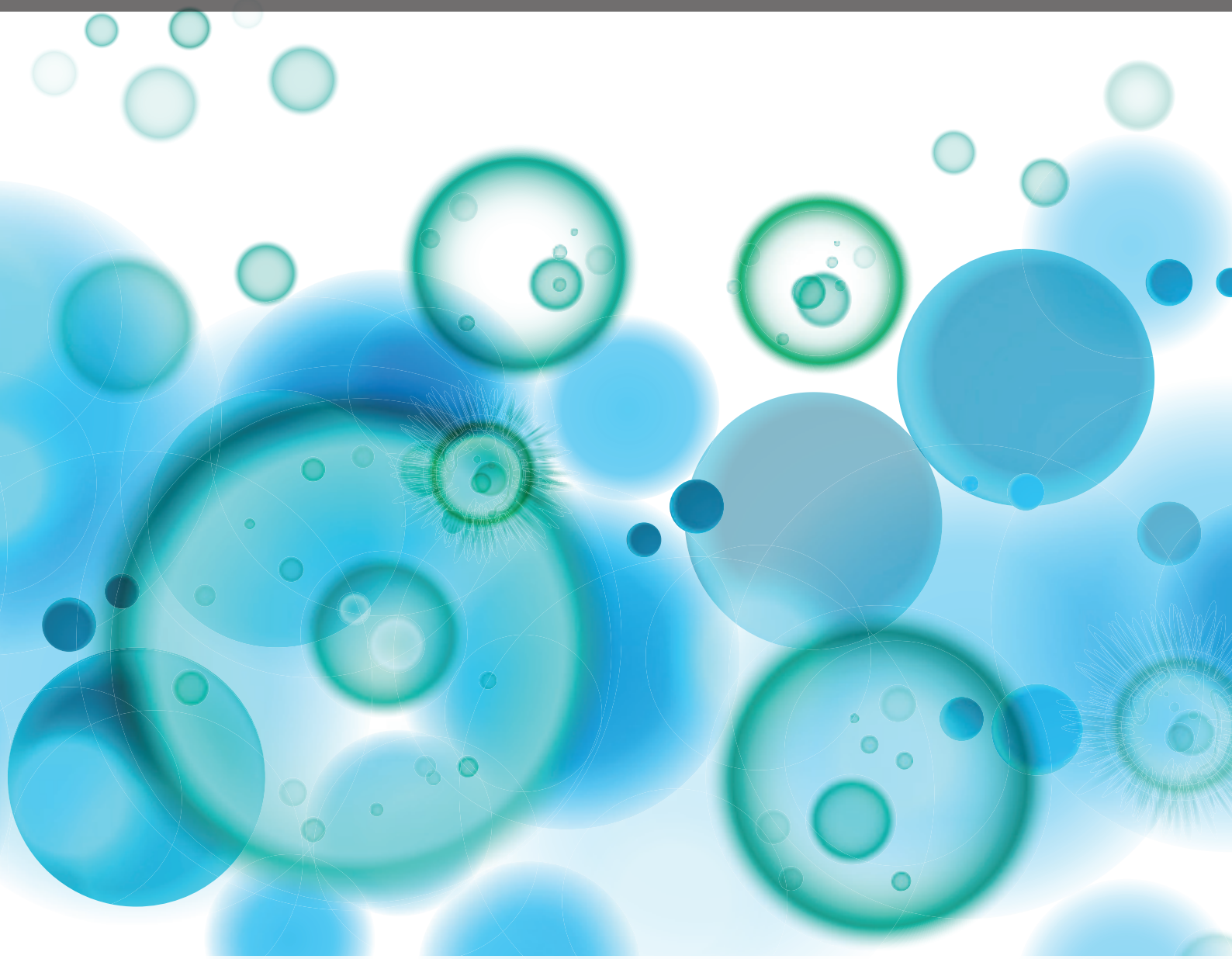
APPLICATION OF ANTIGEN CROSS-PRESENTATION RESEARCH INTO PATIENT CARE

EDITED BY: Marianne Boes
PUBLISHED IN: Frontiers in Immunology



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ISSN 1664-8714
ISBN 978-2-88945-191-3
DOI 10.3389/978-2-88945-191-3

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APPLICATION OF ANTIGEN CROSS-PRESENTATION RESEARCH INTO PATIENT CARE

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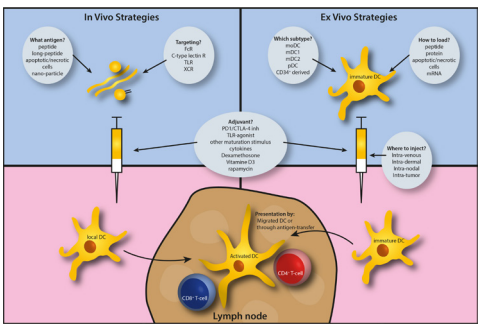

Schematic outline of the considerations to apply antigen cross-presentation research to the clinic, most readily by dendritic cell-based immunotherapy (Copyright: T. W. Flinsenberg and M. Boes, University Medical Center Utrecht, The Netherlands)

The activation of adaptive immune responses requires the processing and presentation of protein antigens to lymphocytes. Especially dendritic cells are effective at display of antigen-derived peptides in the form of immunogenic peptide/MHC complexes to CD4 and CD8-positive T cells, and can stimulate even naive T cells to clonally expand. During the last 40 years, mechanisms that facilitate antigen processing and presentation were clarified, mostly from work in cell lines and mouse models. From mouse-based work, it is now clear that dendritic cells represent a collection of specialized cell subsets that are particularly well endowed to stimulate antigen transport to distinct tissue locations, to transfer antigens between cellular subsets or to trigger T cell responses. Dendritic cell subsets hold great promise for therapeutic application, for example as dendritic cell-based vaccines to bolster immune responses against viruses or malignant growths. Hurdles remain that preclude the efficient application of high quality pre-clinical research into standardized patient care. In this research topic, efforts in dendritic cell research and dendritic cell-based vaccines are discussed, from both pre-clinical and application points of view.

Citation: Boes, M., ed. (2017). Application of Antigen Cross-Presentation Research into Patient Care. Lausanne: Frontiers Media. doi: 10.3389/978-2-88945-191-3

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frontiers in
IMMUNOLOGY

EDITORIAL
published: 17 June 2014
doi: 10.3389/fimmu.2014.00287

Application of antigen cross-presentation research into patient care

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Keywords: immunotherapy, dendritic cell, cancer vaccines, clinical trial, virus infection, autoimmunity, T-cells

Dendritic cell (DC)-based cellular immunotherapy is being explored as a treatment modality for several malignancies, for viral diseases and auto-immune disorders. More than four decades of pre-clinical research on DC biology has cemented a strong foundation for clinical application of DC-based clinical trials, which already have been performed since the 1990s. Although sometimes met with limited patient success, clinical trials do yield better understanding of the requirements for optimal DC-based therapy. Recent advancements in the understanding of human DC biology and subset characteristics now give rise to ample opportunities to explore for a next generation of DC-based immunotherapy. This Research Topic is focused on articles that can help understanding the biology involved in DC antigen presentation, for future DC-based immunotherapy.

Dendritic cells are professional antigen presenting cells (APCs) that are particularly well endowed to elicit adaptive immune responses, via the presentation and cross-presentation of antigen-derived peptide/MHC complexes to T-lymphocytes. These processes decide how the host interacts with its environment, and therefore can be a target for pathogen interruption. van Montfoort et al. (1) provide an overview of cross-presentation features and describe how the study of various viral pathogens can elucidate anti-viral immune strategies. They further describe how DC maturation is crucial in immunity against viruses and how viruses may dampen this response to their own advantage. Understanding these presentation pathways is pivotal to develop effective DC-based immunotherapy.

Dendritic cell-based immunotherapy comes in two flavors. Either DCs are cultured and manipulated *ex vivo* before infusion, or endogenous DCs can be targeted *in vivo*. Concerning the latter approach, local administration of long peptides has proved effective in several diseases giving opportunity for further exploration. Both Delamarre and Cohn (2) and Rosendahl Huber et al. (3) discuss the requirements for improved antigen presentation, providing considerations on $CD4^+$ and $CD8^+$ activation, choice of antigen, and desired adjuvants. Delivering antigen to DCs is a next hurdle to take.

Regarding antigen delivery, it matters to engage responsive receptors, for these receptors can decide the intracellular pathway of antigen routing and enzymatic processing. Antigens are thereby directed toward assembly into peptide/MHC class I (cross-presentation) or peptide/MHC class II complexes, and induction of immunity or tolerance. Fehres et al. (4) describe the biology of receptor-mediated uptake in the context of antigen presentation, with special emphasis on C-type Lectin receptors. They further discuss the possibilities to formulate antigen in order to provide receptor-directed antigen delivery. Another import route of uptake involves the family of Fc receptors, which is discussed by Platzer et al. (5). Here, the role of this receptor family is highlighted in antigen presentation with emphasis on the opposing roles of activating and inhibiting Fc Receptor isoforms. Furthermore, they underscore that mechanisms of antigen presentation in mice are not always identical to the human pathways. Thus, the need for more research on human DC biology is warranted, for DC-vaccination strategies are still heavily based on mouse-biology.

When designing a DC-based immunotherapy, it is relevant to consider the subtype of DCs that one aims for. Boltjes and van Wijk (6) present an overview of all phagocyte subsets that are present throughout the human body in steady-state and under inflammatory conditions. They also emphasize the differences between mouse and human cells, and review cell types that should be considered for immunotherapy. Until recently, monocyte-derived DCs (MoDCs) were used mostly in DC-therapy, for their relative ease to culture in large quantities *ex vivo*. But while MoDCs can be found in human tissue under inflamed conditions, other DC subsets are more prevalent overall and may be more specifically endowed at stimulation of particular T-cell subsets, to be explored in immunotherapy. One subset that was suggested to be superior in $CD8^+$ T-cell priming is the recently identified BDCA-3^+ ($CD141^+$) DC, characterized by CLEC9A and XCR1 expression. Tullett et al. (7) highlight recent findings explaining why these cells are effective at $CD8^+$ T-cell priming and discuss *in vivo* antigen targeting toward these DCs. Wimmers et al. (8) also describe the use of naturally circulating mDCs and pDCs for DC-based immunotherapy. They discuss the division of labor between pDCs and mDCs and the clinical trials that are being performed using these subsets. Interestingly, they highlight that mDCs and pDCs work in synergy, supporting each other to enhance the effector phase of the adaptive immune response. Based on this observation, a next step in DC-based vaccination should include a cocktail of mDCs and pDCs, or *in vivo* antigen targeting to both subtypes.

