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FOCUSSED RESEARCH REVIEW

Toll-like receptor expression and function in human dendritic cell subsets: implications for dendritic cell-based anti-cancer immunotherapy

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Abstract Dendritic cells (DCs) are central players of the immune response. To date, DC-based immunotherapy is explored worldwide in clinical vaccination trials with cancer patients, predominantly with ex vivo-cultured monocyte-derived DCs (moDCs). However, the extensive culture period and compounds required to differentiate them into DCs may negatively affect their immunological potential. Therefore, it is attractive to consider alternative DC sources, such as blood DCs. Two major types of naturally occurring DCs circulate in peripheral blood, myeloid DCs (mDCs) and plasmacytoid (pDCs). These DC subsets express different surface molecules and are suggested to have distinct functions. Besides scavenging pathogens and presenting antigens, DCs secrete cytokines, all of which is vital for both the acquired and the innate immune system. These immunological functions relate to Toll-like receptors (TLRs) expressed by DCs. TLRs recognize pathogen-derived products and subsequently provoke DC maturation, antigen presentation and cytokine secretion. However, not every TLR is expressed on each DC subset nor causes the same effects when activated. Considering the large amount of clinical trials using DC-based immunotherapy for cancer patients and the decisive role of TLRs in DC maturation, this review summarizes TLR expression in different DC subsets in relation to their function. Emphasis will be given to the therapeutic potential of TLR-matured DC subsets for DC-based immunotherapy.

Keywords Dendritic cell vaccination · Myeloid dendritic cells · Plasmacytoid dendritic cells · Toll-like receptors

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Introduction

To date, dendritic cell (DC)-based immunotherapy is explored worldwide in clinical vaccination trials, predominantly in cancer patients (reviewed in [1]). The therapy consists of autologous ex vivo-cultured, antigen-loaded monocyte-derived DCs (moDCs) or $CD34^+$ progenitor-derived DCs that are administered to patients with the intention of inducing antigen-specific T and B cell responses. The generation of anti-tumor immune responses involves the induction of Th1-type $CD4^+$ T cells and $CD8^+$ cytotoxic T lymphocytes. Although DC-based immunotherapy potently induces immunological responses, thus far only a limited number of clinical responses have been observed [2]. It is unclear whether DCs differentiated ex vivo from precursor cells are the most optimal source of DCs for the induction of

potent immune responses. The extensive culture period (8–9 days) and compounds required to differentiate them into DCs may negatively affect DC function, especially migration [3, 4]. Therefore, it is attractive to consider alternative DC sources, such as blood DCs. Two major types of DCs circulate in the blood, which can be distinguished by the presence of different surface markers: myeloid dendritic cells (mDCs) and plasmacytoid dendritic cells (pDCs).

In melanoma patients, we demonstrated that moDC maturation is a prerequisite for the generation of tumor-specific T cell responses [5]. While blood DCs may not require extensive culture, for these cells activation is essential prior to reinfusion, as immature DCs may cause tolerance rather than immunity. To date, most clinical studies use TNFα, IL-1β, IL-6 and prostaglandin E_2 for moDCs maturation. However, it has been suggested that the current protocols used to generate mature moDCs may not result in optimal Th1 responses. Murine studies have shown that activation of DCs by solely pro-inflammatory cytokines yields DCs that support $CD4^+$ T cell clonal expansion, but fail to efficiently direct helper T cell differentiation. In contrast, exposure of DCs to pathogen associated molecular patterns (PAMPs) induces DCs that produce high levels of the Th1 skewing cytokine IL-12p70 and promote efficient T cell help. PAMPs are recognized by pattern recognition receptors (PRRs). MoDCs, mDCs and pDCs express different repertoires of PRRs and respond dissimilarly to extracellular stimuli, suggesting that the DC subsets have specialized and complementary functions [6]. In this review we summarize the expression of the most well-known PRRs, Toll-like receptors (TLRs), on different DC subtypes in relation to their function.

