Dendritic cell maturation: functional specialization through signaling specificity and transcriptional programming

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Abstract

Dendritic cells (DC) are key regulators of both protective immune responses and tolerance to self-antigens. Soon after their discovery in lymphoid tissues by Steinman and Cohn, as cells with the unique ability to prime naïve antigen-specific T cells, it was realized that DC can exist in at least two distinctive states characterized by morphological, phenotypic and functional changes—this led to the description of DC maturation. It is now well appreciated that there are several subsets of DC in both lymphoid and non-lymphoid tissues of mammals, and these cells show remarkable functional specialization and specificity in their roles in tolerance and immunity. This review will focus on the specific characteristics of DC subsets and how their functional specialization may be regulated by distinctive gene expression programs and signaling responses in both steady-state and in the context of inflammation. In particular, we will highlight the common and distinctive genes and signaling pathways that are associated with the functional maturation of DC subsets.

Keywords dendritic cells; homeostasis; immunity; tolerance

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Dendritic cell heterogeneity

Dendritic cells are cells of the mononuclear phagocyte system and develop in the bone marrow from common DC precursors that give rise to plasmacytoid DCs (pDCs) and to intermediate cells known as pre-conventional DC (pre-cDC). After exiting the bone marrow, pre-cDC transiently circulate in the bloodstream and migrate into lymphoid and non-lymphoid tissues where they differentiate into cDC, pDC also enter tissues from the blood stream, constitutively in the case of secondary lymphoid organs but only upon inflammation in the case of non-lymphoid tissues. Analysis of secondary lymphoid organs (spleen, lymph nodes) and of non-lymphoid tissues (skin,

intestine, lung, skeletal muscle and liver) has led to the identification of several distinct populations of cDC. Regardless of their anatomical location or species of origin, cDC can be grouped into two major subsets based on their phenotype, gene expression program, functional specialization and the transcription factors that specify their development (Guilliams *et al*, 2010) (Fig 1).

Xcr1⁺ and CD11b⁺ cDC

The expression of CD8 α or CD103 at the cell surface was originally used to classify a subset of cDC, often referred to as CD8 α -type cDC. However, CD8 α expression does not constitute a “universal” marker of this subset and CD103 is also expressed by intestinal cDC belonging to a second subset known as CD11b⁺cDC (see below). In contrast, the chemokine receptor *Xcr1* has been recently shown to be strictly specific for CD8 α -type cDC (Bachem *et al*, 2012; Crozat *et al*, 2011). The C-type lectin *Clec9a* (also known as *Dngr1*) is also selectively expressed on CD8 α -type cDC, but also on pDC and on a subset of DC progenitors (Schraml *et al*, 2013). On that basis, CD8 α -type cDC will be denoted as *Xcr1⁺*cDC for the purpose of this review. The human DC subset often referred to as CD141 (BDCA3)⁺cDC, is the equivalent of mouse *Xcr1⁺*cDC (Robbins *et al*, 2008; Haniffa *et al*, 2012) and can also be identified by its specific expression of *XCR1* (Bachem *et al*, 2010; Crozat *et al*, 2010; Yamazaki *et al*, 2010) and *CLEC9A* (Caminschi *et al*, 2008; Huysamen *et al*, 2008; Sancho *et al*, 2008).

The other major population of cDC are the CD11b⁺cDC. The phenotypic overlap between CD11b⁺cDC and other cells of the mononuclear phagocyte system—primarily monocyte-derived DC (moDC) and tissue-resident macrophages—has confounded their analysis. All cDC—including CD11b⁺cDC—have a short half-life (approximately 3–5 days in LT and slightly longer in NLT such as the lung or kidneys (Ginhoux *et al*, 2009)) and are continuously replaced from bone marrow progenitors in a manner that depends on the cytokine *Flt3L*, but that is independent of the *CCR2* chemokine receptor. In contrast, the development and maintenance of moDC and tissue-resident macrophages from bone marrow progenitors occurs independently of *Flt3L* but requires *CCR2* expression. Such distinctive developmental requirements allowed the

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Figure 1. Major subsets of DC in mouse and human.

Human and mouse DC subsets can be aligned into four major subsets irrespective of their location in secondary lymphoid tissues or in the parenchyma of non-lymphoid organs. They correspond to Xcr1⁺ cDC, CD11b⁺ cDC, pDC and moDC. The precursors that are found in the blood and give rise to the four major DC subsets are shown. Alternative markers or names used to identify those subsets are also indicated, as well as the proposed conserved functional specialization of these subsets.

unambiguous identification of CD11b⁺ cDC as CD11b⁺Ly6C⁻ CD64⁻ MerTK⁻ cells, which distinguished them from moDC and macrophages (Gautier *et al*, 2012; Tamoutounour *et al*, 2012, 2013). Note that CD64 corresponds to the high-affinity IgG receptor FcγRI. MerTK is a receptor protein-tyrosine kinase that recognizes apoptotic cells and promotes their uptake by phagocytic cells, but inhibits their activation, in particular by antagonizing signaling through the receptor for type I interferons (IFN-I) (Rothlin *et al*, 2007). Human cDC expressing CD1c (BDCA1) have a gene signature that resembles that of mouse CD11b⁺ cDC and are likely to represent functional homologs (Robbins *et al*, 2008; Schlitzer *et al*, 2013).

pDC

Mouse pDC are functionally characterized by their unique ability to produce high levels of IFN-I upon stimulation with viruses (Asselin-Paturel *et al*, 2001; Bjorck, 2001; Nakano *et al*, 2001). Under steady-state conditions, their morphology resembles that of a plasma cell as their name indicates. However, they develop dendrites after activation. Mouse pDC can be unequivocally identified as CD11b⁻CD11c^{int} Bst2^{hi} or as CD11b⁻ SiglecH⁺. Of note, some pDCs express CD8α under steady-state conditions, and the expression of this marker is further induced on activated pDCs (Asselin-Paturel *et al*, 2001; Dalod *et al*, 2002). Thus, care must be taken not to erroneously include CD8α⁺ pDC into the population of Xcr1⁺ cDC, as may happen when using only CD11c and CD8α to define the later.