Dendritic cells are often called "master regulators" of the immune response. Besides firing up immune reactions, DCs play an equally important role in the maintenance of peripheral tolerance, for example by dampening specific T-cell responses or by inducing regulatory T-cell subsets. Loss of tolerance is

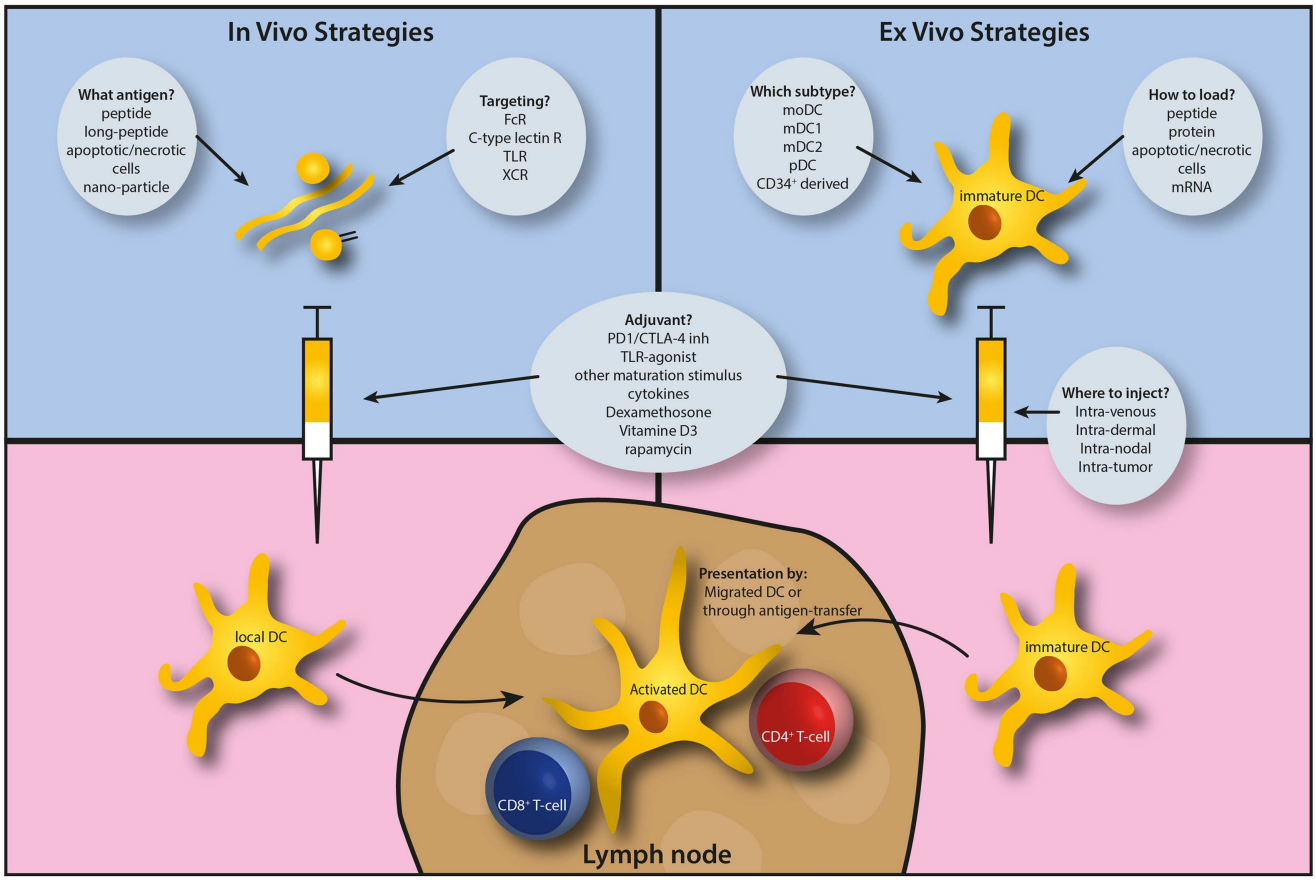

FIGURE 1 | Schematic outline of the considerations to apply antigen cross-presentation research to the clinic, most readily by dendritic cell-based immunotherapy.

of pivotal importance in auto-immune diseases as described by Hopp et al. (9). Their review concerns the presentation of self-antigen, which they discuss in the context of mechanisms in tolerance induction, DC maturation status, DC uptake and processing mechanisms, and tolerance-associated intracellular signaling pathways. The regulation of DC function is also controlled by metabolic pathways, as described by Everts and Pearce (10). Recent advancements concerning regulation of DC metabolism include the identification of key-proteins like PI3K, Akt, and mTOR in DC function. The awareness that manipulation of DC metabolic pathways changes DC function should be explored for designing DC-based cellular therapy, especially since it may give opportunity to steer toward more immunogenic or tolerogenic consequences. This could be of upmost importance in the setting of auto-immune diseases, anti-cancer, or graft-versus-host therapy.

Plantinga et al. (11) finally discuss recent developments in DC-therapy in the setting of allogeneic–hematopoietic cell transplantations (HCT). Such transplantations are considered a last-resort treatment for several malignancies of hematological origin. DCs grown from the same donor background as the HCT are now being explored for their potency to prevent cancer relapses early after allogeneic HCT. The various considerations for such DC vaccinations are discussed, such as the stem cell source, type of tumor antigen, and vaccination strategy.

The breadth and quality of the work discussed in this Research Topic underscores the strong translational push of DC research toward clinical settings (**Figure 1**). Immunotherapy is now being incorporated into standard cancer care, with antibody-based treatments currently being at more advanced stages than cellular therapies. The abundance of currently ongoing DC-based cellular immunotherapy trials should benefit patient care in the near future, as the roots for translational success are implanted in well-established pre-clinical research settings.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 26 May 2014; accepted: 03 June 2014; published online: 17 June 2014.
*Citation: Flinsenberg TWH and Boes M (2014) Application of antigen cross-presentation research into patient care. Front. Immunol. **5**:287. doi: 10.3389/fimmu.2014.00287*
This article was submitted to Antigen Presenting Cell Biology, a section of the journal Frontiers in Immunology.

frontiers in
IMMUNOLOGY

REVIEW ARTICLE
published: 23 April 2014
doi: 10.3389/fimmu.2014.00182

Understanding MHC class I presentation of viral antigens by human dendritic cells as a basis for rational design of therapeutic vaccines

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Effective viral clearance requires the induction of virus-specific $CD8^+$ cytotoxic T lymphocytes (CTL). Since dendritic cells (DC) have a central role in initiating and shaping virus-specific CTL responses, it is important to understand how DC initiate virus-specific CTL responses. Some viruses can directly infect DC, which theoretically allow direct presentation of viral antigens to CTL, but many viruses target other cells than DC and thus the host depends on the cross-presentation of viral antigens by DC to activate virus-specific CTL.

Research in mouse models has highly enhanced our understanding of the mechanisms underlying cross-presentation and the dendritic cells (DC) subsets involved, however, these results cannot be readily translated toward the role of human DC in MHC class I-antigen presentation of human viruses. Here, we summarize the insights gained in the past 20 years on MHC class I presentation of viral antigen by human DC and add to the current debate on the capacities of different human DC subsets herein. Furthermore, possible sources of viral antigens and essential DC characteristics for effective induction of virus-specific CTL are evaluated.

We conclude that cross-presentation is not only an efficient mechanism exploited by DC to initiate immunity to viruses that do not infect DC but also to viruses that do infect DC, because cross-presentation has many conceptual advantages and bypasses direct immune modulatory effects of the virus on its infected target cells.

Since knowledge on the mechanism of viral antigen presentation and the preferred DC subsets is crucial for rational vaccine design, the obtained insights are very instrumental for the development of effective anti-viral immunotherapy.

Keywords: virus, human dendritic cell, cross-presentation, CTL priming, MHC class I-antigen presentation, viral immunity, immunotherapy, virus–host interaction

ROLE OF DENDRITIC CELLS IN THE INDUCTION OF ANTI-VIRAL IMMUNITY

Immune responses to viral infections are a complex interplay between the virus, target cells, and cells of the immune system. Effective viral clearance requires the induction of virus-specific $CD8^+$ cytotoxic T lymphocytes (CTL), which have the capacity to eradicate the virus by direct and indirect mechanisms (1). DC, a low frequent population of white blood cells play a central role in the induction of virus-specific CTL, since they are the most potent antigen presenting cells and unique for their capacity to activate naïve T cells (2). DC are located at strategic positions at sites of pathogen entry, where they continuously sample the environment for invading pathogens. Capturing antigens in combination with encountering danger signals from pathogens induces maturation of DC and their migration to secondary lymphoid organs where they can activate naïve T cells. Activation of naïve $CD8^+$ T cells and polarization toward effective CTL requires presentation of MHC class I–peptide complexes (signal 1) together with co-stimulation (signal 2) and the presence of cytokines (signal 3) such as IL-12 (3) and IFNα (4).

