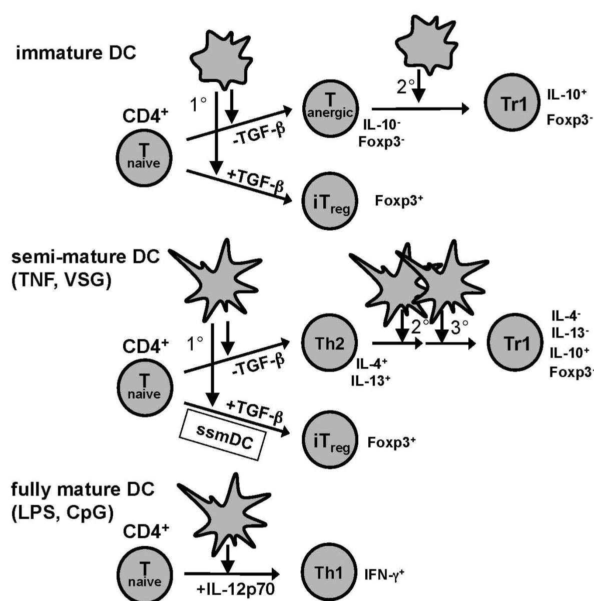

Figure 1. Induction of CD4$^+$ T cell anergy, Treg subsets and polarized Th1/Th2 responses by DC can be directed by their maturation stages and cytokines. Immature DCs induce antigen-specific T cell anergy in naive T cells in the absence of TGF-β, but induce Foxp3$^+$ iTregs when TGF-β is present. A second stimulation of non-regulatory anergized T cells by immature DCs generates regulatory IL-10$^+$ Tr1 cells. A similar T cell phenotype of regulatory IL-10$^+$ Tr1 is generated by repetitive stimulation with semi-mature BM-DCs generated by maturation with TNF or *T. brucei* VSG antigens characterized by a lack of cytokine production. A single stimulation with these DCs in the absence of TGF-β and IL-12 induces a Th2 phenotype that is lost upon repetitive stimulation. *In vivo* steady state migratory DCs (ssmDCs) resemble TNF-matured BM-DCs but capture TGF-β on their surface, thereby inducing naive T cell conversion into Foxp3$^+$ iTreg specific for self-antigens. DCs matured with high doses of LPS or CgG oligonucleotides reach a full maturation stage characterized by cytokine release including IL-12p70 that leads to Th1 induction.

Since the impact of DC maturity, expression of costimulatory molecules and of IL-10 production on the induction of CD4^{+} Treg has been reviewed recently (3), we will not further elucidate on this topic here.

CONVERSION OF NAIVE INTO ANERGIC AND FURTHER INTO Foxp3^{-} Tr1 CELLS BY IMMATURE DCs

T cell tolerance mechanisms include intrinsic and extrinsic mechanisms. Intrinsic control of T cells includes the induction of T cell anergy and T cell deletion, while extrinsic control is mediated by the activity of regulatory T cells (Tregs). The active role of Tregs for extrinsic T cell tolerance has been widely studied and also the result of T cell deletion appears obvious. In contrast, although the molecular details, how anergy is induced and maintained is increasingly understood (26,27), an active functional role for anergic T cells for tolerance or any other usefulness for maintaining such cells in the immune system had not been described.

The term clonal anergy was used to define a specific functional unresponsive state of CD4^{+} T lymphocytes (initially characterized in Th1 T cell clones, i.e. previously activated T cells) achieved by a strong TCR/CD3 signal 1 in the absence of CD28 costimulation as a signal 2 (28,29). Although a variety of experimental approaches have been used to induce T cell anergy *in vitro*, the most consistent hallmarks are defective IL-2 production and lack of proliferation upon TCR/CD3-mediated restimulation even in the presence of costimulation (29-31).