Lymphoid tissue (LT) and non-lymphoid tissue (NLT) cDC

DCs can be also classified according to their anatomical location. For instance, both Xcr1⁺ and CD11b⁺ cDC can either spend their whole life in secondary lymphoid tissues (LT-cDC), or reside first in the parenchyma of non-lymphoid tissues (NLT-cDC). These interstitial NLT-cDC have the ability to migrate via afferent lymphatics to the draining lymph nodes (LN), where they are classified as migratory NLT-cDC (mig-NLT-cDC). Note that the LT-cDC that permanently reside in the spleen are also endowed with migratory properties, in that they are originally located in the red pulp or in the marginal zone where they sense antigens or pathogens that are transported in the blood, and subsequently migrate to the T-cell zone of the periarteriolar lymphoid sheaths.

Monocyte-derived DC

During inflammation, monocyte-derived inflammatory DC (Inf-moDC) develop in inflamed tissues from extravasated Ly6C^{hi} (classical) blood monocytes and disappear once the inflammation resolves. Inf-moDC have been identified in pathological conditions in both humans (Segura *et al*, 2013) and mice (Serbina *et al*, 2003). In some inflammatory settings or during infections by viral, bacterial, fungal and parasitic agents, moDC differentiate into tumor necrosis factor (TNF) and inducible NO synthase (iNOS)-producing DC (Tip-DC) with potent antimicrobial effector functions (Serbina *et al*, 2003; Aldridge *et al*, 2009; Tamoutounour *et al*, 2012; Bain *et al*, 2013).

Under steady-state, non-inflammatory conditions, Ly6C^{hi} blood monocytes are also capable of extravasating into tissues such as the dermis, the intestinal lamina propria and the lung, where they can eventually give rise to moDC that have a short half-life and are continuously renewed (Gautier *et al*, 2012; Tamoutounour *et al*, 2012, 2013; Bain *et al*, 2013; Jakubzick *et al*, 2013). Therefore, moDC and Inf-moDC likely represent alternative context-dependent fates of the same Ly6C^{hi} blood monocyte precursors (Bain *et al*, 2013). Inf-moDC and moDC are both characterized by their CD11b⁺ CCR2⁺ CD64^{lo/+} MerTK⁻ phenotype and show transcriptomic features reminiscent of CD11b⁺ cDC on the top of their monocytic signature, both in mice (Gautier *et al*, 2012; Tamoutounour *et al*, 2012, 2013) and humans (Segura *et al*, 2013). Although moDC represented the major DC subset in the lungs and the dermis upon exposure to house dust-mite and a contact allergen, respectively, they remained the smallest subset among migratory cells within draining LNs, indicating that moDCs migrate poorly as compared to cDC subsets (Plantinga *et al*, 2013; Tamoutounour *et al*, 2013).

DC maturation

One of the most critical features of DC biology is their functional maturation. This is a complex process characterized by the acquisition of a number of fundamental properties: antigen processing and presentation, migration and T-cell co-stimulation (Mellman & Steinman, 2001). However, as will be detailed later, DC maturation is a heterogeneous process that can confer distinctive functional properties.

In steady-state, NLT-cDC and LT-cDC are described as being resting or immature, a phenotype characterized by a low surface expression of major histocompatibility complex (MHC) class II molecules and co-stimulatory molecules (e.g. CD80, CD86 and CD40). In response to activation by infection, injury or vaccination, NLT-cDC and LT-cDC undergo a program of maturation, which renders them capable of inducing the clonal expansion of antigen-specific naïve T cells and their concomitant differentiation into effector T cells. In this context, immunogenic maturation of cDC leads to the upregulation of MHC class II and co-stimulatory molecules at the cell surface, the CCR7-dependent migration to T-cell-rich zones in LNs, and endows the capacity to release cytokines promoting the differentiation of naïve antigen-specific T cells into effector cells, as well as the activation of various other types of immune cells (Probst *et al*, 2003; Sporri & Reis e Sousa, 2005; Gatto *et al*, 2013). Depending on the nature of the stimuli they sense, cDC can produce distinct cytokines and trigger the differentiation of different types of effector T cells, thereby permitting the adaptation of T-cell polarization to the specific nature of the threat.

Importantly, in steady-state, a fraction of LT-cDC and NLT-cDC undergo a constitutive maturation, which we also refer to as “homeostatic maturation” (Lutz & Schuler, 2002). As a result, they upregulate expression of MHC class II molecules at the cell surface, to almost identical levels found under inflammatory conditions, and they migrate to draining LNs and to T-cell zones in a CCR7-dependent manner (Ohl *et al*, 2004). The presence of high levels of MHC class II molecules on these cDC is likely to enhance their capacity to engage T cells. However, this homeostatic maturation program confers tolerogenic rather than immunogenic properties to cDC, in

that they lack the ability to drive the differentiation of naïve self-reactive T cells into effectors and thus result in abortive T-cell responses (Probst *et al*, 2003; Sporri & Reis e Sousa, 2005). Tolerogenic cDC are thought to display processed self-peptide for purging the peripheral T-cell repertoire from those excessively self-reactive T cells that have escaped central tolerance. In addition, these cDC maintain T-cell tolerance to innocuous environmental antigens through the generation of induced regulatory T cells (iTreg). Thus, steady-state cDC maturation is likely to play an important role in peripheral tolerance. As discussed below, the nature of the sterile triggers that result in homeostatic cDC maturation remains an enigma.