Dendritic cells comprise a family of different subsets, diverging in ontogeny, localization, and phenotype. Each DC subset has its own specialized immune functions with regard to the functional interactions with all kind of immune cells, including T cells, B cells, and NK cells, due to differential expression of receptors and intrinsic differences in their ability to produce different cytokines and other membrane-bound and soluble immune modulatory molecules (5). Human DC subsets present in blood, peripheral, and lymphoid tissues can be classified in two main categories: plasmacytoid DC (pDC) and myeloid DC (mDC), which can be further divided into $BDCA1^+$ ($CD1c^+$) and $BDCA3^+$ ($CD141^+$) DC (6). pDC are specialized in the production of high amounts of anti-viral type I interferon (IFN; IFNα/β) upon activation (7), whereas $BDCA1^+$ DC are known for their high production of IL-12 and their ability to induce T cell responses (5). $BDCA3^+$ DC, on the

Abbreviations: DC, dendritic cell; CTL, cytotoxic T lymphocyte; mDC, myeloid dendritic cell; pDC, plasmacytoid dendritic cell; moDC, monocyte-derived dendritic cell; LC, Langerhans cell; PRR, pattern-recognition receptor; VLP, virus-like particle; CLR, C-type lectin receptor; FcR, Fc receptor; TLR, Toll-like receptor.

other hand, can produce high levels of type III IFN (IFNλ) (8), which possess direct anti-viral activity, and induce Th-1 responses (9). In the skin, two additional mDC subsets have been characterized, epidermal Langerhans cells (LC) and dermal interstitial DC (intDC) (10). Since DC represent a very rare population in the human body that hampers isolation of sufficient numbers, *in vitro*-generated DC differentiated from monocytes (11) or hematopoietic progenitor cells (12) are frequently used for functional studies on human DC.

The notion that DC compared to other antigen presenting cells stand out in their capacity to induce strong virus-specific CTL goes back more than 20 years, when it was reported that human blood-derived DC exposed to HIV-1 or influenza virus could induce proliferation of autologous CTL (13, 14). At that time, it was not known whether the efficacy of DC reflected specialized antigen presentation pathways or that other factors were responsible for the efficacy of DC in virus-specific CTL cell induction. At least it was noted that only low numbers of DC were sufficient to induce influenza-specific T cells (14).

Now we know that DC, in addition to their broad expression of pattern-recognition receptors (PRR) and excellent T cell stimulatory capacities, harbor unique specialized antigen presentation pathways, that are of major importance for their central role in the induction of virus-specific immunity; DC can efficiently facilitate MHC class I presentation of endogenously synthesized antigens, a process that is active in all nucleated cells, but also facilitate MHC class I presentation of antigen engulfed from exogenous sources, a process called cross-presentation (15). DC are very efficient in capturing exogenous antigen, because they express a diverse repertoire of receptors and exploit various mechanisms to engulf antigens, including endocytosis, phagocytosis, and pinocytosis. The cross-presentation capacity of DC may be crucial for the induction of virus-specific CTL during infections with viruses that do not infect DC.

Seminal mouse studies have demonstrated the importance of cross-presentation for the generation of virus-specific CTL responses (16–18). In addition, mouse studies have provided important insights into the cell-biological mechanisms underlying cross-presentation by DC (19, 20). However, composition of the human DC compartment and susceptibility to viruses differ largely between mice and men. In addition, the mechanism of cross-presentation by human DC is less well-understood. Therefore, research on MHC class I presentation of viral antigens by human DC is of great importance to understand the induction of virus-specific CTL in humans.

The study into antigen presentation of viruses by subsets of human DC *ex vivo* has been facing several technical challenges, which has hampered the understanding of this process for many viruses. However, some recent technical advancements have become available that empowered this research. For example, the possibility to more efficiently isolate human DC subsets from peripheral blood and other organs and the development of a new generation of protocols to generate human DC subsets *in vitro* (21, 22), as was previously shown for BDCA1$^+$ monocyte-derived DC (moDC) (11) and CD34$^+$ HPC-derived intDC and LC, that resemble mDC found in mucosal tissues including skin (12, 23). These technical advancements have revived the scientific interest in the interactions between viruses and different human DC subsets. Since 2010, a significant body of literature has been published on presentation of viral antigens by different human DC subsets that facilitated this review, which is based for a large part on studies using human DC.

In the present review, the different mechanisms employed by human DC to facilitate MHC class I presentation of viral antigens are discussed. For this purpose, possible sources of viral antigens, essential DC characteristics for optimal MHC class I presentation of viral antigens, and host factors important for virus-specific CTL induction are defined. Furthermore, the roles of the various human DC subsets of human DC in these processes are evaluated. Since knowledge on mechanisms of virus-specific CTL induction by human DC subset is crucial for rational vaccine design, recommendations for development of effective anti-viral immune therapies will be provided based on the insights obtained in this review.

SOURCES OF VIRAL ANTIGEN FOR MHC CLASS I PRESENTATION BY DC

Virus-infected DC can use endogenously synthesized viral proteins as antigens for presentation in MHC class I, whereas non-infected DC need to actively engulf exogenous viral antigens for cross-presentation. Here, we discuss possible sources of viral antigen obtained from different viruses for MHC class I presentation by human DC.

Human moDC are permissive for quite a number of viruses including measles virus (MV), human cytomegalovirus (HCMV), influenza A virus (IAV), human T-cell lymphotropic virus type 1 (HTLV-1), dengue virus (DV), vaccinia virus (VV), respiratory syncytial virus (RSV), herpes simplex virus (HSV), and human metapneumovirus (hMPV) (24–36). Although moDC can take up HIV-1, they are largely refractory to HIV-1 productive infection (37), whereas, productive infection of peripheral blood-derived BDCA1$^+$ DC and pDC has been demonstrated (38). In addition to moDC, RSV also infects BDCA1$^+$ and BDCA3$^+$mDC (39) and IAV infects BDCA1$^+$ mDC, but not pDC (40). LC are permissive for MV, but only after maturation (25). Although LC can take up HIV-1, they are not permissive for HIV-1 replication and transmission, but rather prevent it by degradation (41). Permissiveness to infection indicates that these viruses not only enter human DC, they also induce a certain level of protein neo-synthesis in DC that ranges from restricted synthesis of early viral proteins (33) to extensive synthesis of multiple viral proteins and secretion of viral progeny (26). Intracellular synthesis of viral antigens by DC suggests that these infected DC may facilitate direct presentation of viral antigens in MHC class I and activation of virus-specific cytotoxic T cells (CTL). MHC class I presentation of viral antigens has been reported for DC infected with IAV, MV, HTLV-1, and HCMV, albeit sometimes with low efficiency (14, 25, 27, 31, 42).

Nevertheless, it has been demonstrated in several independent studies, involving IAV, HIV-1, and MV, that the efficiency of MHC class I-antigen presentation of replication-incompetent virus was at least comparable to replication-competent virus (25, 40, 43–46). These heat-or UV-treated replication-incompetent viruses have lost the capacity to induce synthesis of viral proteins, but still efficiently enter DC to act as exogenous sources of viral antigen. It was estimated that MHC class I presentation

of replication-incompetent IAV by BDCA1$^+$mDC was 300 times more efficient than MHC class I presentation of replication-competent IAV (40). These studies clearly show that, at least for the viruses studied, endogenous synthesis of viral antigens is not required for MHC class I presentation and that cross-presentation is an efficient mechanism to facilitate MHC class I presentation of viral antigens.

Thus, cross-presentation is not only an efficient mechanism exploited by DC to initiate immunity to viruses that do not infect DC but also contributes to initiation of anti-viral immunity to viruses that do infect DC. In fact, cross-presentation seems a clever way to bypass direct immune modulatory effects of the virus on its infected target cells. For instance, interference with MHC class I presentation is commonly used by herpes viruses to evade immunity [reviewed by Ref. (47)] and is also exploited by IAV, as was elegantly shown by comparing CMV-specific CTL proliferation by CMV-antigen loaded IAV-infected and uninfected BDCA1$^+$ mDC (40). In addition, early during HIV infection, part of the DC compartment is depleted, which may contribute to decreased activation of adaptive immunity (48). Virus-induced cell death is also reported for RSV (34, 39) and VV (33).

In addition to replication-incompetent viral particles, other sources of exogenous viral antigens for cross-presentation by human DC include virus-like particles (VLP), viral proteins, and virus-infected cells (**Figure 1**). VLP morphologically and immunologically resemble infectious viral particles because they contain the natural viral envelop proteins, however, they are not infectious, because they do not contain the viral genome. Although some VLP naturally occur *in vivo*, they are often man-made, being used as safe representatives of viral particles to study virus–host interactions (49) or in the context of vaccine research (50, 51). VLP can be efficient sources of exogenous viral antigen for cross-presentation by DC, as was demonstrated for hepatitis C virus (HCV) VLP (49), human papilloma virus 16 (HPV16) VLP (50), and VLP composed of the coat protein of papaya mosaic virus (PapMV) (51).

Recombinant proteins such as HCV-derived NS3 (52), HIV-1-derived Nef (53), HCMV-derived pp65 (9, 54), and hepatitis B virus (HBV)-derived hepatitis B surface antigen (HBsAg) (55, 56) are sources of exogenous antigens that are often used to study the mechanism of cross-presentation by DC. Nevertheless, the efficiency of cross-presentation of these recombinant proteins is relatively low compared to other sources of viral antigens. Moreover, with the exception of HBsAg, which is secreted by human hepatocytes and can be measured in peripheral blood, most proteins are not naturally occurring as soluble proteins *in vivo* but are only present in/associated with infected cells.

Cell-associated antigen, i.e., antigen associated to or present in infected target cells, represents another important source of viral antigens that can be encountered by DC. Albert and colleagues contributed the first evidence of this by showing that uptake of apoptotic IAV-infected monocytes by moDC leads to efficient activation of influenza-specific CTL (57). After this study, a compelling number of studies have confirmed that virus-infected target cells can be efficient antigen sources for cross-presentation in many infections. For instance, VV-infected monocytes (45, 58), HTLV-1 infected CD4$^+$ T cells (31), MV-infected B cell lines (25), HCMV-infected fibroblasts (27, 59), and EBV-transformed B cells (60, 61) are reported as efficient sources of viral antigens for cross-presentation by human DC. The latter study nicely illustrated the high efficiency of this mechanism by demonstrating activation of EBV-specific CTL by DC cross-presenting EBV latency antigens that were expressed at low levels in EBV-transformed B cells (61).

