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Dendritic Cells Interactions with the Immune System – Implications for Vaccine Development

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<http://dx.doi.org/10.5772/48513>

1. Introduction

Dendritic cells (DCs) were firstly described by Ralph Steinman and Zanvil Cohn in 1973 [1]. These cells were identified in the spleen of mice and possessed characteristics that were used to name them; they presented uniform pseudopods, like dendrites, so they were called dendritic cells. The advances in the research regarding these cells since then have situated them as key cells that coordinate immune responses. They are distributed along the whole body but especially prevalent in peripheral tissues where antigen capturing might occur. Immature DCs play a central role in activating naïve T cells and directing the subsequent immune response towards a T helper 1 (Th1), Th2, Th17 or T regulatory (Treg) profile [2,3]. Thus, they are the main regulators of the subsequent reaction, producing the optimal response against a given antigen and developing immunity or tolerance.

Despite the increasing research and knowledge acquired in the last years, there are not effective vaccines available against certain pathogens or diseases such as malaria, HIV, hepatitis C, tuberculosis or cancer. These pathogens are intracellular, requiring the induction of strong cellular immunity, including cytotoxic responses (CTL), to remove the infected cells. The development of antibody responses can be stimulated by traditional adjuvants such as alum [4]. However, most of the currently licensed adjuvants lack the ability for inducing cellular or mixed immune responses.

The unique features of the DCs make them ideal target cells for vaccines [5]. They appear in their immature state in the peripheral tissues and once they capture an antigen, they are able to mature and become antigen-presenting cells (APCs) at the same time as they migrate to the lymph nodes. There, they are able to present antigens loaded to major histocompatibility complex (MHC) classes I and II molecules to T cells, glycolipids and glycopeptides to T cells and NKT cells as well as polypeptides to B cells [6]. In addition, it is now apparent that adjuvants are the activators of DCs. For these reasons, a better understanding of the

interactions of DCs with the immune system, antigens and adjuvants is imperative to design new generation vaccines.

In this chapter we will discuss the factors that can influence the interactions of DCs with immune cells for generating an immune response and its applicability in the development of vaccines targeting DCs. Different DC subsets and the alternatives available for triggering their activation will also be explained, such as passive or active targeting using different adjuvants. Finally, the latest approaches combining multiple adjuvants will be described.

2. Dendritic cell subsets

The family of DCs is constituted of several distinct DC subsets that possess common characteristics but differ into their functions related with biasing the immune response towards the appropriate arm in each situation. In general terms, DC subsets are defined based on their expression of surface markers. Traditionally, they have been described as high in Major Histocompatibility Complex (MHC) class II molecules (HLA-DR) and lacking the lineage (Lin) surface markers characteristics of other immune or non-immune cells such as CD14 (monocytes), CD19 (B cells), CD3 (T cells), CD56 (NK cells) or CD34 (stem cells). The expression of the CD11c integrin, as well as co-stimulatory molecules (CD80, CD83, CD86 or CD40) has also been used for defining the immunobiology of DC subsets both in mice and humans. In humans, the expression of CD1c antigen and Blood Dendritic Cell Antigens (BDCA) has also been useful in their definition [7].

There are two main lineages of DCs in humans, the myeloid DCs (MDCs, also named classical or conventional DCs) and plasmacytoid DCs (PDCs). MDCs are originated from a myeloid progenitor in the bone marrow, while PDCs may come from a lymphoid progenitor in lymphoid organs [8]. The DC subsets migrate to different specific locations (tissues) or circulate in the blood. Human DCs in blood and skin are the best characterized, although DCs also can be found in other locations such as lungs or gut.

2.1. Blood dendritic cells

Different DC subsets are classified by the expression of distinct surface molecules. Thus, the PDCs are characterized by the expression of CD303 (BDCA-2), CD123 (IL-3R α) and CD304 (BDCA-4) and MDCs by CD11c. In blood, MDCs are subdivided into three additional subsets, called CD1c⁺ DCs, CD141⁺ DCs and CD16⁺ DCs (Figure 1) [9].

PDCs play a key role in the antiviral immunity derived from their unique ability to produce large quantities of type I interferon (IFN) [10], secrete IP-10, Tumoral Necrosis Factor (TNF) and IL-6 [11]. Through that secretion, PDCs support the function of other immune cells such as MDCs, NK or B cells, thus, linking the innate and adaptive arms of the immune responses. PDCs express several Toll-Like Receptors (TLRs) (TLR1, 6, 7, 9, and 10) but TLR7 and TLR9, recognizing single stranded RNA and unmethylated CpG DNA respectively are the main inducers of type I IFN release.

Under steady-state conditions, PDCs are found in the bloodstream. Following inflammation, PDCs can arrive to the infection site and partake in uptake of antigen and subsequently migrate to draining lymph nodes via high endothelial venules, while most other immune cells enter through lymph vessels [12]. Human PDCs are able to present peptides onto both MHC class I and II molecules [13]. Moreover, it has been shown that mature PDCs are able to effectively develop protective immunity in mice [14].