Dendritic cell subsets

Dendritic cells are the most potent professional antigen-presenting cells (APC) of the immune system. Upon infection or inflammation, immature DCs are activated and differentiate into mature DCs that instruct and activate B and T lymphocytes, the mediators of adaptive immunity [7]. DCs take up and process pathogens and present pathogen-derived peptides to T cells via major histocompatibility complex (MHC) molecules. The recognition of the peptide–MHC complex (signal 1) and stimulation via costimulatory molecules (signal 2) and cytokines (signal 3) lead to the activation of T cells. The immunological outcome strongly depends on the activation state of DCs. Resting DCs or DCs receiving inhibitory signals, such as IL-10 or corticosteroids, induce immune tolerance via T cell deletion or induction of regulatory T cells, whereas mature DCs induce immunity.

The classical view of DCs is that of immature DCs, waiting for pathogens to be recognized, residing mainly in parts of the body that are in close contact with the outside world, such as skin and mucosal tissue. These immature DCs are able to quickly sense and take up pathogens that could harm the host. After recognition of a pathogen, the DCs mature and migrate to lymphoid tissues to present the pathogenic peptides to T cells [7]. DC maturation comprises a tightly controlled series of events, including downmodulation of endocytic and phagocytic receptors, upregulation of costimulatory molecules, such as CD40, CD58, CD80 and CD86, changes in morphology and reorganization of the DC lysosomal and MHC class II compartment. DC maturation is highly complex and should be regarded as a flexible process, the outcome of which depends on the type of signals a DC receives in the periphery.

The DC population is a heterogeneous population. The DC subtypes differ in function, localization and phenotype. In human peripheral blood, two main populations of DCs can be distinguished: CD11c expressing myeloid DCs (mDCs) and CD11c negative plasmacytoid DCs (pDCs) [8]. These DCs express distinct PRRs and respond to different pathogenic stimuli, suggesting that each subset has a specialized function in directing immune responses. However, both mDCs and pDCs have the capacity to initiate suitable T cell responses, depending on the pathogens they encounter.

Plasmacytoid dendritic cells

Human pDCs are a rare subpopulation of cells; they constitute only 0.1% of all blood mononuclear cells. PDCs are devoid of lineage markers and myeloid antigens and do not express CD11c. PDCs express BDCA2 and BDCA4 [9]. In the steady state, they are round, non-dendritic and relatively long-lived cells. After receiving inflammatory stimuli, pDCs develop dendritic cell morphology and function. Most notably, pDCs produce large amounts of type I interferons in response to viruses and are therefore crucial to antiviral immunity [10].

Initially, pDCs were thought to be of lymphoid origin [11]. However, several human and mouse studies pointed out that the cytokine Flt3L is of importance for pDC development and that pDCs can develop out of myeloid precursors under the influence of Flt3L [10, 12, 13]. PDCs reside in blood as well as in several lymphoid organs, and some recent studies suggest functional differentiation between different tissue-residing pDCs [14]. Whether these are actual pDC subsets or whether environmental factors influence pDC function is not clear yet.

Myeloid dendritic cells

Myeloid DCs found in peripheral blood are defined by the expression of myeloid markers, such as CD13 and CD33. They lack lineage-specific markers (CD3, CD14, CD19 and

CD56), but express MHC class II and CD11c. The mDC population can be further subdivided based on differential surface expression of CD1c (BDCA1), CD16 and BDCA3. In addition, another small subset of CD16-expressing DC, termed M-DC8 DCs or SlanDCs, has been described [15]. CD16-mDCs comprise the largest population of mDCs (65–75% of total mDC population), followed by CD1c-mDCs (10–20%) and BDCA3-mDCs (3–5%) [16, 17]. Although CD16-mDCs have the highest frequency in human peripheral blood, CD1c-mDCs have been studied most extensively. The mDC subsets differ in their expression of cell surface markers and potency to stimulate T cells [16–18]. For instance, the recently identified C-type lectin receptor CLEC9a is expressed only by BDCA3-mDCs [19]. According to MacDonald et al. [16] CD1c-mDCs are the most potent T cell stimulators of the three mDC subpopulations.

Monocyte-derived dendritic cells

Since the frequency of circulating mDCs in human blood is very low, many studies exploit in vitro-generated "monocyte-derived dendritic cells" (moDCs) [20]. Monocytes are pre-DCs that originate from myeloid progenitor cells. In vivo, monocytes are capable of transforming into DCs after sensing inflammatory signals and are important for the replenishment of dendritic cells in the host. In vitro, a cocktail of granulocyte/macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4) differentiates monocytes into moDCs [20]. MoDCs are the major DC type used in clinical DC vaccination studies [2, 21]. Whether these moDCs reflect immature "steady state" in vivo mDCs remains to be seen, as most of these moDCs show a dramatically higher expression of MHC molecules and costimulatory molecules compared to freshly isolated immature mDCs [22]. In addition, moDCs and mDCs respond differently to physiologic stimuli and differ in their ability to stimulate T cells [23, 24]. Therefore, although in vitro-generated moDCs share many phenotypic and functional characteristics with circulating mDCs, it is yet unclear to what extend they resemble blood DCs.