DC maturation induced by pattern-recognition receptors (PRR)

All DC subsets, and other antigen-presenting cells such as macrophages, are equipped with a battery of pattern-recognition receptors (PRRs), which can detect molecular patterns of invading microorganisms or endogenous “danger” signals and alert the immune response. These PRRs are expressed both on the cell surface and inside the cell and are extremely diverse, detecting a wide range of molecular species including proteins, carbohydrates, lipids and nucleic acids (Takeuchi & Akira, 2010). The most widely studied family of PRRs on DC are the Toll-like receptors (TLRs). Triggering of TLRs on DC is thought to be critical for their functional maturation to immunogenic DC and the priming of naïve T cells in response to infection, and therefore coupling innate and adaptive immunity. Importantly, some TLRs also recognize host molecules, such as the nucleic acid sensing TLRs (TLR3, TLR7 and TLR9) (Akira *et al*, 2006). Inappropriate DC activation by these endogenous TLR agonists may be linked to the development of autoimmune diseases such as rheumatoid arthritis (RA), psoriasis and systemic lupus erythematosus (SLE). TLR-mediated recognition of commensal microorganisms may also have an important role tissue homeostasis, the most notable example being the intestine where blockade of TLR signaling leads to impaired barrier function and inflammation (Rakoff-Nahoum *et al*, 2004), and it was recently shown that TLR signaling in DC was required to maintain immune homeostasis and tolerance to gut microbiota (Han *et al*, 2013).

Functional specialization of DC through selective TLR expression

Different DC subsets express distinct repertoires of TLRs (Fig 2), which is likely to contribute to their functional specialization. For example, pDC express high levels of TLR7, TLR9 and TLR12. TLR7 and TLR9 detect single-stranded RNA (ssRNA) and unmethylated CpG DNA, respectively. These motifs are found in both bacteria and viruses. TLR12 expression on pDC recognizes the profilin from the intracellular parasite *Toxoplasma gondii* (Koblansky *et al*, 2013). Several studies in mice have shown the importance of TLR7 and TLR9 in the immune response to bacterial and viral pathogens (Akira *et al*, 2006), and of TLR11 and TLR12 in immune responses against *T. gondii* (Yarovinsky *et al*, 2005; Koblansky *et al*, 2013). Mouse Xcr1⁺ cDC uniquely express TLR11 and high levels of TLR3, but do not express TLR4, TLR7 and TLR8, or only very low levels compared to other cell types (Fig 2, (Crozat *et al*, 2009); <http://biogps.org/>). However, rigorous

Figure 2. Comparison of the expression patterns of TLRs across DC and monocyte subsets in mice.

The bar graphs show relative gene expression (mean $\pm$ SD) for individual TLRs across blood monocyte subsets (gray bars), spleen LT-DC subsets from untreated animals (plain color bars) and cutaneous LN (CLN) mig-NLT-DCs from DNFB skin-painted animals (hatched color bars). These data are compiled from our own publicly available datasets and those of the Immgen consortium (Heng *et al*, 2008; Tamoutounour *et al*, 2013). Key: c monocytes, classical blood monocytes characterized as CD11b⁺ Ly6C^{hi} MHCII⁺ cells; nc monocytes, non-classical monocytes characterized as CD11b⁺ Ly6C^{lo} MHCII⁺ cells. For each gene, expression values are normalized to maximal expression across all samples and the mean of 2 to 5 replicates for each cell subset is shown.

examination of the cell-intrinsic responses to TLR4 triggering by XCR1⁺ DC will require pure populations of these cells stimulated *in vitro* with LPS, or comparing *in vivo* responses of WT versus *Tlr4*^{-/-} cells in mixed bone marrow chimeras. Although one report

suggests TLR11 expression is low on Xcr1⁺ cDC (Koblansky *et al*, 2013), several reports have shown that TLR12/TLR11 heterodimers are required for *T. gondii* profilin recognition by Xcr1⁺ cDC (Yarovinsky *et al*, 2005; Andrade *et al*, 2013; Raetz *et al*,

2013). TLR3 recognizes viral double-stranded RNA (dsRNA) (Alexopoulou *et al*, 2001), and its expression by DC is particularly important for promoting cross-priming in the context of viral infection (Schulz *et al*, 2005), which is a defining property of the Xcr1⁺ cDC subset (Guilliams *et al*, 2010). Interestingly, a recent study showed that commensal bacteria in the gut produce dsRNA, which distinguishes them from pathogenic bacteria, and the TLR3-mediated recognition of gut microflora was shown to be important in maintaining immune homeostasis (Kawashima *et al*, 2013). It would therefore be interesting to examine the role of TLR3 signaling in Xcr1⁺ cDC for tolerance to commensal bacteria. The TLRs involved in the recognition of components of bacterial cell walls and flagella (TLR1, 2, 4, 5 and 6) or bacterial rRNA (TLR13) are most strongly expressed on monocytes, neutrophils and to some extent on moDC and CD11b⁺ cDC (Fig 2). However, TLR13 is also expressed in Xcr1⁺ DC (Fig 2) and allows them to respond to bacterial 23S rRNA (Oldenburg *et al*, 2012). Strikingly, while it recognizes ssRNA, TLR8 expression appears to be even more restricted to monocytes and neutrophils.