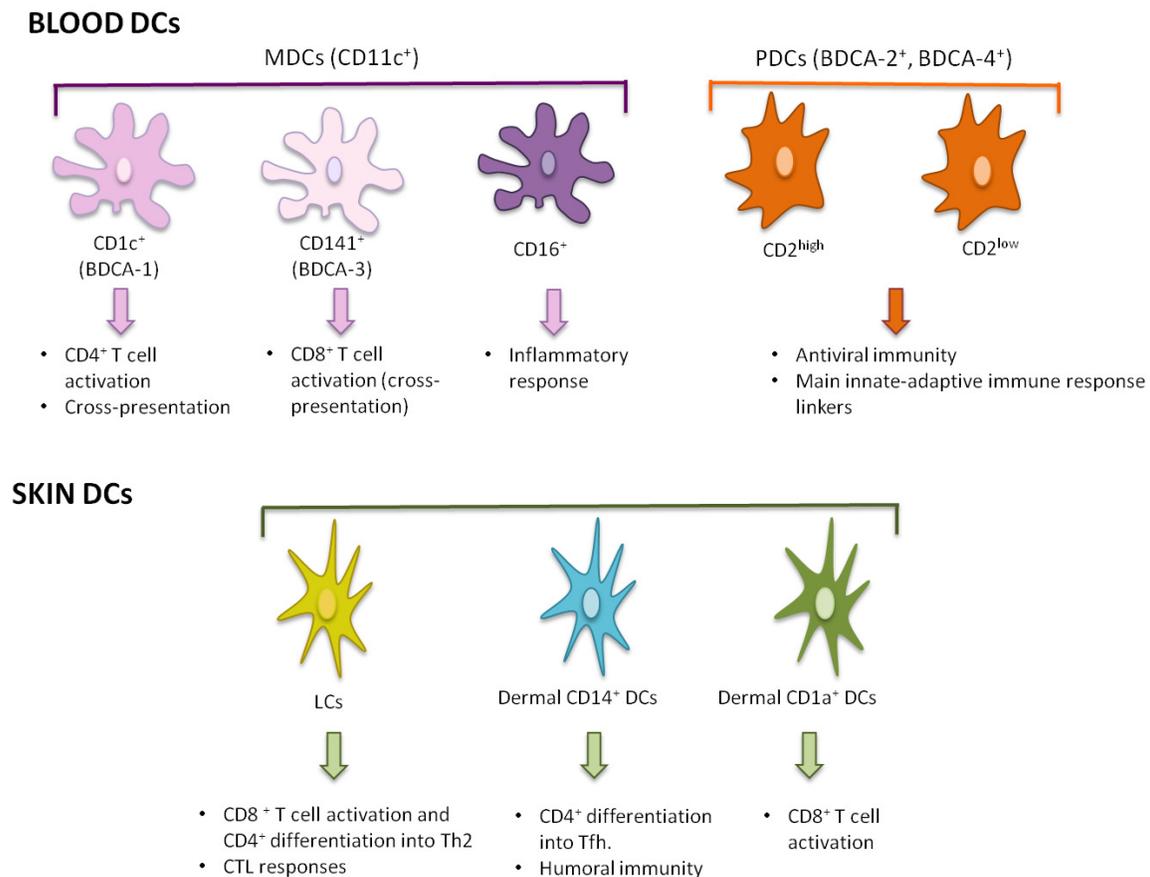


Figure 1. Schematic view of the different human DC subsets in blood and skin and their functions in immunity.

Two PDC subsets can be distinguished based on the CD2 molecule expression on their surface, which also present different functions. Matsui *et al.* [15] showed that DC2^{high} PDCs highly express lysozyme and tend to be present in tonsils and tumors. In addition, they secrete higher amounts of IL12p40, express larger levels of CD80 and trigger the proliferation of naïve T cells more competently than CD2^{low} PDCs.

In contrast to PDCs, MDCs are not so restricted to blood and can be also found in multiple peripheral tissues or in secondary lymphoid organs [16]. They are also superior over PDCs at antigen presentation [17,18]. Immature MDCs express a wide range of TLRs, namely TLR1, 2, 3, 4, 5, 6, 7, 8, and 10 [19,20]. It was recently shown that immature MDCs express mRNA for all TLRs except for TLR9 (they do not express it) and TLR3 in CD16⁺ MDCs [9].

Among the different MDC subsets, CD141⁺ (BDCA-3) MDCs are the most specialized in cross-presentation, i.e. capturing endogenous antigens and presenting them into MHC class I molecules [21,22]. CD1c⁺ (BDCA-1) MDCs are CD4⁺ T cell stimulators, release pro-inflammatory cytokines such as IL-12 and also appear to be chemotaxis inducers [23]. In addition, they also exhibit the ability of cross-presenting antigens. In fact, although CD141⁺ MDCs possess higher ability, they exist in a small percentage (2-5%), which means that other DC subsets are likely also contributing overall to this function [24]. Finally, the few reports so far indicate that the CD16⁺ MDCs show a strong pro-inflammatory function and present a lower antigen presentation ability [9,25] (Figure 1).

2.2. Skin dendritic cells

The skin contains three types of MDCs, the epidermis resident Langerhans cells (LCs) and the dermal CD1a⁺ and CD14⁺ DCs [26], which present phenotypic and functional differences (Figure 1). The TLR panel expression is also different in the skin DC subsets. While LCs express TLR1, 3, 6 and 10, CD14⁺ DCs express TLR2, 4, 5, 6, 8, and 10 [16,27,28]. LCs are efficient at cross-presenting antigens to CD8⁺ T cells and developing CTL responses. Furthermore, they can also interact with CD4⁺ T cells and induce their differentiation into T helper 2 (Th2) cytokine secreting cells (IL-4, IL-5 and IL-13). Conversely, CD14⁺ cells stimulate the differentiation of CD4⁺ T cells into T helper follicular (Tfh) cells that can induce naïve B cells to change their isotype and become IgG and IgA secreting plasma cells. This ability is not shared with LCs. Thus, both humoral and cellular immune responses are achievable with CD14⁺ DCs and LCs respectively. CD1a⁺ DCs are also able to activate CD8⁺ T cells but in a lesser extent than LCs [29].

Although the number of LCs is not very high (they represent around 2% of the total epidermal cells), their dendritic shape helps them forming a network that increases their superficial area in contact with their surrounding space [7]. In addition, it has been recently shown that upon activation, they are able to elongate their dendrites for reaching to the antigens [30].