The phenotype of clonal T cell anergy can be reversed by addition of exogenous IL-2 and CD4^{+} T cell clones express high-affinity IL-2 receptors (IL-2R) (29). Reversal of CD4^{+} T cell anergy can also be performed by exposure to polyclonal stimuli circumventing TCR signaling by phorbol 12-myristate 13-acetate (PMA) and the calcium ionophores. However, in contrast to antigen-experienced T cell clones, naive CD4^{+} T cells have been reported to be resistant to anergy induction *in vivo* as well as *in vitro* upon TCR/CD3 stimulation alone in the absence of any secondary signals or costimulation (32).

Indeed, naive CD4^{+} T cells appear to be dependent on B7 costimulation-driven CTLA-4 engagement for anergy induction (33). CTLA-4 expression is obligatory for tolerance induction *in vivo*, also termed adaptive tolerance (29). This was observed in studies using T cells derived from CTLA-4$^{-/-}$ mice or antibody-mediated blocking experiments (34,35). The exact role and signaling mechanisms of CTLA-4 for the induction of T cell anergy is still a matter of debate (36). Earlier reports suggested that CTLA-4 signaling prevents cell cycle progression through regulation of the cyclin-dependent kinase (cdk) inhibitors p27^{Kip1} and p21^{Cip1} (34,37), although this may not be a strict requirement (38). Engagement of CTLA-4 has also been shown to block IL-2 production and IL-2R expression at least in part through decreased NFAT translocation to the nucleus (37,39,40).

Conversely, anergy induction in antigen-experienced T cells can be induced by Ca^{2+}/calcineurin-dependent signaling through ionomycin only, thereby triggering downstream NFATc2 but not its transcriptional binding partner AP-1 (31,41,42). An NFAT-dependent transcriptional program inducing various anergy-associated genes was identified, which crucially contribute to anergy induction *in vitro* and *in vivo* (31,41,43-45). Several analyses revealed a dominant role for the early growth response genes 2 (Egr2) and Egr-3 as markers of clonal T cell anergy *in vitro* and murine anergy models (46-50). Interestingly, Egr-2 has been reported also to support tolerogenic functions in DCs (51).

After anergy induction the question remains which functional role anergic T cells might have since they appear to persist *in vivo* for relatively long periods of time and can potentially reverse their functional unresponsive state (49,52). It was found that anergic T cells acquire the ability to produce anti-inflammatory cytokines such as IL-10 and suppress naive T cell responses (53-55). However, which signaling pathways or DC-derived instructive molecules are needed to induce IL-10 and regulatory function in anergic T cells remains unknown so far.

T cell anergy induction can be achieved by antigen presentation from immature DCs (12), similar to the generation of Foxp3^{+} Tregs (3) (Fig. 1). Using human monocyte-derived DCs we could show previously that naive allogeneic T cells required two rounds of stimulation *in vitro* to become anergic but without the capacity to release IL-10 or suppressing other T cell responses (18). This may be explained by the source of T cells used for the experiments, i.e. human peripheral blood versus murine lymphoid organs. Thus, while murine T cells receive tonic TCR signals in lymhoid organs increasing their responsiveness to secondary TCR engagements, this tonic activation

state is shut off in human blood T cells an may require restoration before these T cells are fully functional (56). A single intravenous injection of soluble antigens induced T cell anergy as observed initially by neo-self antigen expression or superantigen injection (57-60) or more recently in peptide-specific systems (61). Anergy induction and subsequent IL-10 production have been observed by these anergic T cells after repetitive intravenous injections of peptides, most likely captured and presented by immature DCs in this setting (62). However, the molecular requirements for an anergy-to-Tr1 switch had not been described.