In the above-mentioned studies, apoptotic or necrotic virus-containing cells or cell remnants were used as sources of

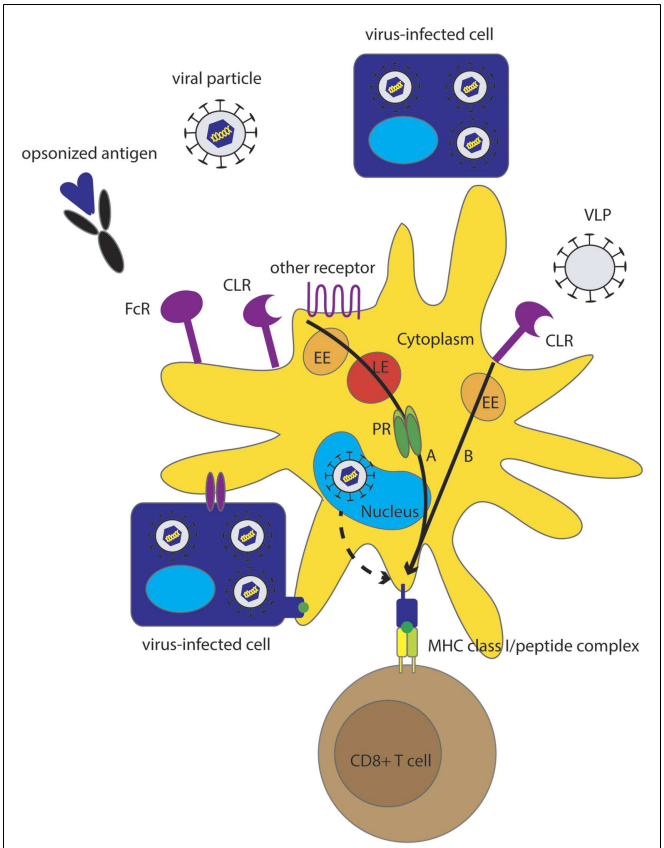

FIGURE 1 | Overview of different pathways underlying MHC class I presentation of viral antigens by human DC. Although direct MHC class I class I presentation may contribute to virus-specific CTL induction (dashed arrow), cross-presentation is an effective mechanism for MHC class I presentation of viruses that do not infect DC but also for those viruses that do infect DC. Sources of viral antigen that can be efficiently cross-presented by human DC include viral proteins, (infectious) viral particles, VLP, and virus-infected cells, also referred to as cell-associated Ag. Endocytic receptors including CLR, FcR and other receptors (**Table 1**) play an important role in the uptake of Ag for cross-presentation. Cross-presentation can be enhanced by opsonization. Two main pathways for cross-presentation have been described that are also relevant for cross-presentation of viruses by human DC and are characterized by differences in the mechanism of protein degradation and differences in kinetics (black arrows). The slower cytosolic pathway, that relies on proteasomal degradation in the cytosol, is important for cross-presentation of viral particles, infected cells, and opsonized viral proteins **(A)**. The relatively fast vacuolar pathway is independent of proteasomal degradation and is important for cross-presentation of VLP **(B)**. Alternatively, DC can obtain viral peptides or MHC class I-peptide complexes by interaction with virus-infected cells. EE, early endosome; LE, late endosome; PR, proteasome.

cell-associated antigens for cross-presentation. Transfer of viral peptides from infected cells to DC could represent an alternative efficient mechanism underlying cross-presentation of cell-associated viral antigens. Two different mechanisms facilitating peptide exchange between cells have been described, including transfer of antigenic peptides via intercellular communication channels, called gap junctions (62), and direct transfer of MHC class I/peptide complexes from infected cells to DC, named cross-dressing (63, 64). The relevance of these pathways in presentation of viral antigens by human DC and induction of virus-specific T-cell immunity should be further evaluated.

In summary, for efficient viral antigen presentation to $CD8^+$ T cells, DC can acquire viral antigens from various sources. Although direct presentation of endogenously generated antigen by virus-infected DC has been reported for some viruses, evidence to support an important role for this mechanism in the induction of virus-specific CTL is lacking. In contrast, there is compelling evidence that cross-presentation of exogenously acquired viral antigen is highly efficient and provides an excellent way for the host to bypass evasion mechanisms that several viruses employ to prevent direct MHC class I presentation in infected target cells.

ENDOCYTIC RECEPTORS INVOLVED IN UPTAKE OF VIRUSES BY DC

Being intracellular parasites, viruses use the host machinery for internalization, proliferation, and transmission. DC are attractive target cells for viral entry because they express numerous receptors at their cell surface and they migrate through the body, which facilitates viral dissemination. Viruses can enter DC via docking with their viral envelop to endocytic receptors expressed at the cell membrane (43, 44, 46). A commonly described receptor used by viruses to enter DC is DC-specific C-type lectin dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN/CD209). DC-SIGN is involved in the infection of moDC by DV (32, 65), HCMV (28), HSV (66), MV (67), and IAV (68) and also in DC-mediated transmission of HIV-1 (69) and HTLV-1 (70) to $CD4^+$ T cells. DC-SIGN is part of the large family of C-type lectin receptors (CLR), comprising Ca^{2+}-dependent receptors that each have unique functions but share the recognition of carbohydrate structures present on micro-organisms (71). Other CLR family members involved in interaction with viruses include Langerin (CD207), involved in the interaction with MV and HIV-1 (25, 41), DC immunoreceptor (DCIR) (72), proposed as an alternative receptor for HIV-1 promoting infection in *cis* and *trans* and macrophage mannose receptor (MMR/CD206), possibly involved in uptake of HBsAg by liver $BDCA1^+$ DC (73). Also non-CLR can be involved in the interaction with viruses or VLP. DC-specific heparin sulfate proteoglycan Syndecan-3 cooperates together with DC-SIGN to facilitate infection of DC and transmission to $CD4^+$ T cells (74) and is involved in the interaction with HPV VLP (75). Since expression of endocytic receptors varies widely between DC subsets (**Table 1**), the different subsets will likely have specialized roles in the interaction with different viruses, determined by the combination of receptors expressed on each DC subset.

Are these CLRs only involved in supporting viruses to enter the host or did they evolve to support activation of the host's immune system through antigen presentation? Langerin is an important receptor for interaction with pathogens in the skin and has been shown to support antigen presentation in MHC class II, but its role in MHC class I-mediated antigen presentation is under debate (25). Moris et al. showed that blocking of DC-SIGN partly reduced MHC class I presentation of internalized HIV-1 by DC, arguing in favor of a role of DC-SIGN in cross-presentation of HIV-1 (91). In contrast, Sabado et al. showed that blocking of DC-SIGN, DEC-205 (CD205), or MR did not reduce MHC class I presentation of HIV-1 antigens (46) whereas Tjomsland and colleagues showed that blockade of MR even promoted cross-presentation of HIV-1 by DC (92). Thus, the physiological role of DC-SIGN in cross-presentation of HIV-1 is thus far inconclusive, which may be explained by differences in experimental set-up such as the HIV-1 strain used. Antibody-mediated delivery of antigen to the CLRs MR, DEC-205 (82), DCIR (81), DC-SIGN (93), and CLEC9A (94) (**Table 1**) on human DCs facilitates efficient cross-presentation. These examples show that CLR can facilitate cross-presentation, however, the physiological role of these receptors in cross-presentation of viral antigens is still under debate.

Whereas CLR can directly recognize viral envelop antigens, complement receptors and Fc receptors (FcR) selectively recognize viral antigens that are opsonized with complement and immunoglobulins, respectively. Antigen immune complexes naturally exist and are formed when pre-existing antibodies bind to blood-borne antigens in the circulation, for example, during HCMV re-infection (85). Binding of immune complexes to Fcγ receptor (FcγR) on DC leads to efficient cross-presentation in MHC class I (85). Strikingly, the observation that FcR-dependent uptake of HBsAg can enhance activation of HBV-specific CTL was made years before the concept of cross-presentation by DC was recognized (95), indicating that opsonization of viral antigens may be important for generating virus-specific CTL. Similarly, opsonization of antigen by complement can efficiently enhance cross-presentation, as was recently demonstrated for HIV-1 by targeting HIV-1 particles to CR3 (92). In addition, although not classically referred to as opsonization, binding of high-density lipoprotein (HDL) to HCV VLP supported efficient Scavenger receptor B-mediated uptake and cross-presentation (96). A similar role for extracellular heat-shock proteins (HSP) has been proposed [reviewed by Ref. (97)], mainly based on mouse studies in the field of cancer immunotherapy. However, the role of HSP in cross-presentation of viral antigens by human DC remains to be investigated.

Although these results indicate that several endocytic receptors may be involved in facilitating cross-presentation, their exact role needs to be determined. Especially recognition of viral antigens by opsonins seems to be an effective way of natural antigen targeting to DC for cross-presentation. Increased knowledge on the receptors used by viruses for infection on the one hand and the receptors that facilitate cross-presentation on the other hand may be of great value for therapeutic interventions.

MECHANISMS UNDERLYING CROSS-PRESENTATION

One of the intriguing aspects of cross-presentation is that processing of incoming antigen needs to be very efficient to compete with the vast amount of endogenous proteins for MHC class I binding.

Table 1 | Summary of receptors that are involved in DC–virus interaction on different DC subsets.