In addition to monocytes, $CD34^+$ precursors in blood are also used to generate DCs for vaccination of cancer patients. They consist of two populations: one with Langerhans cell-like properties and one called interstitial/dermal DCs with properties resembling moDCs. Immunological and clinical responses have been observed in cancer patients vaccinated with $CD34^+$-derived DCs [25]. However, to date most clinical studies use monocytes to generate DCs.

Pathogen recognition by Toll-like receptors

In evolution, the immune system has acquired various receptor families that recognize several crucial molecular components of pathogens. This set of PAMPs recognized by the immune system is limited and constituted mostly of general molecular patterns that are absent on cells of the host and are essential for survival of the microbe. On DC membranes, two main PRR families are present: C-type lectins and Toll-like receptors (TLRs), of which the TLR family is best characterized and recognizes the most diverse group of PAMPs. Nowadays, 15 mammalian Toll-like receptors are found (TLR1-15), of which 10 are in humans [26, 27]. The binding partners of the recently discovered TLR10, TLR12, TLR13 and TLR15 are unknown. TLR11 is only expressed in mice and recent studies suggest that TLR11 associates with molecules originating from uropathogenic bacteria and *Toxoplasma Gondii* [28, 29]. The better-described TLR1-9 can be divided into two main groups: extracellular TLRs that are found on the cell surface (TLR1, TLR2, TLR4, TLR5 and TLR6) and the intracellular TLRs that are located in the endosomal compartments (TLR3, TLR7, TLR8 and TLR9). In general, intracellular TLRs recognize nucleotide-containing structures, for example RNA molecules (TLR3, TLR7 and TLR8) and unmethylated CpG DNA (TLR9), originating from viruses and bacteria. Extracellular TLRs recognize exterior components of bacteria and fungi, e.g., cell wall components. Examples of ligands include lipopolysaccharide (LPS) for TLR4, flagellin for TLR5 and peptidoglycan for TLR2. TLR2 forms heterodimers with either TLR1 or TLR6. The TLR1/2 heterodimer recognizes bacterial triacyl lipopeptides and TLR2/6 recognizes bacterial diacyl lipopeptides. Besides PAMPs derived from pathogens, TLRs have been proposed to recognize endogenous ligands such as heat-shock proteins or necrotic cells [30, 31]. The signaling pathways associated with ligation of the different TLRs are not identical and therefore distinct biological responses are initiated. Ligand binding of TLRs recruits one or more adaptor molecules. The difference in signaling outcome (e.g., variation in cytokine production) can be explained in part by the use of different adaptor molecules by the Toll-like receptors. All TLRs, except TLR3, rely on MyD88 for signal transduction. TLR3 uses TRIF as adaptor molecule, whereas TLR4 is able to recruit both MyD88 and TRIF [32].

TLR expression and functionality in DC subpopulations

DCs show different expression levels of TLRs and respond dissimilarly to TLR ligands. The expression of TLRs varies with species, DC subtype and maturation stage. In this review, we focus on TLR expression and function in human DC subsets. We have divided the human DC population into three groups: in vitro-generated moDCs and circulating mDCs and pDCs. Expression of TLRs and the effects of TLR activation in the three DC subtypes are summarized in Table 1.