2.3. DCs in other mammal models

Among the animal models available, the non-human primates (NHP) are the most similar to humans. However, there exist important functional and phenotypic differences between human and NHP and also among different species of NHP, which makes difficult the establishment of a general rule for comparison. Moreover, these differences are more marked when smaller animals like mice are studied. Furthermore, although phenotypically similar, DCs from different animals may present distinct functionalities. For example, rhesus macaques and human DCs express similar TLR panel. However, signaling through TLR9 produces less IFN- α and negligible IL-12 from PDCs in macaques in comparison to the human counterparts. Furthermore, surface markers used to identify DC subsets in humans are not the same in NHP. This is exemplified by the lack of BDCA-2 and BDCA-4 markers of PDCs in NHP [31]. Skin DCs in NHP have been less studied in comparison with blood DCs but phenotypic and functional differences with the human skin DCs are also evident.

Other mammal models such as pigs [32] have also been used, but the differences with human DCs are higher in comparison with NHP. This variability among species needs to be taken into account when evaluating a vaccine in each animal model and when designing translational studies.

3. Mouse dendritic cells

In mice, DC subsets are also composed by PDCs and MDCs. Splenic MDCs include two main subsets: CD8 α ⁺CD11b⁻CD205⁺ and CD8 α ⁺CD11b⁺CD205⁻, also called lymphoid and myeloid DCs respectively. Moreover, the CD8⁻ subset also expresses the 33D1 marker. Cells of the first subset are able to polarize CD4⁺ T cells to a Th1 response and can also cross-present antigens to CD8⁺ T cells. Cells of the second subset produce large amounts of IL-12 and effectively present antigens into MHC class II molecules so they are specialized in biasing the CD4⁺ T cells toward Th2 responses [33-36]. Recently, it has been reported that there are functional commonalities between mouse CD8 α ⁺ DCs and the human CD141⁺ MDCs [21,22,37]. Although the CD141⁺ MDCs do not express CD8, they share other surface molecules such as CLEC9A, NECL2 or XCR1. More importantly, they also share the high ability of cross-presenting antigens to CD8⁺ T cells [23].

Mouse MDCs can be also classified by the presence or absence of the CD4 surface marker. Thus, three different subsets can be distinguished, CD8⁻CD4⁺, CD8⁺CD4⁻ and CD8⁻CD4⁻ MDCs. The effector function of the CD8⁺ MDCs appears to be clear and is based on the production of CTL responses (due to their ability of cross-presenting antigens to CD8⁺ T cells). However, studies carried out with the CD8⁻ subsets have shown contradictory results. It appears that CD8⁻CD4⁺ MDCs are not able to release cytokines but they effectively present antigens to CD4⁺ T cells, while the CD8⁻CD4⁻ MDCs produce IFN- γ [38,39]. Bialecki *et al.* have recently reported that both subsets demonstrate equivalent ability to present OVA peptide or whole OVA to CD4⁺ and CD8⁺ T cells under steady-state conditions. Conversely, when the invariant Natural Killer T (iNKT) cell agonist α -galactosylceramide (α -GalCer) is used, CD4⁺ and CD4⁻ subsets noticeably differ in their ability to activate and/or polarize iNKT cells both *in vitro* and *in vivo*. Hence, CD4⁻ DCs were more efficient in stimulating iNKT cells, an effect related to their higher ability to secrete cytokines (Figure 2) [40].

Mouse PDCs have CD11c⁺B220⁺ phenotype. As the human PDCs, they also circulate in blood and during inflammation or infection they enter to the lymphoid organs and release large amounts of type I IFNs. In fact, it has been shown that the PDC population decreases from blood 3 days post infection, mainly because they migrate to the spleen [41]. In addition, PDCs have been reported to be involved in induction of tolerance in mice [42,43]. A recent study showed that these different functions could be conducted by two distinct PDC subsets in mice. CD9⁺Siglec-H^{low} PDCs have demonstrated to release IFN- α , activate CTLs and trigger a protective immunity when stimulated with TLR agonists. On the other hand, CD9⁻Siglec-H^{high} PDCs produced trivial amounts of IFN- α , induced Foxp3⁺CD4⁺ T cells and were not able to produce antitumor immunity (Figure 2) [44].

With regard to the skin DCs, two main populations have been identified in mice, epidermal LCs and Langerin⁺ dermal DCs [45]. It has been suggested that these dermal Langerin⁺ DCs could be the mouse counterpart of the human CD1a⁺ dermal DCs (Figure 2) [16].