Family	Name	BDCA1$^+$ mDC	BDCA3$^+$ mDC	pDC	Epidermal LC	Dermal intDC	moDC	Reference
C-type lectin receptors	DEC-205 (CD205)	+	+	+	−	+	+	MacDonald et al. (76), Ebner et al. (77)
	DCIR (CLEC4A)	+	−	+	+	+	+	Bates et al. (78), Lambotin et al. (79), Eklöw et al. (80), Klechevsky et al. (81)
	MMR (CD206)	±	+	−	−	+	+	Chatterjee et al. (82), MacDonald et al. (76), Lambotin et al. (79)
	DC-SIGN (CD209)	−	−	−	−	+	+	Turville et al. (83), MacDonald et al. (76)
	CLEC9A (DNGR1)	−	+	−	−	−	−	Huysamen et al. (84)
	Langerin (CD207)	−	−	−	+	−	−	Turville et al. (83), MacDonald et al. (76)
Toll-like receptors	1	+	+	+	+	+	+	Kadowaki et al. (5), Jongbloed et al. (9), Lambotin et al. (79)
	2	+	+	−	+	+	+	
	3	+	+	−	+	+	+	
	4	+	−	−	−	+	+	
	5	+	−	−	−	+	+	
	6	+	+	+	+	+	+	
	7	−	−	+	+	+	−	
	8	+	+	−	+	+	+	
	9	−	−	+	−	−	−	
	10	+	+	+	−	−	+	
Fcγ receptors	FcγRI (CD64)	+	−	nf	nf	nf	±	Flinsenberg et al. (85)
	FcγRIIA (CD32)	+	+	+	nf	nf	+	Flinsenberg et al. (85), Tel et al. (86)
	FcγRIII (CD16)	−	−	−	nf	nf	−	Flinsenberg et al. (85)
Complement receptors	CR4 (CD11c)	+	+	−	+	+	+	MacDonald et al. (76), Lambotin et al. (79)
	CR3 (CD11b)	±	−	−	±	+	+	Donaghy et al. (87), Lui et al. (88), Poulin et al. (21)
Heparan sulfate proteoglycan	Syndecan-3	nf	nf	nf	nf	nf	+	de Witte et al. (74)
Chemokine receptor	XCR1	−	+	−	−	−	−	Crozat et al. (89), Bachem et al. (90)

pDC, plasmacytoid DC; LC, Langerhans cell; intDC, interstitial DC; moDC, monocyte-derived DC; nf, information not found.

In addition, cross-presentation requires access of incoming antigen to the MHC class I pathway that is mechanistically separated from the uptake vesicles (98).

Dendritic cells harbor unique pathways to facilitate these logistic and mechanistic challenges underlying cross-presentation. Based on research of numerous groups, two main models have been put together for the mechanisms underlying cross-presentation of exogenous antigens, referred to as the "cytosolic" pathway and the "vacuolar" pathway [reviewed by Ref. (20)]. These pathways are not mutually exclusive and may operate together in one cell (99). The most discriminative aspects between the two pathways are discussed below.

In the cytosolic pathway, antigens are degraded by the proteasome, a large enzyme complex situated in the cytosol that makes this pathway sensitive to inhibitors of proteasomal degradation. Alternatively, in the vacuolar pathway, both antigen degradation and MHC class I presentation occur in the endocytic compartment. Involvement of this pathway can be experimentally addressed by confirming resistance to inhibition of proteasomal degradation and sensitivity to inhibition of lysosomal proteolysis.

Lysosomal proteolysis has a detrimental role in the cytosolic cross-presentation pathway. It was experimentally demonstrated that limiting lysosomal proteolysis by chemically increasing the lysosomal pH favors cross-presentation of viral proteins HCV-derived NS3 and HIV-derived Nef by preventing complete degradation of potential MHC class I binding epitopes (53). Several different adaptations on the endocytic compartment, including a differential lysosomal protease activity, mechanisms to control the lysosomal pH, and antigen storage compartments, together endow DC to facilitate cross-presentation via the cytosolic pathway (100–102). Cross-presentation via the cytosolic pathway further requires export of internalized antigens from the endocytic compartment

to the cytosol for proteasomal degradation, which is probably the rate-limiting step in this pathway, at least for protein antigen. Many enveloped viruses can enter the cytoplasm as part of their infection strategy that requires fusion of the viral envelope with the endosomal membrane to release the viral genome into the cytoplasm. This endosomal fusion capacity probably underlies the efficiency of cross-presentation of viral particles, at least for those particles that are able to enter the cytoplasm of DC. The mechanism of cytosolic delivery for other viral antigens and viruses that do not undergo endosomal fusion in human DC is largely unknown. Candidate proteins that may be involved in cytosolic delivery include HSP and p97 and sec61, which belong to the endoplasmic reticulum-associated protein degradation (ERAD) machinery (20), however, the role of these molecules in human DC is poorly studied.

Interestingly, the cytosolic and vacuolar pathway has totally different kinetics, which can be used to determine which pathway is involved (103). Whereas cross-presentation via the vacuolar pathway is fast and can be detected after 20 min (104), cross-presentation via the cytosolic pathway is much slower and formation of MHC class I–peptide complexes via this pathway may take at least 8 h (100), probably because it relies on MHC class I neo-synthesis (20). In contrast, MHC class I loading in the vacuolar pathway occurs in the endocytic compartment and depends on recycling of MHC class I molecules that are constitutively internalized by a highly regulated process (105).

VIRAL ROAD TO CROSS-PRESENTATION

The cytosolic and the vacuolar pathways were largely established based on model antigens and mouse studies. It is important to assess if these models are applicable to cross-presentation of viral antigens by human DC.

As discussed above, viral particles use receptors expressed on the plasma membrane to enter DC and uptake of viruses often involves endocytosis. After receptor-mediated endocytosis, the cargo is transported through the endocytic compartment, a highly regulated network of vesicles with different characteristics and functions (103). An important function of the endocytic system is to sort internalized receptors and cargo to different locations for either degradation or recycling. Viruses use the endocytic system to exert their fusion capacity, however, at the same time DC use it to obtain viral antigen for cross-presentation. For example, when IAV reaches late endosomes, the low pH enforces conformational change, leading to hemagglutinin-mediated fusion of the endosomal and viral membranes and release of the viral RNA and proteins into the cytoplasm (106). IAV is efficiently cross-presented, at least when its fusogenic activity is intact (43, 107). The fusion dependence was also observed for HIV; cross-presentation of HIV-1 was completely absent when fusion-incompetent HIV-1 mutants were used or fusion was inhibited chemically (44, 46). Cross-presentation of HIV-1 viral particles is sensitive to proteasome inhibitors, but enhanced by inhibition of lysosomal proteolysis (46). Taken together, the above-mentioned work suggests a role for the cytosolic pathway in cross-presentation of fusion-competent viral particles, at least by mDC. Interestingly, cross-presentation of IAV by pDC is not sensitive to proteasome inhibitors, but is sensitive to inhibition of endosomal processing. Together with fast MHC class I presentation, this study suggests a role for the vacuolar pathway for cross-presentation of IAV by pDC.

Evidence from different studies involving IAV-infected monocytes (108), HCMV-infected fibroblasts (27), and EBV-transformed B cells (61) suggests that cross-presentation of cell-associated antigen involves uptake by receptor-mediated phagocytosis and that antigen processing is dependent on the proteasome, but also sensitive to inhibition of lysosomal proteolysis (109). Cross-presentation of Ag–Ig immune complexes also requires both proteasomal and endosomal antigen processing (85). Taken together, these data indicate that although cross-presentation of both cell-associated antigen and Ag–Ig immune complexes require proteasomal degradation, they may need some degree of lysosomal proteolysis to facilitate translocation of antigens from lysosomes to cytoplasm. Since these sources of viral antigen do not have intrinsic fusogenic capacity, they rely on functional specializations of DC to export Ag of the endocytic compartment to the cytosol (103).

Interestingly, several lines of evidence suggest that VLP follow a different pathway for cross-presentation. Cross-presentation of PapMV VLP, HCV VLP, and HBV VLP was not affected by proteasome inhibitors but sensitive to reagents that inhibit lysosomal proteolysis (51, 96, 110). Furthermore, it was shown that cross-presentation of HBV VLP by both mouse DC (110) and human DC (our own unpublished observations) is fast and TAP-independent. Together, these studies suggest that cross-presentation of VLP occurs via the vacuolar pathway.

The differences in cross-presentation pathways between fusion-competent viruses and VLP suggest that different vesicles within the endocytic compartment are involved. Chatterjee et al. showed that antigen targeting via MR or DEC-205 both lead to cross-presentation via different compartments (82). Evidence for a process of sorting comes from an elegant study by Lakadamyali et al., where it was shown that after endocytosis, IAV is sorted into a population of dynamic endosomes that rapidly becomes more acidic, which is necessary for the virus to enter the cytoplasm (111). In contrast, an alternative non-viral ligand, transferrin is sorted into a different population of static endosomes that facilitate recycling of antigen and receptors to the cell surface.

Antigen targeting to DC-SIGN can result in trafficking to different cellular compartments, as was shown for HCV envelop protein and Lewis X uptake via DC-SIGN (112). In addition, antibody-mediated antigen targeting to the neck region of DC-SIGN was dramatically more efficient with regard to cross-presentation of the targeted antigen compared to targeting to the carbohydrate-binding domain, and these differences were related to different endocytic trafficking (93). Taken together, these studies suggest that endocytic sorting is important for the fate of antigens and that sorting occurs at the receptor level. The nature of the sorting signal and the role of endocytic receptors and their adaptor molecules in this process remains to be further elucidated. However, an indication that poly-ubiquitination may be involved in sorting and antigen translocation comes from a mouse study involving the MMR (113).

We conclude that both the cytosolic and the vacuolar pathways are applicable to cross-presentation of viral antigen by human DC, depending on the type of viral antigen that is encountered by DC (**Figure 1**). The studies discussed above suggest

that VLP preferentially traffic via the vacuolar pathway for cross-presentation, whereas protein antigen, fusion-competent viral particles, cell-associated antigen, and Ig-opsonized antigen preferentially traffic via the cytosolic pathway for cross-presentation, except in pDC that may preferentially facilitate the vacuolar pathway. Since the above-mentioned studies together suggest that antigen is sorted into pathways with different efficiency of cross-presentation at the receptor level, it is of high importance to gain more knowledge on the receptors used for internalization of viral antigens and their exact role in the sorting of Ag to different pathways in order to fully understand the cross-presentation of viral antigens. Currently, besides VLP, no other viral antigens were found that utilize the vacuolar cross-presentation pathway in human mDC, thus the physiological role of this pathway remains to be further understood. However, since this pathway is highly efficient, as was demonstrated in pDC (114), further understanding of the mechanisms underlying the vacuolar pathway may be of interest for therapeutic purposes.