Overall, with regard to their TLR expression pattern, pDC and Xcr1⁺ cDC appear to be more specifically equipped for detection of intracellular pathogens, either directly or through phagocytosis of material from infected cells, while CD11b⁺ cDC and moDC appear to be more specialized for detection of extracellular pathogens. The analysis of expression patterns of other PRRs across immune cells types has confirmed the notion of functional specialization by DC subsets in the recognition of different pathogens or in the detection of specific danger signals. In particular, moDC, monocytes and neutrophils, but also to some extent CD11b⁺ cDC, express higher levels of cytosolic sensors involved in the recognition of productive intracellular infection by bacteria or viruses (Crozat *et al*, 2009; Lubet *et al*, 2010). Of note, both homeostatic and PRR-induced maturation can lead to dramatic changes in TLR expression on DC, in particular TLR3 and TLR11 are downregulated upon maturation of XCR1⁺ cDC (Fig 2). On the contrary, PRR-induced maturation can induce TLR3 expression on CD11b⁺ cDC (not shown). This emphasizes the dynamic nature of TLR expression in DC subsets and thus of their response to a given stimulus. The expression pattern of PRRs across DC subsets is rather well conserved between mouse and human, with a few exceptions, such as TLR9, which is broadly expressed in the mouse but mainly restricted to pDC in the human (Crozat *et al*, 2009), and TLR8, which in human is not only expressed on monocytes and neutrophils but also on moDC, CD11b⁺ cDC and XCR1⁺ cDC but still absent from pDC (Hornung *et al*, 2002; Lindstedt *et al*, 2005).

Specificity of TLR-signaling pathways in DC

There are three major signaling pathways that dictate the functional consequences of DC activation by TLRs: mitogen-activated protein kinase (MAPK), nuclear factor- κ B (NF- κ B) and interferon regulatory factors (IRFs) (Akira *et al*, 2006). Different TLRs are coupled to distinct downstream signaling pathways by the selective use of different TIR-domain signaling adaptor molecules: MYD88, TRIF, TRAM and TIRAP (MAL). All TLRs, except TLR3, utilize the MYD88 adaptor to trigger activation of TAK1 (TGF β Activated Kinase 1; MAP3K7), which is responsible for downstream activation of both MAPK and NF- κ B, which cooperatively mediate pro-inflammatory

cytokine production, such as TNF α , IL-12 and IL-6. These cytokines have an important role in the polarization of T helper cell subsets during priming by DC, and thus the functional consequences of DC activation. It appears that TAK1 signaling is particularly important in DC maturation and survival, since mice with a specific deficiency of TAK1 in the DC lineage have severe defects in DC development and function (Wang *et al*, 2012). However, specific kinases downstream of TAK1, such as the MAPKs p38 and JNK, and I κ B Kinase (IKK)—which regulates activation of NF- κ B—may have more specific functions in DC maturation. For example, activation of p38 α in DC is particularly important for Th17-mediated immune responses (Huang *et al*, 2012), but not DC survival and homeostasis. However, p38 α signaling was also shown to be important for Treg conversion by migratory CD103⁺ DC in the intestine and mucosal tolerance (Huang *et al*, 2013), indicating that its role is not exclusive to the immunogenic maturation of DC. The specific roles of JNK and IKK signaling in DC maturation have not yet been determined, but it would be interesting to see how they are related to those of TAK1 and p38 α .

TLR3 and TLR4 recruit a specific adaptor called TRIF (Yamamoto *et al*, 2002, 2003a; Hoebe *et al*, 2003), which activates TANK-binding kinase 1 (TBK1) and I κ B kinase ϵ (IKK ϵ) (Hemmi *et al*, 2004), leading to the activation of IRF3 and induction of IFN-I expression. In the case of TLR4, another adaptor called TRAM is required to recruit TRIF for IRF3 activation (Yamamoto *et al*, 2003b), while TIRAP couples TLR4 and TLR2 signaling to MYD88 (Fitzgerald *et al*, 2001; Horng *et al*, 2002). TLR7 and TLR9 also trigger IFN-I induction, selectively in pDC, through a distinct MYD88-dependent signaling pathway that activates IRF7 directly (Honda *et al*, 2005a,b). In pDC, IRF7 is constitutively expressed and forms a complex with TLR7/9 and MYD88 (Honda *et al*, 2005a), sequestered in distinct endosomes where IFN-I production is initiated upon appropriate stimulation. This distinctive expression pattern, and subcellular colocalization of TLR7, TLR9, MyD88 and IRF7 in pDC probably, has an important role in conferring their specific function as a major source of IFN-I upon viral infection.

Although TLR3 does not signal through MYD88, TRIF recruitment can also activate NF- κ B and MAPK through RIP1 or TAK1 (Cusson-Hermance *et al*, 2005). Therefore, NF- κ B and MAPK activation is conserved among all TLR receptors, as well as the induction of pro-inflammatory cytokines such as TNF α and IL-1 β . However, the ability to trigger IRF3 or IRF7 activation, and consequently IFN-I expression, is restricted to TLR4 and the nucleic acid sensing TLRs (TLR3, 7, 8, and 9). IRF3 also directly regulates expression of other genes that have important functions in DC for T-cell priming, including the expression of IRF7, to amplify IFN-I production, and IL-12p40/p35 (Ramirez-Carrozzi *et al*, 2009)—a critical cytokine for Th1-cell polarization. Furthermore, autocrine and paracrine IFN-I signaling in DC is important for the amplification of IL-12p40/p35 expression (Gautier *et al*, 2005). Thus, IRF3 activation may be a critical factor for IL-12 induction in DC and consequently is likely to be important for the polarization of Th1 cell response during naïve T-cell priming. TLR7, TLR9 and TLR3 agonists also selectively promote cross-presentation in DC (Datta *et al*, 2003; Le Bon *et al*, 2003; Schwarz *et al*, 2003; Schulz *et al*, 2005), suggesting that the triggering of IFN-I production by DCs is particularly important for optimal priming of Th1 cells and induction of strong cytotoxic CD8 T-cell responses (Manicassamy & Pulendran, 2009). Therefore, at