Table 1 TLR expression and functionality on DC subtypes

DC subtype	TLR	Expression	Effects of activation	References
moDC	1	+	See TLR2	[35, 39–41, 44, 71]
	2	++	Increased IL-6, IL-8, IL-10, IL-12, TNFα	[35, 38–41, 44, 71]
			Low IFNβ response and no IFNα response	
	3	+	Specific IFNβ mRNA upregulation (not IFNα)	[35, 38–41, 44, 71]
	4	++	Upregulation of CD80, CD86, CD83, CCR7	[35, 38–41, 44, 71]
			Secretion of IFNβ, IFNγ, IL-1β, IL-12p70, IL-13, IP-10	
			Decreased endocytic capacity	
	5	+	Upregulation of CD80, CD86, CD83, CCR7	[35, 39–41, 44, 71]
			Secretion of IFNγ, IL-1β, TNF, IL-8, IL-12p40 (not IL-12p70), IL-13	
			Decreased endocytic capacity	
	6	±	See TLR2	[35, 38, 39, 41, 71]
	7	±	–	[35, 39, 41, 71]
	8	+	Increased TNFα, IL-8, IL-12p40, MCP-1, CCL2, CCL3, CCL4, CCL5	[35, 39, 41, 71]
	9	–		[35, 39, 41, 71]
	10	–		[39, 41]
mDC	1	+	See TLR2	[35–37, 39, 51]
	2	++	Upregulation of CCR7, IL-6, IL-10, IL-12p70,TNFα, no INFα	[35–39, 51]
	3	++	IFNα (intermediate), IL-12p70 (high)	[35–39, 51]
			No TNFα or IL-6	
	4	+	Upregulation of CD80, CD86, CD83, CD40, CCR7	[34–39, 51]
			Secretion or upregulation of CCR7, IL-6, IL-8, IL-10, IL-12p70	
			No IFNα response	
	5	+	Upregulation of CD80, CD86, CD83, CCR7	[35–37, 39]
			Secretion of TNF and IL-8	
			Upregulation of CCR7	
	6	+	See TLR2	[35–37, 39, 51]
	7	+	Upregulation of CD40, CD80 and CD86	[34–37, 39, 51]
			Secretion of IL-12p70	
			No IFNα response	
	8	±	See TLR7	[35–37, 39, 51]
	9	–		[34–37, 39, 51]
	10	+	No ligand known	[36, 39, 51]
pDC	1	±		[35–37, 50, 51]
	2	–		[35–38, 50, 51]
	3	–		[35–38, 50, 51]
	4	–		[34–38, 50, 51]
	5	–		[35–37, 50]
	6	–		[35–37, 50, 51]
	7	++	Upregulation of CD40, CD80, CD86, CCR7	[34–37, 50, 51]
			Very high IFNα response	
			No IL-12p70 response	
	8	–		[35–37, 50, 51]
	9	+++	Upregulation of CD40, CD80, CD86, CD83, HLA-DR, CCR7	[34–37, 50, 51]
			Upregulation of IFNα (very high), IFNβ (lower), IL-6, TNFα (low), IL-8, IP-10	
			No IL-10 secretion	
	10	+	No ligand known	[36, 50–52]

TLR expression and activation: moDCs and mDCs compared

DCs derived in vitro from monocytes (moDCs) have provided immunologists important insights into the biology of DCs. Because of the large amounts of clinical trials using moDCs as an alternative for naturally occurring blood mDCs, we here compare the TLR expression profile in human moDCs and their blood-isolated mDC counterparts. Of the three blood mDC subpopulations, CD1c-mDCs have been studied most extensively. Only a study by Piccioli et al. compared the expression and function of TLRs in the three circulating mDC subtypes, CD16-mDCs, CD1c-mDCs and BDCA3-mDCs. They detected the expression of TLR1-10 at the RNA level in all three mDC subsets, with the exception of TLR3, which was not expressed by CD16-mDCs. In addition, both CD16-mDCs and CD1c-mDCs strongly responded to all TLR agonists, except the TLR9 agonist CpG. Interestingly, CD16-mDCs were responsive to the TLR3 ligand poly(I:C), while TLR3-mRNA was not detected in these cells. The authors suggest that poly(I:C) may activate CD16-mDCs through TLR3-independent mechanisms [17], such as cytosolic RNA sensors like RIG-like helicases [33]. Another possibility may be that minor contaminations of, for instance, endotoxin in poly(I:C) have activated other TLRs. Due to the low frequency of BDCA3-mDC in peripheral blood, no functional studies were performed on this mDC subset.

Since other studies on TLR expression and function in mDC subtypes are lacking, we focused on CD1c-mDCs when comparing moDCs with blood mDCs. Human moDCs and CD1c-mDCs show very similar TLR expression profiles (Table 1). Both DC subsets express the extracellular TLRs (TLR1, TLR2, TLR4, TLR5 and TLR6) and the endosomal TLRs (TLR3 and TLR8) (Table 1). In addition, both moDCs and CD1c-mDCs respond to specific ligands of these TLRs, leading to a mature phenotype and production of proinflammatory cytokines [34–42]. However, some significant differences between moDCs and CD1c-mDCs in TLR expression and ligand reactivity were found.