DC MATURATION AS A CRITICAL FACTOR FOR CTL INDUCTION

Antigen presentation in MHC class I can lead to CTL priming or tolerance, depending on the context in which DC encounter the antigen (15). Sensing of danger signals by PRR on DC (**Table 1**) induce DC maturation, a differentiation process initiated after innate immune recognition that regulates key functions involved in CTL induction, including migration, antigen presentation, co-stimulation, and production of cytokines. Co-stimulation lowers the threshold for antigen recognition by the T-cell receptor and is important for proliferation, survival, effector function, and memory formation of T cells. Changes in antigen presentation after DC maturation include upregulation of MHC class I molecules (42), enhanced proteasomal activity (115), and reduced lysosomal antigen degradation (116) due to lower expression of lysosomal proteases (107). It is well-accepted that matured human DC have an enhanced capacity to activate virus-specific CTL (25, 42, 56, 60, 117, 118). Importantly, however, the experimental stimuli used for induction of DC maturation are often not representative for the type of danger signals that are encountered by DC during viral infection *in vivo*.

Which danger signals can be naturally encountered by PRR on DC during viral infection? Viruses can display danger signals of various nature including viral nucleic acids, replication intermediates, carbohydrate structures, and proteins on the envelop, that can be sensed by PRR on DC (**Table 1**). IAV and RSV, both ssRNA viruses, induce maturation of different human DC subsets including moDC, BDCA1$^+$ mDC, and pDC (34, 39, 42, 119, 120). Also VLP have been shown to induce DC maturation (49, 50, 75), which is not dependent on TLR but may be mediated by a recently identified innate recognition mechanism (121). In addition to virus-derived danger signals, virus-induced danger signals produced by the host in response to viral infection can induce DC maturation. Examples of such virus-induced host-derived maturation signals include cytokines such as IFNα/β and TNFα secreted by virus-infected cells (122) and damage-associated molecular patterns (DAMP) released by damaged or dying cells (123). During interaction of DC with cell-associated Ag, DC can encounter both virus-derived danger signals and host-derived maturation signals (27, 124, 125) or host cell-derived DAMP, such as TLR4 ligand high-mobility group box 1 (HMGB1) (126) or CLEC9A ligand F-actin (127).

The induction of DC maturation by virus-derived and virus-induced stimuli suggests that these factors also enhance CTL priming, however, direct experimental evidence on the contribution of virus-induced DC maturation on CTL induction by human DC is limited. IAV-infection of DC is associated with strong DC maturation and efficient antigen-specific CTL proliferation (42, 117). Similarly, TLR agonist poly I:C that mimics viral double-stranded RNA (dsRNA) is a strong inducer of DC maturation and effectively enhances cross-presentation of recombinant viral antigen by several subsets of human DC (9, 56, 128, 129). Also TLR7/8 agonists have been shown to enhance DC-induced CTL expansion and effector function *in vitro* (81). In contrast, cross-presentation of cell-associated antigen was inhibited when polyI:C or IAV were present in the captured dead cells, suggesting that virus-derived danger signals may also have a detrimental effect on cross-presentation, which may be specific for cross-presentation of cell-associated antigen (130). IFNα, a widely studied representative of virus-induced signals, can exert multiple effects on human DC that promote CTL cross-priming [reviewed by Ref. (4)]. For example, moDC differentiated in the presence of IFNα, so called IFNα-DC, have superior cross-presentation capacity compared to classical moDC (52, 131). In conclusion, although it is widely accepted that virus-derived and virus-induced stimulatory signals are required for effective cross-priming of virus-specific CTL, it has been difficult to experimentally address this hypothesis in the currently used *in vitro* models. Challenges include the low precursor frequency of naïve virus-specific CD8$^+$ T cells and dissection of the separate contributions of DC maturation and antigen presentation to CTL induction.

Interference with DC maturation and thereby subverting the development of effective CTL induction is an important mechanism of immune evasion used by many viruses. Examples of viruses that interfere with DC maturation are MV (132), VV, via the production of cytokine receptor homologs (33), HSV, via destabilization of host mRNA (35, 133) and HCMV, which prevents upregulation of co-stimulatory molecules and production of cytokines (134) and induces TGFβ production by its target cells (124). Furthermore, DC isolated from patients with chronic HIV, HBV, and HCV infections showed functional impairments in the capacity to produce IL-12 or induce T-cell activation, which may be a direct effect of the virus on DC and thereby the cause of the failing adaptive immune response, but could also be the consequence of the chronic infection (135, 136).

The connection between innate immune recognition of viruses by human DC and the induction of virus-specific CTL is an important subject for further study. In addition, the PRR and pathways underlying recognition of viruses by DC and the mechanisms by which viruses circumvent these pathways needs to be further explored. Novel molecular techniques such as the ability to knock down PRR in human DC will empower this research, which is important for the development of therapeutic interventions.

DC SUBSETS INVOLVED IN CROSS-PRESENTATION OF VIRAL ANTIGEN

Before 2010, the large majority of studies on cross-presentation of viral antigen by human DC were performed with *in vitro*-generated moDC, however, more recently a number of groups have succeeded in obtaining sufficient numbers of DC from blood or other organs to assess the ability and mechanism of cross-presentation of viral antigens by different human DC subsets.

BDCA3$^+$ DC were initially recognized as a subset with superior cross-presentation capacity compared to other human DC subsets (9, 21, 89, 137). Comparison of transcriptional profiles revealed that BDCA3$^+$ DC represent the human equivalent of murine CD8α$^+$ and CD103$^+$ DC (56, 138), which have a superior intrinsic cross-presentation capacity compared to other DC subsets (139). In parallel, selective expression of CLEC9A (84), a receptor that senses dead cells (140) and facilitates cross-presentation by mouse (141) and human DC (94), suggested that human BDCA3$^+$ DC would excell in cross-presentation of dead cell material. Superior capacity to cross-present cell-associated antigen by BDCA3$^+$ DC was demonstrated by several independent studies (9, 21, 89, 102, 137), however, not observed in all studies (118). Although BDCA3$^+$ DC are highly capable of cross-presenting cell-associated antigen, cross-presentation of cell-associated antigen has also been demonstrated for BDCA1$^+$ DC (102), pDC (89, 118), and moDC (31, 57). Also for other types of antigen, cross-presentation is not restricted to the BDCA3$^+$ DC subset. Cross-presentation of protein antigen was shown for peripheral blood and tissue-derived BDCA1$^+$ DC (9, 128), BDCA-2$^+$ pDC (102, 128), and BDCA3$^+$ DC (9, 56, 102, 128, 137), as well as for *in vitro*-generated CD34$^+$-derived DC (102) and moDC, as discussed above. Although BDCA3$^+$ DC are highly capable of cross-presenting cell-associated antigen, cross-presentation of cell-associated antigen has also been demonstrated for BDCA1$^+$ DC (102), pDC (89, 118), and moDC (54).

Both BDCA3$^+$ and BDCA1$^+$ DC share the specialized machinery that is associated with efficient cross-presentation capacity, i.e., high phagosomal pH, production of ROS within endocytic compartments, and efficient transfer of exogenous antigens into the cytosol (102). Both subsets have a similar efficiency of endogenous MHC class I presentation after transfection, a similar efficiency of cross-presentation of heat-inactivated IAV that can egress to the cytosol at low pH and a similar efficiency of cross-presentation of antigen that is selectively delivered to early endosomes (107). Nevertheless, BDCA3$^+$ DC were superior compared to BDCA1$^+$ DC at cross-presentation of antigen that was artificially targeted to lysosomes by using antigen conjugated to DEC-205 targeting antibodies (107). This suggests that although both DC subsets can efficiently cross-present Ag delivered to early endosomes, BDCA3$^+$ DC may exhibit a specialized machinery to transfer Ag from late endosomes and lysosomes to the cytosol. This DC characteristic might explain the superior capacity to cross-present IgG-opsonized antigen targeted to FcγR that could not be attributed to superior FcγR expression and/or antigen uptake in these cells (85).

Plasmacytoid DC contribute to anti-viral immune responses by producing large amounts of IFNα/β, however, their role as professional antigen presenting cell in the initiation of virus-specific T-cell responses was initially questioned based on controversial results in mice (86). Direct comparison of intrinsic characteristics that can influence cross-presenting capacity, such as phagosomal pH and ROS production, between pDC and BDCA1$^+$ and BDCA3$^+$ mDC was hampered due to inconclusive data for pDC (102). However, pDC express a broad repertoire of antigen-uptake receptors on their cell surface such as FcR and CLR BDCA-2, DEC-205, DCIR that can facilitate the uptake and cross-presentation of viral antigens (116) (**Table 1**). In addition, pDC can efficiently transfer exogenous Ag into the cytosol suggesting that they may be capable of cross-presenting antigen via the cytosolic pathway (102). Numerous functional studies showed that human pDC can cross-present recombinant protein antigens, long peptide antigens, IAV-derived antigens, and cell-associated antigens (88, 118, 119, 142). In addition, it was also demonstrated that pDC can efficiently cross-present viral antigen via the vacuolar pathway, which may be facilitated by MHC class I storage in recycling endosomes (114). Taken together, we conclude that human pDC can efficiently facilitate cross-presentation of a wide range of viral antigens. Direct comparison of cross-presentation efficiency between human pDC and mDC was thus far inconclusive, with one study showing a higher efficiency of cross-presentation by pDC (114), another study showing superior MHC class I-restricted IAV presentation by BDCA1$^+$ mDC (40) and three studies concluding that pDC and BDCA1$^+$ or BDCA3$^+$ mDC have similar cross-presentation efficiencies (118, 119, 142).

Although blood DC required DC maturation for efficient cross-presentation, skin or lymph node DC can cross-present under steady state conditions, which might be due to a more mature/activated status of these tissue DC compared to circulating DC (56, 102, 143). In addition to BDCA1$^+$ and BDCA3$^+$ DC, skin contains Langerin$^+$ LC and dermal intDC, often referred to as CD14$^+$ DC. Comparison of CD14$^+$ DC to other skin DC subsets indicated that this subset showed the least cross-presenting capacity among skin subsets (10, 56), which may be related to the finding that these cells express immunoglobulin-like transcript receptors that antagonize CTL development (144). Cross-presentation capacity of LC cells is under debate and may vary upon the source of LC and type of antigen used in experiments. Cross-presentation of recombinant protein antigen by *in vitro*-generated LC has been demonstrated in several independent studies (10, 102, 145), however, cross-presentation of replication-incompetent MV and MV-infected cells by skin-derived LC was absent (25). Sine LC are potentially interesting vaccine target cells, because of their presence at mucosal sites such as skin and higher respiratory tract (25), further studies on the cross-presentation capacity of primary LC are required.