least in the context of Th1 and CD8 T-cell priming, IFN-I induction represents an important functional distinction between different TLR agonists by DC in their role in the immune response. Much less is known about the role of specific TLR-signaling pathways in other polarized T-cell responses. As mentioned above, p38 MAPK activation in DC has been specifically implicated in Th17-cell polarization (Huang *et al*, 2012), which may be due to the important role for p38 in MYD88-dependent IL-6 expression.

Besides the distinct expression pattern of different TLRs by DC subsets, there is also selective use of specific signaling pathways by different DC. For example, while TLR9 engagement in pDC leads to direct activation of IRF7 (Honda *et al*, 2005a,b), and downstream induction of IFN-I (IFN- α and IFN- β) and IFN- λ , in mDC and cDC TLR9 triggers a distinct pathway leading to activation of IRF1 and specific induction of IFN- β (Schmitz *et al*, 2007; Hoshino *et al*, 2010). In addition to the differential induction of IFNs, other important differences between the genes regulated by IRF1 and IRF7 may have functional consequences for pDC versus cDC activation by TLR9. Interestingly, TLR7- and TLR9-mediated induction of IFN-I in both pDC and cDC is dependent on the same upstream kinase; IKK α (IKK1; CHUK) (Takeda *et al*, 1999). In Flt3-derived pDC stimulated with TLR7 or TLR9 agonists, IKK α directly phosphorylates IRF7 leading to induction of IFN-I (Hoshino *et al*, 2006). Similarly, in Flt3-derived cDC stimulated with CpG DNA, IKK α is also responsible for IRF1 activation and induction of IFN- β (Hoshino *et al*, 2010). Furthermore, we recently showed that IKK α is required for TRIF-dependent IRF3 activation and IFN- β induction in GM-CSF-derived DC (Mancino *et al*, 2013), which represent mDC. These studies suggest the role of IKK α in IFN-I induction by DC is conserved among DC subsets and different TLR ligands.

Another conserved element in IFN-I induction by TLR3, TLR7 and TLR9 is the signaling adaptor TRAF3 (Hacker *et al*, 2006; Oganessian *et al*, 2006). The TRAF signaling adaptors are important regulators of innate immune signaling pathways, they act as ubiquitin ligases and couple receptor signaling to downstream kinases such as TBK1/IKK ϵ in the case of TRAF3, and TAK1 in the case of TRAF6—which is required for both MAPK and IKK/NF- κ B activation by TLRs (Akira *et al*, 2006). Interestingly, while TRAF3 is required for MYD88-dependent and TRIF-dependent IFN-I induction, TRAF3 negatively regulates MYD88-mediated NF- κ B activation and pro-inflammatory cytokine expression, at least in the context of TLR4 signaling (Hacker *et al*, 2006). By regulating the balance between pro-inflammatory cytokine production and IFN-I expression, TRAF3 could have a key role in modulating DC activation. IKK α has also been shown to inhibit pro-inflammatory cytokine production in macrophages (Lawrence *et al*, 2005), suggesting a similar role for IKK α in modulating cytokine production in response to TLR signaling in favor of IFN-I production. We have shown that IKK α activation in DC is critical for Th1 cell priming and acquired immunity to intracellular pathogens (Mancino *et al*, 2013). But the specific role of TRAF3 in DC activation *in vivo* has yet to be determined.

Homeostatic DC maturation

While the mechanisms of TLR-induced DC maturation have become increasingly well characterized, we still understand little about the

signaling pathways and upstream regulators that drive steady-state DC maturation. A concept of “limited,” or “partial,” maturation has been proposed to distinguish the homeostatic (tolerogenic) DC maturation in steady-state, from TLR-induced (immunostimulatory) DC maturation (Lutz & Schuler, 2002). Recent studies suggest that genes, which regulate the NF- κ B signaling pathway, play critical roles in restraining DC maturation in steady-state (Dissanayake *et al*, 2011; Hammer *et al*, 2011; Kool *et al*, 2011). In the absence of such factors, steady-state DC maturation becomes immunostimulatory, leading to inappropriate priming of naïve T cells and autoimmunity. A20, the product of the *Tnfrsf3* gene, is a negative regulator of NF- κ B and MAPK signaling and restricts pro-inflammatory signals through the TNF receptor, TLRs, nucleotide-binding oligomerization domain protein (NOD) and CD40 (Ma & Malynn, 2012). Targeted deletion of A20 in DC, using CD11c-Cre transgenic mice, led to marked splenomegaly and lymphadenopathy within 3 weeks of birth associated with the accumulation of large numbers of myeloid and lymphoid cells (Hammer *et al*, 2011; Kool *et al*, 2011). A20 was shown to restrict MyD88-dependent signals that drive IL-6 and TNF secretion by DCs and subsequent T-cell expansion. But, A20 also restricted MyD88-independent signals in DC that upregulate T-cell co-stimulatory molecules, and blockade of CD80 and CD86 in mice with A20-deficiency in DC significantly antagonized the increased activation of adoptively transferred naïve T cells. These studies suggested that the absence of A20 expression in DC led to unrestrained MyD88-independent signals that increase co-stimulatory molecule expression and cause spontaneous T-cell activation. In addition, enhanced MyD88-dependent signals in DC increase secretion of cytokines such as IL-6 that drive T-cell and myeloid expansion. The A20-binding protein, ABIN-1 (encoded by the *Tnfrsf1* gene), has also been shown to be an important negative regulator of TLR-induced NF- κ B and MAPK activation. Deletion of *Tnfrsf1* in CD11c-expressing cells leads to exaggerated DC activation in a mouse model of psoriasis, associated with increased MyD88-dependent IL-23 expression (Callahan *et al*, 2013). One should make a cautionary note here as to the DC-specific nature of these observations, as with any Cre-transgenic system there is often off-target deletion of the “floxed” gene, in the case of CD11c, this promoter is also expressed prominently in macrophages, NK cells and even in activated T cells. In fact, the targeted deletion of A20 in myeloid cells, using lysozyme M (*Lyz2*)-Cre mice, also leads to spontaneous autoinflammatory disease (Matmati *et al*, 2011), although *Lyz2* is not strongly expressed in cDC.