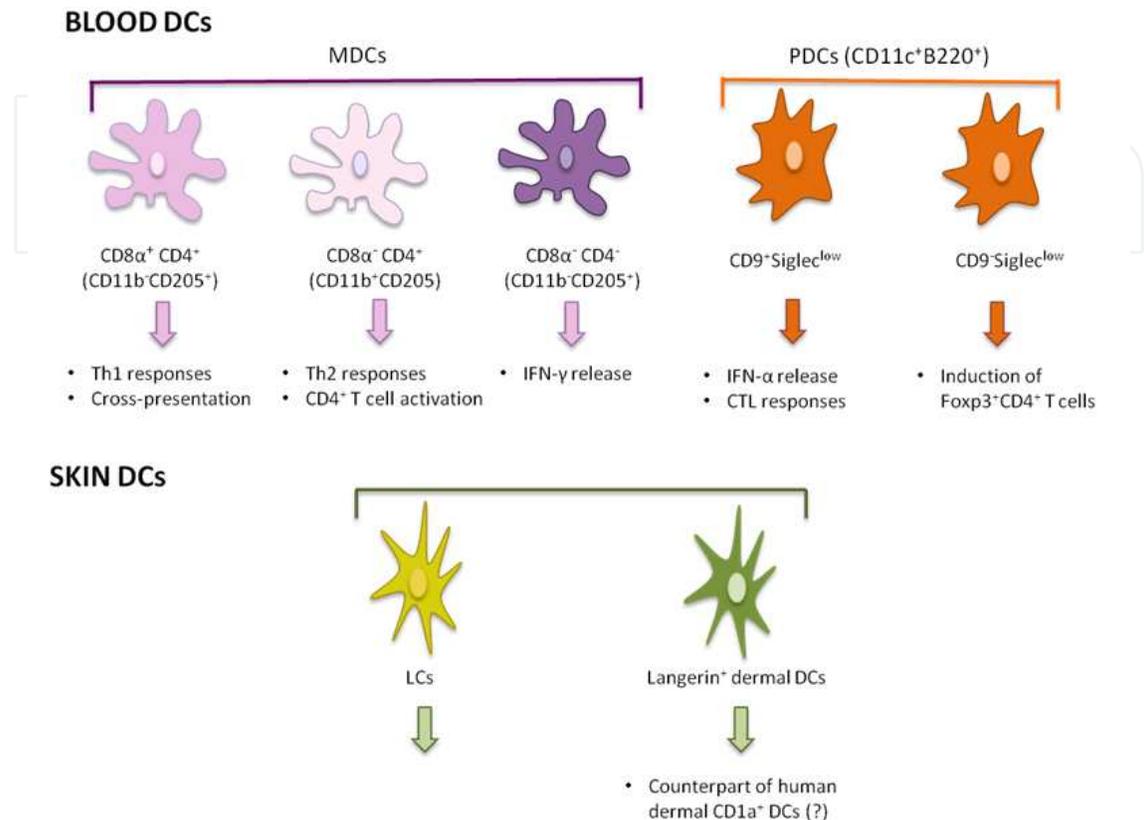


Figure 2. Schematic view of the different mouse DC subsets in blood and skin and their functions in immunity.

Therefore, the research on mouse DC subsets as a model to aid data for human vaccination should be carefully considered because of the important functional and phenotypic differences between DCs in these two species. Vaccines effective in mice could not be effective in other species or vice versa. Although some human DC subsets seem to have their mouse counterpart, translating mouse studies results to humans should be made with caution. For example, although mouse CD8⁺ DCs and human CD141⁺ share the ability for cross-presentation, there are other human DCs that have the same function, such as CD1c⁺ DCs or LCs. Other animal models such as non-human primates could represent a good alternative to mice since they present more similarities with humans. However, there exist important differences mainly in the surface molecule expression and also in maturation and some functions [46]. Thus, they can represent a better tool for preclinical studies than mice, but again, the results should be translated with caution to humans.

4. Activation of the immune response by dendritic cells

DCs are the main linkers of the innate and adaptive immune responses and depending on the context they are able to induce immunity or tolerance. They drive naïve T cells into

different effector cells or into Treg cells that suppress activated T cells. These different functions of DCs require a fine orchestration of stimuli to be adequately directed.

Either circulating or in the peripheral tissues, DCs are found in an immature or resting state specialized in capturing antigens. In this state they express low levels of MHC class I and II and costimulatory molecules in their surface. They have the ability to produce MHC class II molecules but they are directed to endosomes and lysosomes. When MHC class II molecules are loaded to a peptide (from the DC itself or a captured antigen), the formed complexes can be directed to lysosomes for degradation or be stably expressed into the cell membrane. This different ways are determined by the maturation status of the DCs. When they are immature, MHC class II molecules-peptide complexes are ubiquitinated, which finally drives the complexes to the lysosomes for being degraded. When DCs are activated by microorganisms, malignant cells or by an inflammatory stimuli, the MHC class II-peptide complexes are not successfully ubiquitinated and they are guided to be expressed into the plasma membrane [47]. Those stimuli are the signals that make DCs to undergo their maturation process.

While DCs are undergoing their maturation process, they reduce the ability for capturing antigens and they also increase the expression of MHC class I molecules, costimulatory molecules (CD80, CD86) and T cell adhesion molecules (e.g. CD48, CD58). Furthermore, maturation process also involves a migration of the DCs to the T cell areas of lymphoid organs. This process becomes DCs into antigen-presenting cells (APCs). The APCs are able to present processed antigens to effector cells, undergoing to the pathogen-specific immune response. In fact, DCs can present peptides into MHC molecules I and II to T cells as well as glycopeptides and glycolipids to T cells and NKT cells, and polypeptides to B cells [6]. These processes are highly regulated and influenced by the different stimuli that interact with DCs.

The development of antibody responses is an easily achievable feature and it is produced by most of the current licensed vaccines. Antibodies exert their function by neutralizing the pathogens, binding to the complement, allowing phagocytosis after opsonization, and/or producing antibody-dependent cellular cytotoxicity [48]. However, the induction of potent cellular immune responses, including cytolytic responses (CTL), remains a challenge in vaccinology. For accomplishing a CTL response, cytolytic cells have to recognize processed peptides presented into MHC class I molecules or into the CD1-lipid complex. Thus, the CD8⁺ T cells, CD4⁺ Th1 cells and NKT cells become activated and release the cytolytic molecules that will destroy infected cells at the same time as they develop inflammatory reactions and some humoral responses. The presentation of antigens into MHC class I molecules is a process called cross-presentation. Although the MHC class I molecules expression is increased upon maturation, the degree of expression may vary among different DC subsets [49].