To answer the latter question, we used immature BM-DCs to induce T cell anergy *in vitro*. We stimulated naive TCR-transgenic CD4^{+} CD25^{-} OT-II T cells with immature DCs in the presence of OVA peptide antigen *in vitro*. In the absence of TGF-β the induction of Foxp3^{+} iTregs could not be observed. Instead a single stimulation with immature DCs resulted in anergic, non-regulatory Egr2^{+} IL-10^{-} CTLA-4^{+} CD25^{low} T cells. Of note, immature DCs were superior in CTLA-4 induction as compared to mature DCs. A second stimulation of the anergized T cells with immature DCs thus triggered CD28 and CTLA-4 and by using blocking antibody Fab fragments our data indicate that both simultaneous signals are required to convert the anergic cells into regulatory T cells. The resulting population resembled Tr1 cells since they appeared as proliferating Egr2^{+} IL-10^{+} CTLA-4^{+} CD25^{high} cells with regulatory capacity. Suppressor activity was facilitated by highly effective IL-2 deprivation, enabled through CD28-mediated CD25 upregulation and the simultaneous CTLA4-mediated inhibition of nuclear translocation of NFATc1 and block of IL-2 production. Thus, Tr1 cells proliferated at the expense of IL-2 produced by effector T cells without producing IL-2 themselves. Together, two rounds of antigen-specific stimulation of naive T cells by immature DCs providing moderate CD80/CD86 signals in the absence of TGF-β induce IL-10^{+} Tr1 cells (63). This notion adds to the current opinion that Tr1 cells can be generated from repetitively stimulated Th1 or Th2 cells (1,22) as mentioned above but also from anergic T cells, however by different types of DCs (Fig. 1).

CONVERSION OF NAIVE CD4^{+} T CELLS INTO Foxp3^{+} iTregs BY RelB^{+} ssm DCs

In addition to their role in pathogen defense, migratory dendritic cells (DCs) are also critical for maintaining tolerance to self-antigens (64). Although the etiology of most autoimmune diseases remains obscure, abundant progress in this field has been made. It is now clear that immature DCs have tolerogenic properties by inducing T cell anergy or Tregs *in vitro* and *in vivo* (3). Especially the identification and characterization of Tregs has opened a new area of research, which promises to acquire sufficient knowledge for the development of new strategies against autoimmunity (65). To promote antigen-specific tolerance, DCs must capture, process and present self-antigens in a "steady state" phenotype within lymphoid tissues. This can occur either by lymph node resident immature DCs that capture soluble antigens through the lymph node reticular conduit system (66) and thus may induce T cell deletion, anergy or Tregs. In addition, also ssmDC display a partially mature (semi-mature) phenotype (Fig. 1, ssmDCs). They express CCR7 to migrate and transport self-antigens from peripheral organs such as the skin to the draining lymph nodes under homeostatic conditions (12). A murine transgenic model expressing OVA as a neo-self-antigen in the epidermis (K5-mOVA) showed that this antigen is carried by ssmDCs displaying a semi-mature phenotype and is then cross-presented for CD8^{+} T cell deletion or de novo conversion of naive CD4^{+} T cells into Foxp3^{+} Tregs (19,67).

The question remained which transcription factor in DCs would force them to induce iTregs *in vivo* when self-antigens are presented by ssmDCs under physiological conditions. Members of the NF-kB family such as RelA, RelB and c-Rel have been associated with inflammation or immunogenicity for many cell types (68). However, the functional role of these molecules in tolerogenic DCs is not fully understood. Tolerance to self-antigens expressed in peripheral organs is maintained by CD4^{+} CD25^{+} Foxp3^{+} Tregs, which are generated as a result of thymic selection (nTregs) or peripheral induction (iTregs). We demonstrated that ssmDCs from the skin mediated iTreg conversion in draining lymph nodes of mice. These DCs displayed a partially mature MHC II^{int} CD86^{int} CD40^{hi} CCR7^{+} phenotype, used endogenous TGF-β/latency-associated peptide (LAP) complexes for conversion of naive T cells into Foxp3^{+} iTregs and showed nuclear RelB translocation. Heterozygous deficiency of the alternative NF-κB signaling pathway (RelB/p52) reduced steady state migration of DCs. These DCs transported and directly presented