We conclude that essential mechanisms of cross-presentation are present among most human DC subsets, with the exception of CD14$^+$ DC. Superiority of cross-presentation among DC subsets can be attributed to the repertoire of uptake receptors and adaptations in the endocytic compartment and may vary depending on the type of antigen.

TECHNICAL LIMITATIONS AND NOVEL APPROACHES

Although several technical advancements have potentiated the study of MHC class I-antigen presentation by human DC, several important questions remain to be addressed.

One of the current technical challenges is to measure antigen presentation at the level of DC. The purest read-out would be to measure MHC class I-antigen complexes at the surface of DC (signal 1 only), however, tools are lacking (20). The best current available method to quantify MHC class I-antigen presentation is a read-out involving activation or *in vitro* induction of virus-specific T cells. However, it should be taken into account that activation of virus-specific T cells results from a combination of TCR ligation by MHC class I–peptide complexes (signal 1) and other stimuli provided by DC such as cytokines and co-stimulation (signal 2 and 3).

The study of induction of human $CD8^+$ T cells by DC is also hampered by the extreme low frequency of naïve virus-specific T cells in peripheral blood. As discussed above, MHC class I presentation by human DC has been most frequently studied for IAV, HIV-1, and CMV. For these viruses, it has been possible to obtain sufficient numbers of "memory" T cells from peripheral blood and use T-cell expansion and IFNγ production as read-outs for antigen presentation in an autologous setting (13, 14, 54). Virus-specific T-cell clones to other viruses can be obtained by several rounds of antigen-specific expansion *in vitro*. However, performance of such *in vitro*-generated clones in cross-presentation studies is complicated due to their limited life span and the allogenic bias present in experiments because DC and T cells are not from the same donor. A novel promising approach for the study of cross-presentation of viruses by human DC is the use of T-cell receptor transfer to generate autologous virus-specific T cells (146, 147). Such T cells are evaluated in the context of immunotherapy of patients but may also be exploited as tools to monitor antigen presentation by DC.

RECOMMENDATIONS AND CONSIDERATIONS FOR DEVELOPMENT OF THERAPEUTIC VACCINE STRATEGIES

Chronic viral infections such as HIV, HBV, and HCV are a big health burden and affect 100 millions of patients worldwide. Viral persistence is associated with a failure of the patient's immune response to eradicate the virus (136). In addition to chronic persistent infections, reactivation of latent infections including HCMV, EBV, and HPV is a major threat for immune compromised patients. In addition, a high proportion of these chronic and latent infections including HIV, HBV, HCV, EBV, HPV, and HTLV is related to the development of malignancies later in life (148). Immunotherapy represents an attractive therapeutic intervention to combat such infections and prevent virus-related malignancies by using the body's own defense mechanisms. To accomplish this, immunotherapy is directed to improve virus-specific immunity and eradicate the virus but also generate protective memory responses to prevent re-infections. Moreover, immunotherapy should overcome T-cell exhaustion and anergy, often observed in patients with chronic infections (148).

Insights into the mechanisms underlying effective priming of virus-specific CTL by human DC are instrumental for the development of effective virus-specific immunotherapy. We identified cross-presentation as a crucial mechanism for the induction of virus-specific CTL and embrace the concept to utilize the effective cross-presentation mechanisms naturally present in DC for immunotherapy. In line with this concept, antibody-mediated antigen targeting to endocytic receptors is an emerging approach employed by numerous groups to target antigen to DC for cross-presentation. Endocytic receptors that efficiently facilitate cross-presentation by human DC include FcγRIIA, CLEC9A, DEC-205, and DCIR (81, 85, 94, 116, 149). An advantage of antigen targeting to specific receptors is the possibility to select receptors that are uniquely expressed by distinct subsets of DC (**Table 1**), such as proposed for XCR1 (150) or CLEC9A (94). Selective targeting to DC prevents antigen consumption by irrelevant cells, which may lead to reduced availability of antigen to DC and improper T-cell activation.

As discussed previously, DC maturation is crucial for virus-specific CTL induction. Although the endocytic receptors are very potent in internalizing antigen, their role in promoting DC maturation is less clear. Therefore, the combination of antigen targeting with adjuvants is an important field of study. FcγR have been shown to facilitate both efficient antigen uptake and DC maturation, however, it was recently shown that FcγR-dependent DC maturation in human DC is less strong than was previously observed in mice DC (85, 151). Other interesting approaches that combine antigen targeting to DC and DC maturation in one cargo include TLR-ligand–peptide conjugates (152) and nanoparticles that contain both antigen and adjuvant (116).

Since DC comprise a heterogeneous family of subsets that differ in location, frequency, receptor expression, and functional specializations, it is important to design a therapeutic vaccine with the desired DC subset in mind. Based on accumulated evidence from *in vitro* studies on antigen presentation by human DC subsets, we conclude that most human DC subsets have the basic capacity to cross-present, as long as the antigen is efficiently targeted to an endocytic compartment that favors cross-presentation. Nevertheless, DC subsets do have unique functional characteristics, such as type of cytokine production, which can have high impact on the type of immune response induced. Moreover, DC subsets express different PRR (**Table 1**) and only adjuvants for a selected number of TLRs are currently available at clinical grade.

In addition to antigen targeting to DC *in vivo*, recruiting of DC precursors may represent an attractive immunotherapeutic approach, as was recently proposed for monocytes, which can contain a natural reservoir of HBsAg that can be presented in MHC class I upon differentiation of these monocytes to moDC (153).

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Based on two decades of research into MHC class I-restricted presentation of viral antigen by human DC, we conclude that cross-presentation of viral antigens is a highly efficient mechanism for defense against viruses. Furthermore, cross-presentation of viral antigens seems not only pivotal for defense against viruses that do not infect DC, but also for those that infect DC, as demonstrated by *in vitro* studies using replication-incompetent IAV, HIV-1, and MV. Since these viruses represent a selection of all viruses that can productively infect human DC, the contribution of direct presentation by human DC infected with other viruses cannot be completely ruled out. Nevertheless, as discussed in this review, cross-presentation has many conceptual advances compared to direct presentation by infected DC.

So far, knowledge on the presentation of viral antigens by human DC is mainly derived from *in vitro* studies. Whether these studies faithfully represent the *in vivo* situation is of course difficult

to predict. Several caveats from these *in vitro* studies include the use of *in vitro*-generated DC, which may behave differently than their *in vivo* counterparts, the use of laboratory adapted virus strains, and pseudo-typed viruses, which may have tropisms that may not represent the *in vivo* situation, and the use of recombinant viral proteins and TLR ligands that are not fully representative for antigens or danger signals that can be encountered *in vivo*. Nevertheless, taking these limitations into account, together these studies have given us an important understanding of the mechanisms underlying MHC class I presentation of viral antigens by human DC. This knowledge is an important basis for the rational design of therapeutic vaccines for chronic viral infections.

Interesting venues for further research include identification of DC receptors involved in viral infection and initiation of immune response, elucidation of the molecular signals underlying sorting of viral antigen to endocytic compartments that favor cross-presentation and the role of virus-derived danger signals and virus-induced maturation stimuli in cross-presentation and CTL priming.

A more detailed knowledge of these key factors in virus–host interaction will further empower the design of novel therapeutics for infectious diseases.

ACKNOWLEDGMENTS

This study was supported by a VIDI grant (project 91712329) from the Netherlands Organisation for Scientific Research (NWO) to Andrea M. Woltman.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 31 January 2014; paper pending published: 03 March 2014; accepted: 07 April 2014; published online: 23 April 2014.
*Citation: van Montfoort N, van der Aa E and Woltman AM (2014) Understanding MHC class I presentation of viral antigens by human dendritic cells as a basis for rational design of therapeutic vaccines. Front. Immunol. **5**:182. doi: 10.3389/fimmu.2014.00182*
This article was submitted to Antigen Presenting Cell Biology, a section of the journal Frontiers in Immunology.

frontiers in
IMMUNOLOGY

REVIEW ARTICLE
published: 30 May 2014
doi: 10.3389/fimmu.2014.00255

Dendritic cell-targeted vaccines

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Despite significant effort, the development of effective vaccines inducing strong and durable T-cell responses against intracellular pathogens and cancer cells has remained a challenge. The initiation of effector $CD8^+$ T-cell responses requires the presentation of peptides derived from internalized antigen on class I major histocompatibility complex molecules by dendritic cells (DCs) in a process called cross-presentation. A current strategy to enhance the effectiveness of vaccination is to deliver antigens directly to DCs. This is done via selective targeting of antigen using monoclonal antibodies directed against endocytic receptors on the surface of the DCs. In this review, we will discuss considerations relevant to the design of such vaccines: the existence of DC subsets with specialized functions, the impact of the antigen intracellular trafficking on cross-presentation, and the influence of maturation signals received by DCs on the outcome of the immune response.

Keywords: dendritic cells, MHC class I, $CD8^+$ T cells, vaccination, adjuvants, immunologic

INTRODUCTION

Vaccination is the most effective way to prevent the spread of infectious diseases. We classify vaccines into two main types: preventative or therapeutic. Preventative vaccines typically elicit generation of specific antibodies and memory B cells. They are designed to block the spread of infection through these humoral immune responses (1). Alternatively, therapeutic vaccines are designed as a treatment to eradicate the cause of disease. Therapeutic vaccines are typically intended to activate or induce cytotoxic antigen-specific $CD8^+$ T cells to eliminate virally infected cells or cancer cells. There are many conditions for which vaccination has diminished the devastating effects of disease, and the discovery of these vaccines has largely resulted from successful trial and error. However, there are many diseases for which no vaccine exists; e.g., human immunodeficiency virus, hepatitis C, malaria, and cancer. It is likely that cytotoxic $CD8^+$ T-cell activity will be required to protect patients from these chronic conditions. For this reason, efforts are required to develop carefully designed therapeutic vaccines that will derive from our increasing understanding behind the mechanisms of the human immune system. Dendritic cells (DCs) are the antigen-presenting cells that initiate and direct adaptive immune responses, and thus are critically important in our consideration of vaccines designed to induce cellular immunity.