Most notably, moDCs showed negligible TLR10 expression, whereas blood mDCs did express TLR10. An extensive qPCR expression profiling by Means et al. [39] revealed that mDCs have 20-fold higher expression levels of TLR10 mRNA compared to moDCs. MRNA expression of TLR10 in mDCs was even comparable to that of TLR5. Unfortunately, the ligands and functionality of TLR10 are still unknown, so this difference between moDCs and mDCs could not be explained. However, since TLR10 is also expressed by regulatory T cells [43], TLR10 activation on DCs may induce tolerogenic DCs. The fact that moDCs have a more mature phenotype [22] may thus explain the absence of TLR10 in moDCs.

Iwasaki and Medzhitov [27] concluded that human mDCs totally lacked TLR4 expression. The authors mainly base their conclusion on a study performed by Visintin et al. [44]. However, their results were contradicted by Ito et al. [34] and Matsumoto et al. [38] in 2002 and 2003, respectively, who showed that freshly isolated human blood mDCs expressed TLR4. This was confirmed by experiments in the same study of Ito et al. [34] which showed that stimulation of mDCs with LPS induced the production of IL12. According to Means et al. [39] moDCs secrete type I IFNs on LPS administration, whereas this ligand did not provoke type I IFN secretion in mDCs. While this was confirmed by Matsumoto et al. [38], the function of type I IFN secretion after LPS challenge remains unclear, as type I IFNs have anti-viral rather than anti-bacterial properties. The disparity in IFN secretion may also be caused by the difference in maturation stage between moDCs and CD1c-mDCs. Muzio et al. [40] showed that as differentiation of monocytes progresses toward moDCs, the expression of TLR 3, 4 and 5 increases. Eventually the expression of TLR4 in moDCs is higher than that in CD1c-mDCs. This higher number of TLR4 molecules per cell may explain the difference in type I IFN secretion between moDCs and CD1c-mDCs. A high number of TLR4 molecules might induce a broader immune response: a low number of TLR4 molecules is able to only activate the MyD88 pathway, resulting in an immune response without an IFN response, whereas a higher amount of TLR4 molecules might be able to activate both the MyD88 and TRIF pathway, leading to a broader immune response, including production of type IFNs [45]. This is in line with the finding that the TRIF adapter protein in non-plasmacytoid DCs is mainly responsible for the activation of transcription factors that induce type I interferons [45].

TLR1, 2 and 6 are expressed by both moDCs and mDCs (Table 1) [35–39, 41]. Through the formation of heterodimers with TLR1 or TLR6, TLR2 gains the capacity to bind a wide variety of bacterial and yeast-derived ligands. Consequently, this plays a central function in pathogen recognition by DCs. TLR1/2/6 activation leads to DC maturation and secretion of several cytokines important in immune system activation, especially IL-6, IL-8, IL-10, IL-12 and TNF-α. Differences in expression between moDCs and mDCs in TLR1, TLR2 and TLR6 expression and reactivity are negligible.

TLR5 expression was evident in both moDCs and CD1c-mDCs as indicated in Table 1. Means et al. [39] especially investigated the role of TLR5 and its main ligand, bacterial flagellin, in moDCs and to a lesser extent in mDCs. They found increased surface expression of maturation markers for both, without the induction of a type I IFN response, which is expected considering the bacterial origin of flagellin.

Studies on TLR7 expression on moDCs or mDCs show conflicting results. This inconsistency was also noted in the review by Iwasaki and Medzhitov [27]. It was especially fueled by the contradictory results of Jarossay et al. and Krug et al. [35]. Using three different primer sets for RT-PCR on TLR7, Ito et al. [34] concluded that human mDCs do express TLR7. For TLR7 expression in moDCs, there is still disagreement between studies; however, the most recent findings suggest that moDCs indeed express TLR7 [39]. For moDCs, a thorough study of TLR7 mRNA/protein expression, as was performed for mDCs by Ito et al., is lacking. Confirmation of TLR7 protein expression is difficult due to low expression and a lack of good antibodies. Therefore, TLR7 expression in moDCs is still unclear, as indicated by the "+/−" in Table 1. TLR8, the "near cousin" of TLR7, was found to be consistently expressed in lower amounts in both moDCs and mDCs. As TLR7 and TLR8 share the same ligands, it was impossible to attribute TLR8 as the binding receptor responsible for the secretion of cytokines or DC maturation after, for example, R848 challenge [34].