soluble OVA provided by subcutaneously implanted osmotic mini-pumps, as well as cell-associated epidermal OVA in transgenic K5-mOVA mice to CD4$^+$ OVA-specific TCR-transgenic OT-II T cells. The Langerin$^+$ dermal DCs subset, but not epidermal Langerhans cells, mediated conversion of naive OT-II x RAG-1$^{-/-}$ T cells into proliferating CD4$^+$ CD25$^+$ Foxp3$^+$ Tregs. Thus, these data showed that Langerin$^+$ dermal ssmDCs mediate peripheral iTreg conversion for epidermal self-antigen in skin-draining lymph nodes via RelB and TGF-β (67,69,70). Of note, homozygous RelB$^{-/-}$ mice lack peripheral lymph nodes (71), which does not allow to study ssmDCs in these mice and the results we obtained from heterozygous RelB$^{+/-}$ (67) mice may still involve indirect effects from other cell types. However, the efficient conversion of CD4$^+$ T cells to Foxp3$^+$ iTreg by Langerin$^+$ ssmDCs was corroborated by antibody-mediated specific targeting of the self-antigen myelin oligodendrocyte glycoprotein (MOG) to different ssmDC subtypes (72). More recently, also a critical role for the classical NFκB pathway for ssmDCs and tolerance has been found as demonstrated by spontaneous development of autoimmunity in mice with specific IKKβ deficiency in CD11c$^+$ cells (73).

The phenotype and transcriptional signature of TNF-matured BM-DCs generated with GM-CSF *in vitro* is highly similar as compared to ssmDCs from peripheral skin-draining lymph nodes. Both types show only a moderate to high expression of MHC II and costimulatory with the complete absence of detectable cytokine production, but with the exception to express LAP on their cell surface, which captures TGF-β to store it in its inactive form. Whether ssmDCs produce TGF-β by themselves, is unclear. However, the release of active TGF-β from ssmDCs by αVβ8 integrins is critical to maintain self-tolerance in mice (74). Thus, despite a similar semi-mature phenotype of TNF-matured BM-DC and ssmDCs these cells may be functionally distinguished by the capacity to provide TGF-β or not (Fig. 1).

INFLAMMATORY GENE SIGNATURES IN DCs MARK THEIR Th2 PROGRAMMING CAPACITIES

Helminths and other parasites represent prototype Th2-inducing pathogens. The way DCs sense type-2 pathogens ranges from pattern recognition receptors to tissue damage and to metabolic changes (75). Key factors released by DCs upon recognition of type-2 pathogens to instruct Th2 cells have not been identified so far. Although IL-4 represents a key factor for development and maintenance of Th2 responses, this cytokine is not produced by DCs. Since the absence of IL-12p70 production alone may allow Th2 polarization, a default pathway would induce Th2 cells. However, the failure of DCs to induce Th2 immunity in the absence of exogenous IL-4 produced by basophils does not support the concept of a default mechanism (76). In any case a common feature of Th2-inducing DCs is their semi-mature state, characterized by the up-regulation of antigen presenting molecules (MHC I, MHC II, CD1d) and costimulatory markers (CD80, CD86, CD40) on their surface but their lack to release polarizing cytokines such as IL-12p70 (Th1) or IL-6 and IL-23 (Th17) (77).

Is there a factor that could block full DC maturation while allowing partial maturation? The production of IL-10 by DCs has been associated with their propagation of Th2 immunity (78), however a clear and direct effect of IL-10 on naive T cell conversion to a Th2 phenotype is missing. IL-10 has been shown to fully block DC maturation and induce anergic or regulatory T cells mostly of the Tr1 type (79,80). Thus, although not fully clear, it seems most likely that a partial activity of IL-10 would suppress the production of polarizing cytokines such as IL-12p70 in an autocrine and paracrine fashion (81,82) and promote Th2 development indirectly via modulation of DCs. Since Th1 immunity can eradicate helminths and other parasitic infections but Th2 polarization allows for their persistence, many helminths developed mechanisms to promote IL-10 release from macrophages, DCs and other cells such as regulatory T cells (83) as mechanisms of immune evasion.

In this respect, deviation into Th2 immunity seems to represent a mild form of pathogen-directed immune evasion. When testing different developmental stages of the fox tape-worm *Echinococcus multilocularis* on BM-DCs, the early vulnerable stages of the larvae such as oncospheres and metacestodes induced more drastic forms of immune evasion, namely DC apoptosis or inhibition of DC maturation. Thereby the induction of immunity at the DC level is completely blocked, while the successor protoscolex stage that is protected by its laminated layer "allowed" partial maturation of DCs (84) for induction of Th2 immunity, similar to what has been observed for

Echinococcus granulosus (85).