In contrast to the immune response against pathogens, the development of tolerance has traditionally thought to occur because of the absence of infection or inflammation stimuli. However, there is evidence that suggest that there are also some endogenous signals that

favor its development [50,51]. Moreover, there are differences in the regulation of the self-tolerance and the environment-tolerance. The development of the central (thymic) tolerance needs the recognition of self-antigens in the thymus. Some soluble antigens can reach the thymus by the blood and be presented by resident DCs or by medullary thymic epithelial cells. In addition, PDCs have also the ability to transport self-antigens to the thymus, a process regulated by the expression of the CCR9 chemokine receptor [52]. Alteration of this balance leads to autoimmune diseases. On the other hand, the mechanisms involved in the immune tolerance to allergens are distinct, because this process requires the development of peripheral T cell tolerance, modifying the Th and Treg balance. Treg cells control the allergic responses by several mechanisms, which include the suppression of effector immune cells that act in allergic processes (DCs, Th cells, mast cells, eosinophils and basophils) and the migration of inflammatory cells to tissues. In addition they also decrease the allergen-specific IgE/IgG4 ratio, a process regulated by IL-10 and TGF- β [53]. DCs seem to have an important role on immune tolerance. In fact, both in autoimmune diseases and allergy, it has been shown that DCs are present in the site of inflammation. This process is accompanied by a decrease in the number of circulating DCs, consistent with the migration from the periphery to the inflammation site [54]. However, the role of each specific DC subset in diverse mechanisms of tolerance remains to be well established.

5. Targeting dendritic cells

In the field of vaccines against infectious diseases generation of any tolerogenic responses should be avoided. The best way for developing high immune responses tends to be the stimulation of DCs in the same way that pathogens do. DCs possess immune receptors, called pathogen recognition receptors (PRRs), specified in recognizing highly conserved pathogen associated molecular patterns (PAMPs) from the microorganisms. Currently, methods for detecting PRRs are based in the use of stained antibodies specific for each PRR [55]. There are several PRRs, including TLRs, C-type lectin receptors (CLRs), cytoplasmic retinoic acid-inducible gene-I-like receptors (RLRs), nucleotide oligomerization domain-like receptors (NLRs) and others [56]. Upon recognition of antigens, especially via PRRs DCs undergo their maturation process. However, the nature of the subsequent immune response will depend on the PRR or combination of them that are stimulated.

TLRs are the best-characterized PRRs. They are a family of transmembrane receptors. Except TLR3, they possess a major adaptor protein required for signaling, the myeloid differentiation protein 88 (MyD88). MyD88 is a cytosolic adaptor protein consisting of a N-terminal death domain (DD) and a C-terminal Toll/Interleukin-1 receptor (TIR) domain, connected by a short linker. The TIR domain is composed of three highly conserved motifs, known as box 1, 2 and 3, which play a key role in the initiation of the immune signaling upon ligand coupling to TLRs [57, 58]. Engagement of PAMPs to TLRs makes MyD88 recruit the IL-1 Receptor-associated kinases (IRAK4, IRAK1, IRAK2 and IRAK-M), which enable the induction of interferon regulatory factor (IRF)-responsive genes, activation of mitogen-associated protein kinases (MAPKs), and nuclear factor κ B (NF- κ B). Finally, these factors modulate the production of type I IFN and inflammatory cytokines such as IL-6 and

IL-12p40 [59]. The adaptor molecule for TLR3 is called Toll/IL-1R domain-containing adaptor-inducing interferon (IFN)- α -(TRIF). Moreover, TLR4 signals through both MyD88 and TRIF pathways.

Up to date, 10 different TLRs have been identified in humans (TLR1-TLR10), while in mice there have been recognized 12 (TLR1-TLR9 and TLR11-TLR13). These signaling receptors are specialized in recognizing bacteria, viruses, fungi and protozoa components not present in mammals. TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10 are expressed in the cell membrane, thus recognizing microorganism's surface molecules. In contrast, TLR3, TLR7, TLR8, and TLR9 are intracellular receptors, which recognize pathogen derived DNA and RNA [60]. In addition, an eleventh human TLR has been recently identified. TLR11 senses profilin, present in *Toxoplasma gondii*. Profilin has homologous proteins in human and also in plants. However, it has been proposed that the structure of the TLR11 in human does not allow the response to endogenous components [61].

The interactions of the TLRs with their ligands and the downstreaming effects have been elegantly reviewed by Dzopalic *et al* [58]. Briefly, TLR2 exerts its function by forming heterodimers with TLR1 and TLR6. Thus, it is the receptor with the highest spectrum of ligands, recognizing molecules such as peptidoglycan or bacterial lipoproteins. TLR3 interacts with double-stranded RNA; TLR4 recognizes LPS and its derivatives; TLR5 recognizes flagellin protein; TLR7 and TLR8 are receptors for single-stranded RNA; and TLR9 interacts with unmethylated CpG DNA motifs. There is low evidence about the ligands binding TLR10. A study carried out by Govindaraj *et al.* [62] showed that PamCysPamSK4, a di-acylated peptide, might activate the human TLR10/1 hetero and TLR10 homodimer, while Pam3CSK4 might be recognized by the TLR10/2 heterodimer.

The CLRs can be presented as both soluble and transmembrane receptors and are composed of at least one carbohydrate recognition domain (CRD). Thus, these receptors interact with carbohydrate structures like mannose, fucose or glucans [63]. There are several types of CLRs such as mannose receptor, comprising CD206 and DEC-205 (CD205) [64], DC-SIGN (CD209), Langerin (CD207), dectin-1 family and DC immunoreceptor family (DCIR) [65]. Triggering CLRs produces a signaling pathway that commonly produces NK- κ B. However, one of the potential problems of targeting CLRs is that some of them are able to recognize endogenous ligands, decreasing the specific response against pathogens.

NLRs and RLRs are cytoplasmic receptors that recognize components of the microorganisms. Upon PAMP stimulation, many NLRs form the inflammasome, a protein complex that activates IL-1 β and IL-18. On the other hand, RLRs (RIG-I and MDA5) interact with double-stranded RNA and stimulate NK- κ B and IRF3/7 signaling pathways. Stimulation of RIG-I also produces the activation of the inflammasome [66].

5.1. *In vivo* targeting of antigens and adjuvant-activation of dendritic cells

In vivo targeting of antigens to DCs has shown to be a promising approach for vaccines, particularly for enhancing the stimulation of cellular immune responses. Targeting of

vaccine antigens involves the administration of an antigen linked to a specific antibody or to a specific marker expressed on DCs with the objective to more efficiently deliver the antigen to the DCs.