In another study, the NF- κ B protein p105 (NFKB1) was found to be crucial for maintaining the resting state of cDC (Dissanayake *et al*, 2011). Upon adoptive transfer, self-antigen-pulsed, bone marrow-derived DC lacking Nfkb1 were able to activate CD8⁺ T cells directed toward self-antigens and trigger autoimmunity in the absence of TLR signals. NFKB1 is the precursor for the p50 subunit of NF- κ B, which lacks a transactivation domain, and is therefore transcriptionally inactive. The formation of p50 homodimers has been shown to repress NF- κ B-dependent expression of pro-inflammatory genes and is thought to be a particularly important mechanism for endotoxin (LPS) tolerance in chronically activated macrophages (Bohuslav *et al*, 1998). The ability of p50 to inhibit IFN- β expression in this context has been shown to be critical for the tolerogenic phenotype (Cheng *et al*, 2011). Although not tested by Dissanayake and colleagues, it would be interesting to evaluate

the role of enhanced IFN- β production in the immunostimulatory phenotype of Nfkb1-deficient DC, given the strong association between IFN-I signaling networks and TLR-induced DC maturation (Baranek *et al*, 2012; Vu Manh *et al*, 2013; Pantel *et al*, 2014).

Gene expression signatures of steady-state and TLR-induced DC maturation

To investigate the immunogenic maturation of DC subsets in different contexts, we recently performed a genome-wide analysis of gene expression signatures in several DC subsets stimulated with various TLR agonists. We then applied bioinformatics tools to identify signaling pathways and transcription factors associated with DC maturation under these conditions. A major conclusion of our analysis was that different DC subsets undergo a convergent transcriptional reprogramming during TLR stimulation. Furthermore, the signaling pathways regulating activation of NF- κ B and IRF3, or IRF7, were strongly implicated in this common program of TLR-induced DC maturation (Vu Manh *et al*, 2013). Here, we have extended this strategy to investigate the differences between TLR-induced “immunogenic” DC maturation and homeostatic “tolerogenic” DC maturation. We analyzed how the expression of the core sets of genes, identified as modulated by TLR-induced immunogenic DC maturation, was affected during homeostatic—tolerogenic—DC maturation. For this, we compared NLT-cDC with their migratory counterparts in tissue-draining LNs (mig-NLT-cDC) (Miller *et al*, 2012). At first glance, the corresponding heatmap shows that most of the core genes that were downregulated upon TLR-induced DC maturation (“CORE DOWN” genes), such as in splenic DC from mice infected with mouse cytomegalovirus (MCMV) or in lung CD103⁺ DC from mice challenged with dsRNA (PolyI:C), were also strongly down-modulated during homeostatic DC maturation (Fig 3A). Furthermore, many of the core genes that were upregulated upon TLR-induced DC maturation (“CORE UP” genes) were also strongly induced upon homeostatic DC maturation. These data show that converging changes occur in gene expression between homeostatic and TLR-induced DC maturation, suggesting

that overlapping instructing signals are driving both processes. Next, we used ingenuity pathway analysis to determine whether the gene sets regulated upon homeostatic DC maturation were enriched for targets of the same transcription factors, activation receptors or cytokines previously identified as major regulators of TLR-induced DC maturation (Fig 3B–C). The genes down-modulated both upon TLR-induced and homeostatic DC maturation were strongly enriched for targets of the E2F1 network (Fig 3C) and for annotations linked to positive regulation of mitosis (not shown), showing that homeostatic DC maturation is characterized by the repression of cell cycle genes, as we previously reported for the maturation of spleen DC subsets upon MCMV infection *in vivo* (Baranek *et al*, 2012). As expected, the genes induced in lung CD103⁺ DC from PolyI:C-treated mice were very strongly enriched for targets of the IRF/IFN-I network (Fig 3D, ❶), as well as the NF- κ B/TNF/IL-1 β network (❷) and the CD40/CD40LG (❸) or IFN γ (❹) networks. Surprisingly, this was also the case for the genes commonly induced during homeostatic maturation. However, many genes within these networks were induced specifically during either TLR-induced or homeostatic maturation (Fig 3D and Supplementary Table S1). This suggests that the outcome of IRF, NF- κ B, CD40 or IFN γ signaling during DC maturation is dictated by instructive signals depending on the tissue localization and the inflammatory context, which must ultimately determine the functional consequences of DC maturation.