Such a partial maturation profile can be observed also in BM-DCs after treatment with the pro-inflammatory cytokine TNF or products of *Trypanosoma brucei* (77). The question remained whether DC maturation by inflammatory TNF is qualitatively or quantitatively different for maturation markers and T cell programming as compared with maturation by different types of variant surface glycoproteins (VSGs) of *Trypanosoma brucei*. Microarray analyses revealed a common inflammatory signature of 24 genes regulated by all stimuli. Only few differences were observed between TNF and two types of VSGs, although being endogenous and inflammatory as opposed to foreign and pathogen-derived. TNF and the VSGs induced similar partial DC maturation as marked by high MHC II, costimulation and Jagged-2 expression but low cytokine profiles and their functional instruction of Th2 responses. In contrast, DC stimulation by LPS regulated almost 5000 genes, resulted in Delta-4 and IL-12p70 induction and polarization towards Th1. All partially matured DC showed comparable effects after injection on Th2-mediated asthma in mice and therapeutic influences on Th1/Th17-mediated experimental autoimmune encephalomyelitis (EAE) with respect to antibody isotype switching or regulatory IL-10 producing Tr1 induction, respectively. Full maturation by LPS regulated the same 24 inflammatory genes as observed after treatment with TNF or VSGs; but in addition numerous other genes, which led to Th1 skewing. In sum, few genes regulated in DCs allow their Th2 polarization, while additional (the same plus more) genes are regulated in DCs inducing Th1 responses. Thereby these data add to the concept of quantitative determination of Th polarization at the DC level (9,77). Thus, after years of searching for specific DC factors directing Th2 responses, quantitative effects and the default concept in determining Th2 polarization have come back into focus (2,9).

Th1 INDUCTION BY DCs AND THE SOURCES OF IL-12p70

The textbook knowledge suggests that IL-12 production by DCs directs optimal activation of polarized T helper type-1 cell (Th1) responses (Fig. 1). The following will encourage to have a closer look on the published results that leave decisive questions open. Several unexpected findings about IL-12 production reward a more systematic analysis. For example, the typical Th2 cytokine IL-4 could enhance bacteria-induced IL-12 production by DCs (86,87). Also IL-10, well established as an immunosuppressive cytokine, could enhance the IL-18-mediated IFN-γ release by $CD8^+$ T cells (88). While the three-signal-theory for differentiated T cell responses has been first established for $CD4^+$ Th1 cells (1/TCR, 2/costimulation, 3/IL-12), this could be clearly adapted also to CTLs (89) and IL-12 as signal 3 is considered as one of the most critical adjuvants for tumor cell killing (90).

The production of IL-12p70 is accepted as the critical event for Th1 polarization (91, 92). The cytokine is encoded by the two genes Il12a (p35) and Il12b (p40) resulting in the synthesis of a IL-12p70 disulfide-linked heterodimer. Major inducers of IL-12 in DCs are pathogens triggering Toll-like receptors (TLRs) (93,94). TLRs can also cooperate with each other to enhance IL-12 release by human (95) and mouse DCs (96). Although the IL-12-independent Th1 induction has also been described via IL-18, type-I interferons or CD70 (97-99), IL-12 is considered the most potent Th1 inducer (100). Reversely, human DCs treated with PGE_2 were blocked for IL-12 production and failed to generate Th1 responses (101). For murine DCs we found that dose and timing of the mast cell-derived PGD_2 exposure of DCs are critical to exert this effect (102). Depending on the infection IL-12 was necessary for microbial elimination as in murine *Leishmania major* models (103) or dispensable such as in Chagas' disease or mouse hepatitis virus infection (97,104). Vaccination strategies against *Leishmania* infection using DCs from IL-12-deficient mice indicate that Th1 responses may rely mainly on IL-12 by sensitizing Langerhans cells (105). The same group also showed that using BM-DCs as vaccines, bystander IL-12 production from the recipient mice could compensate for the lack of DC-vaccine-derived IL-12 (106). It is possible that DCs transmit cytokine production signals to other DCs, such as from migratory to lymph node-resident DC subtypes (107). In addition, two waves of DC migration into lymph nodes have been observed and are necessary to fully prime T cell responses (108). These date indicate that different migratory and lymph node-resident DC subsets may directly interact or coordinately work to generate polarized Th1 responses.