For an efficient vaccination, the vaccine candidate also needs to stimulate the immune system in the same way as pathogens do, i.e. signaling through PRRs. However, the immune system is not always able to respond to certain microorganism and thus, alternative ways for stimulating the immune response should be investigated in order to develop an effective vaccine. For example, unlike HIV in humans or SIV in rhesus macaques, sooty mangabeys act as a reservoir for SIV and do not develop AIDS. It has been demonstrated that those differences are derived from the distinct activation of the sooty mangabeys PDCs upon TLR7 and TLR9 activation by the virus [67]. Consequently, PDCs of the reservoir lack the characteristic chronic immune activation producing a much lower amount of IFN- α . In addition, some pathogens have the ability of modulating the expression of the TLRs, such as the hepatitis B virus, which is able to reduce the TLR9 depending IFN- α secretion by PDCs, although not the one dependent on TLR7 [68].

For developing an effective vaccination, inactivated or subunit protein based vaccine antigens need to be accompanied by adjuvants. Adjuvants are components added to the vaccine antigens that are able to modulate the bias, the intensity and the duration of the immune response interacting with DCs and also with other immune cells. Some adjuvants have been shown to directly be able to interact with receptors on APCs for regulating the immune response. Traditionally, adjuvants have been classified as immunostimulatory or carrier systems. The first ones interact with the PRRs of the APCs. The latter were supposed to form a depot that allowed the long-term release of the antigens or to passively drive antigens to DCs. However, it is nowadays clear that carrier systems are also able to directly activate the immune response [69]. For example, it has been shown that alum, which has been used in human for more than 80 years and was thought to act by a depot effect [70], interacts with NLRs and activates NALP3 inflammasome [71]. This interaction with PRR has also been shown for other molecules. Ariza *et al.* have shown that the previously known anticancer drug Bryostatin-1 exerts its activity by the interaction with TLR-4 present in MDCs, which triggers the activation of NF- κ B and release of cytokines (IL-5, IL-6, and IL-10) and chemokines [72].

The first approaches to specifically deliver antigens and immune-stimulatory adjuvants to DCs were carried out directing antigens to molecules present in all DC subsets, such as CD11c or MHC class II [73,74]. However, the most recent studies have developed more specific strategies. Thus, targeting a specific PRR, particular DC subsets are being targeted and leading to different activation of the immune system. Guo *et al.* have shown an improvement of the response elicited in a porcine model by the traditional vaccine for foot-and-mouth disease incorporating a CpG-enriched recombinant plasmid into the formulation [75]. On the other hand, activation of PRRs is not a universal science, and should be studied for each pathogen. Cheng *et al.* have reported that the TLR2/TLR6 agonist Pam₂CSK₄ increases the immunogenicity of a *Chlamydia trachomatis* vaccine, producing strong

humoral and cellular immune responses as well as better protection after challenge in mice. However, other adjuvants such as polyinosinic-polycytidylic acid (poly (I:C), TLR3), monophosphoryl lipid A (MPLA, TLR4), flagellin (TLR5), imiquimod R837 (TLR7), imidazoquinoline R848 (TLR7/8), CpG-1826 (TLR9), M-Tri-DAP (NOD1/NOD2) and muramyl dipeptide (NOD2) elicited lower immune responses and were less efficient for eliciting protection upon a challenge, demonstrating that Pam₂CSK₄ is the most suitable adjuvant for further evaluation of a vaccine against *Chlamydia trachomatis* [76].

It has also been proposed that targeting specific DC receptors should be accompanied by an appropriate route of immunization. Thus, directing antigens to LCs or dermal DCs should be improved by subcutaneous or intradermal routes, and the intravenous route should be better for targeting blood circulating DCs [77]. However, it has not been clearly demonstrated.

Nowadays, it is clearer that achieving an effective immune response needs the activation of multiple PRRs. There are several adjuvants that are able to interact with more than one PRR. For example, poly(I:C), a synthetic analogue of double-stranded RNA, binds TLR3, MDA5 and RIG-I [78]. In addition, it has been shown that for an efficient immune activation, interaction with both TLR3 and RLRs is necessary [79]. This multiple activation has been thought to be a possible reason for the development of strong immune responses.

The other alternative for efficient activation of more than one PRR at the same time is the co-administration of antigens formulated with a combination of adjuvants. Raman *et al.* have demonstrated that achieving strong and effective T cell responses against cutaneous leishmaniasis in mice required the combination of MPLA and CpG along with the antigen. However, the administration of the antigen with each adjuvant separately did not produce an optimal response [80]. In the same way, Zhu *et al.* found that mice immune response upon vaccination against HIV using envelope peptide along with macrophage-activating lipoprotein 2 (MALP2, TLR2/6), poly(I:C), CpG was importantly improved [81].

On the other hand, combination of TLR and CLR agonists appears an interesting approach [82]. Klechevsky *et al.* have evaluated the CD8⁺ T cell responses developed in humans upon targeting antigens to DCs *in vitro* using different adjuvants. Even at low doses, antigens conjugated to antibodies against DCIR were able to trigger antigen specific CD8⁺ T cell responses as well as memory responses by *ex-vivo* generated DCs, LCs, MDCs and PDCs. Although the use of monoclonal antibodies to target DC-SIGN did not produce such high response, the addition of the CL055 (TLR7/8) enhanced the cross-presentation and cross-priming developed by DCIR activation. This activation produced a higher CD8⁺ T cell expansion, a high release of IFN- γ and TNF- α and production of low levels of Th2 type cytokines [83].

In addition, as discussed below, combination of immunostimulatory adjuvants with carrier adjuvants is another alternative for PRR selective targeting.