What distinguishes “tolerogenic” from “immunogenic” DC maturation? Certain “CORE UP” genes encoding for immune activation molecules (*Mx1*, *Rsad2*, *Il15*, *Il15ra*, *Irf1*), thought to be selectively upregulated during TLR-induced immunogenic DC maturation, were also strongly induced during homeostatic maturation of tolerogenic DC (Fig 3A). Conversely, the *Tnfrsf3* gene, encoding the A20 protein previously reported to preserve immune homeostasis by preventing DC activation in steady-state, was highly induced under all conditions of TLR-induced maturation but was down-modulated upon homeostatic maturation of skin CD11b⁺ cDC. These observations prompted us to further examine the changes in the expression of genes involved in activation versus inhibition of immune responses during homeostatic or TLR-induced DC maturation (Fig 3E). A

Figure 3. Comparison of homeostatic versus PRR-induced DC maturation.

(A) Converging changes in gene expression between homeostatic and PRR-induced DC maturation. Heatmap showing the fold change between immature and mature DC for two sets of genes previously reported to be, respectively, decreased (“CORE DOWN”) or increased (“CORE UP”) upon TLR-induced maturation; irrespective of DC subset, stimuli and species of origin. For homeostatic maturation, fold change in gene expression levels was computed by comparing mature DC having migrating in cutaneous or lung lymph nodes under steady-state conditions (CLN CD11b⁺ mig-NLT-DC and LULN CD103⁺ mig-NLT-DC) to their immature counterparts from skin or lung. For TLR-induced maturation, fold change in gene expression levels was computed by comparing TLR-stimulated DC (lung CD103⁺ DC isolated from PolyI:C-treated animals, spleen CD11b⁺ DC and CD8 α ⁺ DC isolated from MCMV-infected mice, and spleen CD8 α ⁺ DC isolated from PolyI:C-treated animals) to their immature, unstimulated, counterparts from the same tissue. Genes that showed a statistically significant and similar regulation in their expression in homeostatic maturation and in PolyI:C-induced maturation of lung CD103⁺ DC are shown in bold, black font. Genes that showed a statistically significant regulation in these conditions but with a reciprocal change between homeostatic maturation versus PolyI:C-induced maturation of lung CD103⁺ DC are highlighted in bold, red font. (B–D) Overlapping instructive signals drive homeostatic and PRR-induced DC maturation. Venn diagrams were drawn for comparing the sets of genes significantly induced (UP) or repressed (DOWN) upon TLR-induced maturation of lung CD103⁺ DC isolated from PolyI:C-treated animals, and upon homeostatic maturation of CLN CD11b⁺ mig-NLT-DC or in LULN CD103⁺ mig-NLT-DC. Ingenuity pathway analysis was used to search whether resulting gene lists were enriched for targets of known transcription factors, activation receptors or cytokines for repressed (C) genes or induced (D) genes. The results of the most significant enrichments obtained are shown as bar graphs, with regulators regrouped by functional network according to IPA classification (❶, IRF/IFN-I network; ❷, NF- κ B/TNF/IL-1 β network; ❸, CD40/CD40LG network; ❹, IFN- γ network). Red bars indicate p-values for TLR-induced maturation (red circle of the Venn diagrams) and blue bars for homeostatic maturation (specifically for the genes commonly regulated upon both conditions of homeostatic maturation, area circled by a blue line on the Venn Diagrams). The corresponding gene lists are given in Supplementary Table S1. (E) Changes in the expression of genes involved in activation versus inhibition of immune responses upon homeostatic or PRR-induced DC maturation. The heatmap shows the relative expression of individual genes in immature versus mature DC, normalized to the mean expression levels in immature DC. Genes that showed a statistically significant and similar induction in their expression in homeostatic maturation and in TLR-induced maturation are shown in bold, black font. Genes that showed a statistically significant induction in TLR-induced maturation reaching levels higher than those observed in homeostatic maturation are highlighted in bold, red font. Conversely, genes that showed a statistically significant induction upon homeostatic maturation reaching levels higher than those observed in TLR-induced maturation are highlighted in bold, blue font.

Figure 4. Key components of homeostatic versus PRR-induced DC maturation.

The scheme illustrates commonalities and differences in the signaling pathways and downstream regulation of gene expression triggered in DC during homeostatic (tolerogenic) versus TLR-induced (immunogenic) maturation. The stronger and broader activation of signaling by the IRF/IFN-I and NFκB/TNF/IL-1β networks in TLR-induced maturation might not only result from activating receptors on DC but also from differences in the kinetics of the activation of negative regulators including A20 and p105, with a very early induction during homeostatic DC maturation contrasting with a delayed, although even stronger induction occurring during TLR-induced activation, as a late negative feedback loop in response to autocrine/paracrine inflammatory cytokine signaling.

number of “activation” genes, shown in red on the upper heatmap of Fig 3E, were expressed to higher levels in TLR-stimulated DC. However, many of these genes were induced equally, or even more strongly, in DC during homeostatic maturation (shown in bold black and in blue, respectively, on the upper heatmap of Fig 3E). Some “inhibitory” genes, shown in blue on the lower heatmap of Fig 3E, were expressed to higher levels upon homeostatic DC maturation. However, many of these genes were induced in TLR-stimulated DC to similar, or even higher levels (shown in bold black and red, respectively, on the lower heatmap of Fig 3E). For example, *Itgb8* was induced to similar levels upon homeostatic maturation in migratory dermal CD11b⁺ cDC found in CLN and upon PolyI:C challenge in CD103⁺ lung DC, although it was induced to even higher levels upon homeostatic maturation in migratory CD103⁺ DC from lung-draining lymph nodes (LULN). The strong induction of inhibitory genes during TLR-induced DC maturation are likely to reflect the development of potent negative feedback loops that limit DC activation even under strong inflammatory conditions to tightly control the intensity and duration of DC activation and thus prevent unbridled, deleterious immune responses.