While $CD8\alpha^+$ splenic DCs did not require other signals than pathogen to produce IL-12p40, macrophages needed an IFN-γ priming prior to the pathogen signal (109). This

IFN-γ priming to enable IL-12p70 release has been proposed also for some DCs (110). Recently, monocyte-derived DCs were found to release IL-12p40 upon entry to an infection site, which was dependent on NK cell-derived IFN-γ (111). Of note, in this study the IL-12 production by DCs at the infection site appeared not to depend on phagocytosis of pathogens, pointing to bystander effects.

It also became apparent the last years that early during Th1 development NK cell cooperation with DCs plays a critical role (112-114). IL-12 production by DCs stimulates the IFN-γ production by NK cells and in a feedback loop represents a major cofactor for further IL-12 production by DCs and macrophages. In addition, IFN-γ signaling via STAT1 into developing Th1 cells enhances subsequent IL-12 signals by inducing STAT1 and STAT4 binding to the promoter region of Tbx21, the gene encoding T-bet, which is the master transcription factor of Th1 cells (115). Skin infection of *Leishmania major* led to the recruitment of NK cells from the blood into lymph nodes and their IFN-γ production starts 9h after infection (113). Whether soluble IL-12 can be transported via the afferent lymphatics and lymph node conduit system to high endothelial venules (HEV) to meet T cells (116) or whether it is provided by the lymph node migratory DCs, is not known.

The widely accepted view about $CD4^+$ T cell activation and differentiation into functional subsets proposes that three signals from DCs are critical to induce Th1 cells. However, some more recent data on DCs and Th cell differentiation indicate that this model may not be complete, pointing out major problems with this all-three-signals-from-one-DC concept.

Time points of IL-12 secretion by DCs

DCs reside at relatively high densities in epithelial tissues such as the skin at an immature state. Typically, microbial infection after skin injury will lead to pathogen uptake and DC maturation. One consequence of microbe-induced DC activation/maturation at the infection site is their subsequent migration to the draining lymph nodes where they process and present the transported antigens to T cells. This migration capacity to T cell areas of secondary lymphoid organs is one hallmark of DC biology as opposed to macrophages, which remain at the infection site to clear the microbes from the local environment. The chemokine receptor CCR7 is a strict requirement for this migration, which is up-regulated continuously after DC activation reaching half-maximal levels only after 24 hours *in vitro* (117), whereas DC immigration in draining lymph nodes of mice can be observed after 8h (118). *In vitro* and *in vivo* data indicate that also IL-12 secretion by human monocyte-derived DCs and murine DCs within the spleen can be detected after 10h or 4h, respectively (109,119). Others showed IL-12 secretion by DCs into the synapse formed with T cells 5h after DC activation (120). Thus, after pathogen recognition, DCs produce IL-12 with a fast kinetics but up-regulate CCR7 to migrate to lymph nodes later at 24-48h, a time period when IL-12 release is already terminated (118). How can DCs then coordinate the two functions of migration and IL-12 secretion for Th1 induction?

Mutual exclusiveness of migration and cytokine release

Analyses of human monocyte-derived DC populations indicate that mutually exclusive subsets of migratory and IL-12 cytokine producing cells exist in these bulk cultures (121). This finding was supported by our *in vivo* data. When murine *in vitro* generated BM-DCs were matured, fluorescence-labeled with CFSE and injected s.c. into mice, the $CFSE^+$ migratory DC which arrived in the lymph nodes were completely negative for intracellular cytokine detection, while endogenous $CFSE^-$ DCs appeared positive for various cytokines (107). Thus migration and cytokine production might not occur simultaneously in individual DCs. Experiments using IL-12p40-YFP^+ reporter mice indicated that YFP^+ BM-DCs were still able to migrate to the draining lymph nodes after s.c. injection (116). This would indicate IL-12 production at the infection site followed by DC migration to the lymph node. Such DCs have been reported as "exhausted" DCs that were unable to prime Th1 responses but induced Th2 cells (119). The reverse situation, that DCs become IL-12^+ after migration to the lymph node has not been demonstrated so far. To achieve this, genetic manipulation leading to the expression of lymph node homing receptors in DC was necessary to demonstrate their cytokine-producing and lymph node-homing potential (122,123). Although these genetic manipulations improved T cell responses, they do not contribute to understand the physiological series of events needed for Th1 cell priming.