Human moDCs and mDCs do not express TLR9 and do not respond to TLR9 ligands [34, 37]. TLR9 is only expressed by pDCs in the human setting and is responsible for a very high type I IFN response [46].

In summary, data suggest that both moDCs and mDCs express TLR1-8 and not TLR9, and only mDCs express TLR10. The mRNA expression of these receptors was confirmed by DC reactivity studies using TLR ligands, as summarized in Table 1.

TLR expression by human pDCs

Evidence suggests that pDCs are specialized in the recognition of viral antigens, as they largely lack expression of extracellular TLRs and only express TLRs that recognize and respond to viral antigens. PDCs abundantly express TLR7 and TLR9 in their endosomal compartments. In addition, triggering of TLR7 and TLR9 on pDCs leads to high type I IFN secretion and a typical mature DC phenotype, i.e., upregulation of expression of major histocompatibility molecules and the costimulatory molecules, CD80 and CD86.

Interestingly, it has been suggested that in human pDCs, TLR9 displays a unique feature that is not shared by the other described TLRs. Depending on the stimulus, activation of TLR9 on human pDCs can have a different outcome. The dual function of TLR9 is attributed to the distinct intracellular locations where TLR9 can be triggered. Upon encountering nucleic acids, TLR9 will traffic from the endoplasmatic reticulum through the Golgi to the early endosomes [47, 48]. There, TLR9 triggering may induce the recruitment and phosphorylation of IRF7, which in turn induces IFNα secretion, thus activating an innate immune response. In contrast, in the late endosomes, TLR9 triggering preferentially activates NF-κB, which results in phenotypical maturation of DCs and secretion of IL-6 and TNFα, hence activating an adaptive immune response [49].

Especially IFNα is produced after TLR7 or TLR9 triggering [34–37, 50]. IL-12, a cytokine commonly produced by mDCs and moDCs after TLR stimulation, is not secreted by pDCs as found in the majority of the studies. Only Jarrossay et al. [35] mentioned a weak IL-12 response by pDCs, which was possibly due to a polluting mDC subset in the samples tested.

Some reports showed a minimal expression of TLR1 on pDCs [36, 37, 51]. However, the possible function of TLR1 on pDCs remains ambiguous, especially since its heterodimeric partner TLR2 is not expressed by pDCs and pDCs do not respond to TLR1/TLR2 ligands [35–38].

Interestingly, pDCs do not express TLR3 and TLR8. Since both TLR3 and TLR8 bind viral ligands and pDCs are specialized in the recognition of viral pathogens, it would not be unlikely for pDCs to express these receptors. Possibly, TLR8 lost its function during evolution as TLR7 binds the same ligands and shares the same signaling pathway, leading to similar effects. However, why pDCs lack TLR3 expression is still unknown.

Surprisingly, pDCs also express TLR10, both at the mRNA and protein level [37, 51, 52], but the function and binding partner of TLR10 are yet to be identified. Overall, human pDCs only express TLR7, 9 and 10 and react on TLR7 and TLR9 activation by secreting type I IFNs, especially IFNα.

Practical limitations

When comparing data obtained by different groups, we noticed inconsistencies between studies, which may be caused by technical limitations. For instance, several different techniques to isolate fresh mDCs and pDCs from blood were used by different groups, including cell sorting by FACS and magnetic beads. Even small impurities in the isolated cell population can interfere with the results, as was demonstrated by the IL-12 secretion by a small contaminating mDC subpopulation, which was first contributed to the pDC population [34, 37, 50]. In addition, most expression profiling of TLRs on DCs is performed by RT-PCR, because of a lack of antibodies directed against TLR molecules. But when antibodies were used, several of them proved to be unreliable as demonstrated by Matsumoto et al. [38] who showed that a TLR3-specific antibody also bound to a centrosomal protein that shared an epitope with TLR3 and incorrectly suggested TLR3 protein expression in pDCs, although TLR3 mRNA expression was absent.