In the course of this analysis, differences were observed between the two conditions of homeostatic maturation and even between steady-state immature DC subsets, for example *Il12b* was induced upon homeostatic maturation of lung CD103⁺ but not skin CD11b⁺ DC, and immature skin CD11b⁺ DC expressed the highest levels of *Il10*, compared to lung resident or splenic DC. These observations

suggested that the polarization of DC functions toward tolerance, during homeostatic maturation, versus immunogenicity upon TLR-induced maturation, is very complex. It might not simply correlate with the respective induction of a universal set of immunosuppressive versus pro-inflammatory genes as previously proposed (Miller *et al*, 2012). Rather, a complex array of molecules involved in the activation or inhibition of immune responses is induced in DC both during homeostatic and TLR-induced maturation, but differences in their nature and kinetics of induction might contribute to distinct functional outcomes. Indeed, it appears that the consequences of signaling through the IRF/IFN-I and NFκB/TNF/IL-1β networks differ between homeostatic and TLR-induced maturation, with a stronger and broader activation in the later condition, especially of IRF/IFN-I network genes (Fig 4). Further studies integrating more complex and better-controlled datasets, including kinetic studies of models of TLR-induced versus homeostatic DC maturation, will be necessary to address these issues in more depth. Finally, it is important to consider post-translational control of gene expression and subcellular localization of molecules during DC maturation. It is well documented that MHC and CD86 expression in DC are not only controlled at the transcriptional level but also through regulation of their intracellular trafficking to the plasma membrane by molecules such as MARCH1 (De Gassart *et al*, 2008; Tze *et al*, 2011), and it is likely that other important aspects of DC cross-talk with T cells are controlled at the post-transcriptional level. For example, a recent report has shown that TLR-driven early glycolytic reprogramming in

DC is necessary to promote *de novo* synthesis of fatty acids for the expansion of the endoplasmic reticulum and Golgi, required for the production and secretion of proteins that are integral to DC activation. Blockade of glycolytic reprogramming in TLR-stimulated DC selectively inhibited the translation but not the transcription of genes encoding cytokines and co-stimulatory molecules (Everts et al, 2014). Hence, it would be interesting to examine whether the “activation” genes induced at the mRNA level upon homeostatic DC maturation are actually translated or not, and if so whether this is correlated with differences in metabolic reprogramming between TLR-induced and steady-state DC maturation.

Perspectives

Our review highlights the complex processes leading to the functional maturation of DC that integrate the intrinsic properties of different DC subsets, dictated by their specific gene expression programs, and contextual signals received from the tissue micro-environment. It is clear that different DC subsets are equipped to respond to specific signals by virtue of their distinctive gene expression programs. However, there are also some common “core” elements to the maturation pathways of different DC subsets that are triggered irrespective of the tissue or inflammatory context.

In our analyses reported here, we have uncovered a reciprocal regulation of new candidate genes (*Idh1*, *Cat* and *Jdp2*) between PRR-induced versus homeostatic DC maturation. It will be interesting to examine the potential roles for these genes in the functional switch between tolerance and immunogenicity during DC maturation. For example, *Idh1* encodes for the metabolic enzyme isocitrate dehydrogenase 1 and gain-of-function mutants are frequently found in glioma, acute myeloid leukemia (AML), melanoma, thyroid cancer and chondrosarcoma patients where they are proposed to overproduce 2-hydroxyglutarate (2HG) leading to inhibition of epigenetic regulators and consecutive aberrant histone- and DNA-hypermethylation. While nothing is known about the function of *Idh1* in DC, it is tempting to speculate that its downregulation upon PRR stimulation might play an important role in the regulation of DC metabolism and the epigenetic landscape regulating a switch in cellular functions from tolerance to immunogenicity.

To further delineate the specific genes that differentiate steady-state “tolerogenic” DC maturation from PRR-induced “immunogenic” DC maturation, irrespective of DC subset, tissue localization or animal species, it will be important to generate broader gene expression datasets comparing tolerogenic versus immunogenic DC subsets isolated from the same tissue in steady-state and after PRR-challenge, respectively. The lack of these direct comparisons is a major limitation of the existing data and analysis presented here.

The implication of NF- κ B and IRF-signaling pathways in both steady-state and inflammatory DC maturation is intriguing. It is of no surprise that these pathways are important regulators of genes induced during TLR-induced DC maturation, and this is supported by a wealth of the literature. However, the role of these pathways in steady-state DC maturation is less obvious. Recent studies suggest that negative regulators of NF- κ B activation, A20, TNIP and NFKB1, have an important role in restraining DC activation in both

steady-state and during inflammatory conditions, to prevent the inappropriate or uncontrolled activation of DC and the subsequent unfolding of pathological immune responses. Indeed, these pathways also appear to be important in the development of autoimmune and autoinflammatory diseases, as genome-wide association studies in humans have strongly linked polymorphisms in the A20 and TNIP1 loci to the development of SLE and psoriasis, respectively (Ma & Malynn, 2012). However, expression of these genes does not distinguish steady-state and TLR-induced DC maturation.

While NF- κ B- and IRF-regulated genes feature strongly in both steady-state and PRR-induced DC maturation, the strong induction of IRF-dependent genes appears to be a distinguishing feature of PRR-induced “immunogenic” DC maturation, at least in mice (Fig 4). This is in keeping with the potent adjuvant properties of IFN-I inducing TLR agonists for priming T-cell responses. However, the specific roles of these pathways in steady-state versus PRR-induced DC maturation have yet to be ascertained.

Supplementary information for this article is available online: <http://emboj.embopress.org>

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Conflict of interest

The authors declare that they have no conflict of interest.

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