Time window of IL-12 susceptibility of $CD4^+$ T cells

During T cell priming an early wave of IL-12 production may not be detectable for T cells. After arrival of the DCs

in the lymph nodes, T cell priming is initiated with subsequent polarization for CD4$^+$ T cells. However, at the early time points human and mouse CD4$^+$ T cells do not express a functional IL-12R and its upregulation requires TCR signals (124-128). After intravenous *Plasmodium* injection into mice IL-12Rβ was upregulated only at days 3∼4 after infection (129). Thus, early IL-12 production may occur but may not elicit polarization signals in T cells at these early time points in the lymph node. These investigations on the polarization of Th1 cells further revealed that after initial T cell priming via TCR and CD28, TCR down-regulation is required to allow IL-12R expression for IL-12-mediated Th1 polarization. Thus, after initial antigen-specific T cell priming the subsequent polarization signal may occur ideally in the absence of antigen presentation.

In addition, IL-12 may act on the DCs itself. IL-12 has been shown to induce NF-κB in DCs, DC maturation as well as IL-12 production (130,131). A second wave of late IL-12 production potentially occurs in a bystander fashion and may allow full differentiation of Th1 cells.

Bystander activation

Although DCs release cytokines after pathogen contact, also endogenous signals can initiate cytokine production. The interaction of 4-1BBL on mature DC with 4-1BB on co-cultured immature DCs has been shown to induce IL-12 production by the immature DCs (132-134).

Mature CFSE-labeled BM-DCs injected s.c. into mice remained negative for intracellular cytokines when re-analyzed from the draining lymph nodes 24h later, whereas the endogenous lymph node-resident CD11c$^+$ DC population showed the production of various cytokines (107). It requires clarification, whether migratory DCs transmit bystander activation signals (e.g. via 4-1BB-L to 4-1BB) or secrete factors that contribute to the bystander cytokine production (e.g. tissue injury after DC injection). In the case of a bystander activation signal 4-1BB/4-1BB ligand/receptor trimerization is required (135) that cannot be detected by simple surface staining. The latter possibility on the role of extrinsic factors is based on the findings that TNF-matured DCs are neither able to produce IL-12 themselves (22,77) nor to stimulate IL-12 via bystander activation *in vivo* (136). It is also conceivable that mature migratory DCs simply transport and then "present" the pathogens itself to lymph node resident immature DCs via DC-DC interactions, similar as reported for presentation of intact antigen by migratory DCs to B cells (137,138).

Together, the available data indicate that the common model on IL-12 production by DCs is not sufficient to explain all the published findings mentioned above. Despite a plethora of publications on IL-12 production by DCs, the precise mechanisms and time points of IL-12 production still remain elusive. A recent report may point to a unifying concept. While the conventional DC subsets were found to promote proliferation but poor polarization, secondarily generated monocyte-derived DCs during allogeneic immune responses showed the reverse functions by promoting Th1 and Th17 polarization and suppressing T cell proliferation, partially by release of nitric oxide (139). Although the IL-12 production by DCs in this setting has not been tested, it is tempting to speculate that the first wave of T cell stimulation by conventional DCs may occur largely in the absence of their IL-12 production to stimulate T cell proliferation whereas the second wave of inflammation-induced monocyte-derived DCs may release IL-12 to polarize T cells into Th1.

ACKNOWLEDGEMENTS

Research projects of the author that are published or mentioned in this article, were funded by the Wilhelm-Sander-Foundation and the German Research Council, DFG (LU851/8-1, TR52 TP-B7, IRTG1522).

CONFLICTS OF INTEREST

The authors have no financial conflict of interest.

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