5.2. *Ex vivo* loading of antigens to dendritic cells

Ex vivo antigen-loaded and/or matured DCs have been developed starting from monocytes or CD34⁺ precursors in the presence of GM-CSF and IL-4. Afterwards, the antigen is added

to the immature DC culture, generally accompanied with various maturation stimuli (adjuvants) to ensure the ability of cross-presentation and the capacity to develop strong immune responses. Finally, these *ex vivo* matured DCs are administered to the patient.

Ex vivo loading of tumor antigens to DCs for developing anticancer vaccines has become the most important application of this approach [84]. The main objective in vaccination against cancer is to trigger potent CLT responses, able to eliminate tumoral cells and to develop memory responses capable of avoiding tumor relapses. This strategy is safe and well tolerated, as well as capable of inducing cellular immune responses, including the expansion of CD4⁺ and CD8⁺ tumor-specific T cells. However, most of the patients do not develop clinical responses [24].

Palucka *et al.* have addressed the most important immunological parameters that determine the effectiveness of this strategy in *ex vivo*-vaccinated patients [24]: (1) the quality of the elicited CTLs, (2) the quality of induced CD4⁺ Th response, (3) the abrogation of Treg cells, and (4) the breakdown of the immunosuppressive tumor microenvironment. In fact, the ability of *ex vivo* cultured DCs for migrate to the tumors is low, impairing the possibilities of activating T cells [85,86]. Moreover, tumors possess mechanisms able to impair the maturation of DCs, and they also have the ability of generating immune reactions that favor their own growth or development [87].

The selection of the antigen for DC-loading can affect the efficacy of the vaccination. Mainly, two kinds of antigens have been used for this approach, mutated (unique) antigens and shared non-mutated antigens. Using mutated antigens makes necessary the personalization of the treatment. In this regard, idiotypic-pulsed DCs are emerging as a promising tool. The idiotypic is the combination of the multiple antigenic determinants of the immunoglobulins for each individual. This approach has been evaluated in a phase III clinical trial in patients with follicular lymphoma, showing a significantly prolonged duration of the remission induced by chemotherapy treatment [88]. However, tumor-specific idiotypes have demonstrated disappointing results in some diseases such as multiple myeloma [89]. Alternatively, the use of shared non-mutated antigens will open a window for developing largely applicable vaccines, but pre-existing Tregs could impair the efficacy of these vaccines [87].

Finally, there are some limitations in the use of *ex vivo* matured DCs. The vaccines need to be prepared for each individual, which increases the time and the costs of vaccination. In addition, the manipulation of the cell cultures increases the risk of endotoxin contamination. Besides, there is a lack of universal protocols for this purpose, so the results of the vaccination may vary depending on the clinic centers.

6. Particulate adjuvants for targeting dendritic cells

Traditionally, adjuvants that were supposed to act by a depot effect were called particulate adjuvant or carriers. They were thought to sediment at the site of injection from which they released the antigen during prolonged periods of time [70]. Nowadays it is clear that,

although they play a role as passive-delivery systems, their adjuvant activity is also due to their ability to interact with the immune system.

Conventional vaccines are composed of whole organisms. Although these vaccines present higher immunogenicity, they have some disadvantages that make the vaccination with antigens derived from the pathogens a better alternative [90-92]. These antigens are usually peptides and tend to be low immunogenic. The main reason is that the uptake and presentation by APCs is not well performed, so effective T cell responses are not developed. In fact, proteins and peptides are easily degraded by proteases and have limited bioavailability due to their low ability to cross biological membranes [93,94]. Carrier adjuvants present some advantages that make them very useful for the development of peptide-based vaccines. First, they protect labile antigens from the effect of hydrolytic enzymes. Secondly, they can be passively or actively delivered to APCs. Moreover, they allow the incorporation of multiple adjuvants and antigens into the same system, as well as APC-targeting molecules, leading to the development of multicomponent vaccines. Thus, finding the optimal combination between antigens and adjuvants acting by different mechanisms is the major goal of vaccination.

Although the most used adjuvant is still alum, other strategies based on particulate systems have emerged and have been licensed for human use. For example, vaccines against influenza (Inflexal[®]) or hepatitis A (Epaxal[®]) are composed of virosomes [95,96] and another vaccine against influenza (Fluad[®]) is formulated with MF59, a squalene-based oil in water emulsion [97]. There are also licensed vaccines composed of more than one adjuvant systems, such as Cervarix[®] or Gardasil[®], both against human papillomavirus [98-100]. The first one is adjuvanted by a mixture of virus like particles (VLPs) and AS04, a combination of MPLA and alum. The second comprises VLPs and alum. The fact of being licensed vaccines means that this strategy is effective, and more research is needed in order to find the appropriate formulations for diseases lacking a vaccine.

6.1. Interactions of particulate adjuvants with dendritic cells

A wide variety of particulate adjuvants have been evaluated as DC-targeting vaccines, which have particular characteristics such as size, shape or composition. The antigen or additional adjuvants can be incorporated by different strategies, such as encapsulated [101], and covalently [102] or non-covalently coupled to the particles [103]. In addition, the delivery of the products can be controlled, allowing a slow and continuous release, by pulses, or triggered by factors such as pH [104], electric or magnetic fields [105], temperature [106] or ionic strength [107]. Thus, the interaction of these systems with DCs is not universal and the adjuvant effect is developed by different mechanisms.

There are two main mechanisms by which carrier adjuvants interact with APCs. i.e. passive and active targeting. Derived from their particulate nature, all of them are directed to DCs by passive targeting. Thus, endocytosis and pinocytosis by APCs is spontaneously favored. Active targeting can also be developed incorporating different ligands into the particulated system. Those ligands can be antibodies, polysaccharides, peptides and drugs that bind to

receptors on DCs. Recently, we have designed an adjuvant system that includes poly lactic-co-glycolic acid based microparticles (PLGA MPs) combined with different adjuvants such as poly(I:C), MPLA, and α -galactosylceramide, co-encapsulated with bovine serum albumin (BSA). Intradermal vaccination of mice with these MPs showed higher IgG titers than those obtained by the administration of the soluble peptide mixed with the same combinations of poly(I:C), MPLA, and α -galactosylceramide used in the MPs. In contrast to the soluble peptides, MPs were able to develop cellular immune responses. Furthermore, the combination of MPLA and α -galactosylceramide triggered the higher cellular response, demonstrating the usefulness of this approach [108].