Even small impurities in the isolated cells (~1–5%) can cause problems. Furthermore, interpretation of mRNA expression level is highly subjective and does not always correlate with the expression level of the encoded protein. In addition, mRNA levels do not reveal the localization of the protein. Perhaps TLR proteins residing in intracellular compartments and showing high mRNA expression are only effective when transported to another compartment or to the extracellular membrane after stimulation. For instance, it has been demonstrated that the ectodomain of TLR9 is cleaved in the endolysosome before TLR9 is transported to endosomal compartments and that only the cleaved form of TLR9 is functional after ligand binding [53]. In addition, post-translational modification, such as ubiquitination, may affect TLR expression and functionality [54]. Therefore, next to TLR expression profiling, functional studies using TLR ligands are important in establishing whether the TLR expressed in a given DC subtype is functional. However, cytokine responses and DC maturation may also be caused by non-TLR receptors, such as C-type lectins or undiscovered TLRs that recognize the same pathogenic ligands. In addition, it is very important to use highly purified TLR ligands, since minor contaminations of, for instance, endotoxin may obscure the results.

Implications for immunotherapy of cancer

The above summarized data suggest that mDCs mainly recognize and respond to bacterial and fungal antigens, whereas pDCs are specialized for viral recognition. However, although pDC and mDC subsets have different functional specialization, as they express a different repertoire of TLRs and respond dissimilarly to microbial stimuli, both pDCs and mDCs may be of importance for the induction of anti-tumor responses in DC vaccination therapy. MDCs as well as pDCs can induce Th1 responses after TLR stimulation [34] and anti-tumor responses induced by pDCs have been reported in animal models [55, 56].

Reactivation of tumor-infiltrating DCs

Several studies suggest that human pDCs have the ability to infiltrate solid tumors since they are found in a wide variety of human cancers, including breast cancer [57], head and neck cancer [58] and ovarian cancer [59]. Soluble factors secreted by the tumor create a suppressive environment, thus preventing differentiation and maturation of infiltrating DCs [60–62]. Despite their ability to infiltrate solid tumors, pDCs on the site are unable to sense DNA via TLRs and become activated. These findings have been correlated with poor prognosis [57] and are linked to the induction of regulatory T cells [63]. Although pDCs at the tumor site have a pre-pDC phenotype and maintain the immunosuppressive environment, several studies have now demonstrated that topical TLR7-agonist (Imiquimod) treatment led to enhanced pDC recruitment and type I IFN production by resident pDCs at the tumor site, which in turn generated an inflammatory environment resulting in tumor regression [64, 65]. Furthermore, activation of resident tumor pDCs can also be achieved by intratumoral injection of CpG motifs inducing TLR9 triggering. This strategy has therapeutic potential in patients with basal cell carcinoma and melanoma skin metastases [66]. Since TLR9 is believed to be expressed only on pDCs, CpG injection will not directly activate mDCs. Therefore, intratumoral injection of other TLR ligands, such as the TLR7/8 ligand R848 that activates both mDCs and pDCs, will be even more potent in reactivating DCs at the tumor site and might favor the induction of anti-tumor immune responses.

DC vaccination with pDCs and mDCs

Recent studies suggest that pDCs and mDCs cooperate and act synergistically (Fig. 1). In mice, pDCs not only directly induce tumor antigen-specific $CD8^+$ T cell immune responses, but also enhance the ability of mDCs to present tumor antigens to T cells [67]. Moreover, human mDCs and pDCs activate each other after specific stimulation of only one of the DC subsets with appropriate TLR ligands in vitro [68]. These studies suggest that also in a clinical setting, such as DC vaccination in cancer patients, vaccination with both pDCs and mDCs may generate stronger anti-tumor responses than vaccination with moDCs alone.

We recently completed a clinical trial with tumor antigen-loaded, TLR ligand-matured pDCs in stage IV melanoma patients, which appeared feasible and safe (manuscript in preparation). In the majority of patients vaccinated with pDCs, we found responses against the monitoring protein, demonstrating that even small numbers of naturally occurring DCs can induce immunological responses. When mDCs and pDCs would be used in combination, both DC subsets will need stimulation with carefully selected TLR ligands, due the fact that they express a different repertoire of TLRs (Table 1; Fig. 1). Since TLRs act in synergy [69], the combination of different TLR ligands may even be more potent (Fig. 1). It is of importance to find TLR ligands that either induce optimal maturation of both mDCs and pDCs, or at least do not interfere with maturation of either DC subtype. Future studies will address whether mDC-pDC cross talk can improve anti-tumor responses in cancer patients.