Important advances of knowledge on the mechanism of action of particulate adjuvant have been done in the last years. Recent data demonstrate that alum targets NALP3 (also known as NLRP3) inflammasome, a kind of NLR, and mediates the delivery of IL-1 β and IL-18 [71], although other studies show that activation of these receptor is dispensable for the effect of alum [109], or that it can activate NALP3 inflammasome by indirect mechanisms [110]. PLGA MPs also activate NALP3 inflammasome, although it is not clear if that activation is or not directly triggered by MPs [111,112]. Other adjuvant systems include an immunomodulatory adjuvant in their structure, such as ISCOMs or ISCOMATRIX[®]. They are cage-like structures comprising cholesterol, phospholipids and a saponin, being the last one the main responsible of the adjuvant effect [113].

In other cases the mechanism of action is not well defined. For example, non-targeted liposomes are thought to act by passive targeting derived from their particulate nature [114], which is more easily detected by the immune systems than smaller soluble antigens and can resembles the structure of the pathogens.

6.2. Factors influencing the efficacy of particulate vaccines

There are some aspects that are crucial for this effect, such as particle size and surface charge of the particles, or their route of administration. The particle size is one of the most important parameters; the smaller the size, the higher the surface area for antigen delivery [115]. Moreover, DCs have different phagocytic ability depending on the particle size. Chua *et al.* have evaluated the differences between chitosan micro and nanoparticles (NPs) for being taken up by DCs [116]. They have found that the uptake of both micro and NPs by bone marrow derived mouse DCs depends on particle concentration. NPs were faster than MPs for reaching to the lymph nodes, although this fact did not affect the quality of the immune response, which lead to similar antibody responses than the administration of the soluble antigen emulsified in Freund's adjuvant. In addition, it has been shown that small particles can reach to the lymph nodes and be engulfed by resident DCs, a fact not achievable by larger particles (500nm-2 μ m) [117,118]. However, other studies show contradictory results and suggest that MPs are better for biasing the immune response towards the humoral arm while NPs can stimulate cellular responses [115]. In addition, coupling ligands to MPs and NPs for targeting DC-SIGN has shown that only NPs are able to actively target DCs, while MPs are non-specifically taken up [119]. The distinct results in

these studies can also be explained because different delivery systems and protocols have been used. Thus, each case should be studied individually.

Modifying the surface charge has also showed differences in the uptake of polystyrene MPs by DCs. Covalently coupling of polyaminoacids/proteins became the particles positively charged and lead to a high increase in the uptake by monocyte derived human blood DCs [120].

The evaluation of different routes of administration has also lead to contradictory results. We have previously reported that PLGA MPs encapsulating the SPf66 malarial peptide produced higher humoral and cellular immune responses when they were administered by the intradermal route rather than when the subcutaneous route was used. Furthermore, the intradermal route allowed a 10 fold reduction of the dose without compromising the immune response [121]. A study by Mohanan *et al.* was designed to address the differences in the immune response of mice vaccinated with ovalbumin-loaded liposomes, N-trimethyl-chitosan NPs and PLGA MPs by subcutaneous, intradermal, intramuscular and intralymphatic routes. They found that only Th1 immune responses were sensitive to the route of administration, but not the Th2 responses [117]. On the other hand, Speiser *et al.* have carried out a phase I/II clinical trial where they have not found significant differences upon subcutaneous or intradermal vaccination with a vaccine against melanoma comprising VLPs and CpG [122].

7. Conclusion

DCs are the cells that join the innate and adaptive immune responses. The interaction of these cells with T or B cells regulates the immunity against pathogens. Moreover, the interaction of antigens and/or adjuvants with DCs also influences the immune response. These unique characteristics of DCs make them an ideal target for vaccination purposes. However, there are several fundamental concerns when designing vaccines directed towards DCs. Studying the human immune system and understanding its complexity is essential for this purpose. Animal models play an important role in this field, but the results obtained in those studies have to be carefully interpreted and translated to humans. The fact of having similar immune effector functions is not enough for accepting any specie as a good model. For example, both human and mouse PDCs have an important role on antiviral immunity by releasing high amounts of type I IFN. Nevertheless, the phenotypic differences between human and mice are going to condition the response. CpG motifs can be used for selective activation of human PDCs with TLRs agonist, but in mice MDCs would be also activated because they do express TLR9. Differences in the expression of uptake receptors such as BDCA-2 will also limit the comparison of studies that target antigens to BDCA-2, as mouse DCs do not present this C-type lectin. Furthermore, it is also important to take into account that mice and humans have distinct susceptibilities to some diseases (for example mice do not develop HIV and they do not have a similar counterpart like SIV for monkeys), impairing the translation of studies addressing immunological responses against pathogens.

In conclusion, there are a lot of factors influencing the interaction of DCs with the immune systems for activating an effective immune response. Consequently, the use of distinct approaches for stimulating DCs would lead to a different activation of the immune system. Thus, the study and understanding of these relations is essential for developing useful vaccines against malaria, HIV, or tuberculosis, or to treat allergies, autoimmunity disorders or rejection of transplanted organs.

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Acknowledgement

This project was partially supported by the “Ministerio de Ciencia e Innovación” (SAF2007-66115), “University of the Basque Country (UPV/EHU)” (UFI 11/32) and FEDER funds. A. Salvador thanks the “University of the Basque Country” for the Fellowship Grant.

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