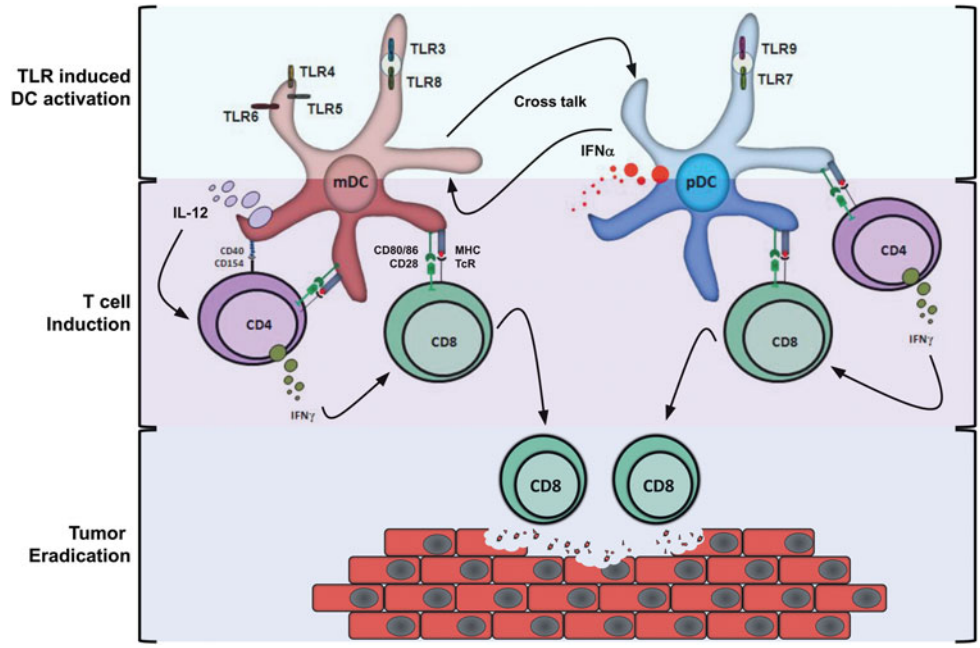

Fig. 1 TLR activation of human DC subsets can enhance anti-tumor responses in vivo. DC subsets express a wide repertoire of TLRs, which upon triggering induce the activation of the DC. Where pDCs predominantly express TLRs in endosomal compartments, mDCs have a broader TLR expression pattern, both at the endosomal and extracellular membranes. By cross talk between mDCs and pDCs, either by cell–cell contact or soluble factors such as type I IFNs, TLR-induced activation of one subset can lead to the activation of the other subset. Type I IFNs appear to yield more potent mDCs in terms of IL-12 secretion, induction of tumor-specific cytotoxic T cells and Th1 responses in vitro. On direct TLR activation, mDCs gain the ability to secrete large quantities of IL-12, which is beneficial for the polarization of a Th1 response. Both mDCs and pDCs have the capacity to evoke T helper cell responses. Moreover, pDCs can promote the ability of mDCs to cross-prime $CD8^+$ T cells. Consequently, TLR activation of mDCs and pDCs and the cross talk between those two subsets can strongly enhance anti-tumor responses in vivo

Concluding remarks

To date, most clinical studies apply moDCs for DC vaccination of cancer patients. As described above, culturing procedures required to obtain moDCs may affect their ability to induce anti-tumor immunity. We hypothesize that TLR-matured blood DCs may be a potent alternative, since these cells do not require extensive culturing and can be isolated in a closed system in accordance to GMP standards. Despite their low frequencies, human naturally occurring DCs may have superior migratory and antigen-presenting capacities compared to in vitro-generated moDCs, especially when combining different DC subsets. Alternatively, the potency of in vitro-generated moDCs could be improved by electroporation with mRNA-encoding proteins involved in DC maturation, migration and T cell activation, as was suggested by Bonehill et al. [70]. Future studies will need to prove the feasibility and potency of TLR-matured DC subsets for DC-based immunotherapy.

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