DCs induce and regulate immunity against pathogens, and tolerance against self-antigens and commensal microorganisms (2–4). In their immature state, DCs reside in the periphery where they are situated to recognize and capture antigens. Upon receiving an activating stimulus, DCs migrate to lymphoid organs whereby they present processed peptides derived from captured antigens to T cells in the context of major histocompatibility complex (MHC) class I or II (5). The immune response initiated by the DCs is dependent upon the context in which the antigen was captured. DCs induce tolerance under steady-state conditions, in the absence of infection or inflammation – generally in this case it is self-antigens that are processed and presented. The exact nature and state of tolerogenic DCs remain elusive. However, there is an increasing body of evidence suggesting that microenvironmental signals condition DCs to become tolerogenic (6). In this process, beta-catenin activation appears to play a central role (7–10), although other mechanisms also contribute to tolerance induction (8). In the presence of inflammatory signals, such as microbial products, proinflammatory cytokines, and other endogenous signals, DCs undergo a process called maturation. DC maturation is associated with dramatic functional and morphological changes that lead to an optimized ability to initiate T-cell immunity. It is characterized by an increase in cell surface expression of MHCI and MHCII molecules and accessory/costimulatory molecules, increased antigen processing, and induction of specific cytokine production (5). Maturation depends on both the nature of the stimuli and its extent and combination (11). Additionally, the DC compartment is diverse and contains different cell types with both conserved and unique functions and specialties. Indeed, different DC subsets possess different capacity for antigen presentation, cytokine production, and microbial sensing (12). Thus, it seems that different types of immune responses are initiated by specialized DC subsets.

The critical role of DCs to activate $CD8^+$ T cells makes them an attractive target for vaccination against intracellular pathogens and diseases for which cellular immunity seems to be a crucial part of the immune response. One approach is cell-based immunotherapy with *ex vivo* generated DCs loaded with antigens (13). This approach however is laborious and expensive, and thus far clinical results have been limited. Another more promising approach to direct DCs involves selective targeting to DC-specific endocytic receptors by monoclonal antibody coupled or fused to a desired antigen. These complexes are internalized by the DCs, trafficked through the intracellular vesicular system, processed, and the antigenic peptides are loaded onto MHC and presented to T cells (14, 15). In mice, in the presence of adjuvant, these antigen–antibody conjugates induce robust immune responses (16). However, in the

absence of adjuvant, these conjugates can promote a tolerogenic state (17). This *in situ* targeting strategy is in its infancy in human patients. The first clinical trials to evaluate this vaccine approach are in progress and their preliminary results are encouraging (18–20). Recent progress in understanding the biology of DCs should further help with optimization of a DC-targeted vaccine strategy: (1) identification of the human DC subsets with superior capacity at initiating $CD8^+$ T-cell responses if any, (2) selection of the receptors based on expression pattern to target the desired DC subset(s), and also their ability to deliver antigen to intracellular compartments for processing and loading on MHC and (3) choice of the adjuvant(s) to induce the desired immune response. In this review, we will discuss the issues relevant to human vaccination through *in vivo* DC targeting: the existence of multiple DC subsets with specialized functions, how DCs handle external antigen for presentation on MHCI and the intracellular targeting that induces optimal immune responses, and finally the role of DC maturation signals in orchestrating the immune outcome.

DENDRITIC CELL SUBSETS

Increasingly it has become apparent that there exists a division of labor among DC subsets in both mice and in humans (12, 21, 22). The number of DC subsets identified, and the functional studies performed both *in vivo* in mice and *in vitro* using isolated DC subsets from humans yield evidence for specialization in T-cell priming and induction of immune responses, although the functions of the different DC subsets can partially overlap.

While the mouse DC network has been quite well characterized, until recently thorough studies with human blood DCs have been difficult due to their paucity in the blood and the difficulty to access human tissues. However recent genome-wide expression profiling studies helped identify the potential human counterparts to the mouse DC subsets (23, 24).

Human and mouse DCs can be divided in two main subsets: plasmacytoid DCs (pDCs) and conventional/myeloid DCs (mDCs) (**Figure 1**). pDCs play a crucial role against viral infection by producing vast amounts of type I interferon in response toll-like receptors (TLR) 7 and 9 and intracellular sensor triggering (25). pDCs have been shown to be rather poor at antigen presentation in comparison to mDCs (26–28), although recent studies suggest that efficient antigen delivery to pDCs via endocytic receptors can lead to robust presentation on both MHCI and MHCII (29–31). However, the influence of antigen presentation by pDCs *in vivo* has yet to be understood. Additionally, in mice there is evidence that suggest pDCs play a major role in the generation of tolerance (32, 33). Whether this is true for human pDCs is still unknown.

Human mDCs can be divided into two main subsets based on the surface markers BDCA1/CD1c or BDCA3/CD141. A transcriptional comparison of mDCs has shown genetic similarity between human $BDCA1^+$ DCs and $BDCA3^+$ DCs from various tissues to murine $CD11b^+$ and $CD11b^-$ DCs, respectively (23, 34–36). Human $BDCA3^+$ DCs express a number of markers unique to mouse $CD11b^-$ $CD8\alpha^+$ and $CD11b^-$ $CD103^+$ DCs including the lectin receptor Clec9A/DNGR1, the chemokine receptor XCR1, and Necl2 (37–39). Further, human $BDCA3^+$ DCs and mouse $CD11b^-$ $CD8\alpha^+$ DCs share the expression of the transcription factors IRF8, BATF3 essential for their development (35, 40–43). Conversely, the transcriptional programing of mouse $CD11b^+$ $CD8\alpha^-$ DCs and human $BDCA1^+$ is dependent on IRF4 (44, 45). Functional studies of the mouse and human mDCs revealed differences between the two species, however. A clear division of labor exists among the two mDC subsets in mice with $CD11b^-$ $CD8\alpha^+$ DCs and $CD11b^-$ $CD103^+$ DCs being far superior and essential at priming $CD8^+$ T-cell responses, while $CD11b^+$ $CD8\alpha^-$ DCs are specialized for presenting antigen on MHCII to stimulate helper T-cell immunity (12, 46, 47). This division of labor does not appear as clear between $BDCA3^+$ DCs and $BDCA1^+$ DCs at least in *in vitro* studies. Indeed both $BDCA1^+$ DCs and $BDCA3^+$ DCs can effectively cross-present antigens on MHCI (28, 31, 37, 38, 40, 41, 48–52). In addition, $BDCA1^+$ DCs also produce high levels of IL-12 upon stimulation, a cytokine essential to inducing Th1 response and cross-priming of $CD8^+$ T cells (28, 44, 48, 53, 54). $BDCA3^+$ DCs and $BDCA1^+$ DCs also exhibit a comparable capacity to present antigen on MHCII (28, 31). The skin contains two additional DC subsets that have been functionally characterized, the Langerhans cells (LCs) and the $CD14^+$ DCs (36, 55). $CD14^+$ DCs appear specialized in initiating humoral immune responses, while *in vitro*-derived LCs cross-present antigen on MHCI and prime $CD8^+$ T cells of higher avidity as compared to $CD14^+$ dermal DCs *in vitro* (26, 55). A side-by-side comparison of *in vitro*-derived LCs with $CD14^+$ DCs suggests the two DC subset have similar capacity for cross-presentation (36). Importantly, LCs isolated from skin are incapable of cross-presentation of captured antigen, while they can present antigen on MHCII to $CD4^+$ T cells (36, 56). Whether this deficiency is the result of the isolation procedure or a true characteristic of LCs remains to be confirmed.

Finally, the human equivalent of mouse inflammatory DCs was recently identified (57, 58). This DC subset is found in inflammatory microenvironments and can be divided into two main populations: $CD16^+$ $BDCA1^+$ DCs or $CD16^-$ $BDCA1^+$ DCs. They have characteristic gene patterns similar to that of DCs and macrophages, and thus are likely derived from monocytes. Although there are limited data on the functional specialization of human inflammatory DCs, they appear highly plastic like their murine counterparts (57, 58).

One limitation of the studies aimed at characterizing the functional capacity of human DCs is that they are performed *in vitro* using T-cell lines or memory T cells. These assays permit to evaluate the DCs' capacity for antigen presentation. However, other factors are also important for DC function *in vivo* and priming of immune responses. The enhanced capacity of LCs to prime $CD8^+$ T-cell responses may at least partially result from their ability to express IL-15 upon maturation (59, 60). The costimulatory molecule CD70 also promotes the priming of $CD8^+$ T-cell responses and the generation of $CD8^+$ T-cell memory (61–63). CD70 has been found to be expressed on LCs and all three blood DCs subsets upon maturation [(64, 65); Delamarre, personal communication]. Finally, DC function may depend on environmental cues, resident $BDCA3^+$ DCs constitutively produce IL-10, possibly in a vitamin D3-dependent manner, and thus mediate T-cell tolerance rather than immunity at steady-state (66). Granulocyte–macrophage colony stimulating factor (GMCSF) has

A HUMAN

	blood DC subsets			skin DC subsets		non lymphoid tissue
						
	BDCA1	**BDCA3**	**BDCA2/PDC**	**LC**	**CD14**	**Inflammatory DC**
Pattern Recognition Receptors	TLR 1-6,8, 10, MDA5, RIG-I	TLR 1-3, 6, 8, 10, MDA5, RIG-I	TLR 1, 2,6, 7, 9, 10, MDA5, RIG-I	TLR 1, 3, 5, 6, 7, 9, 10	TLR 1-10	TLR 1,2,4, 9
MHCI presentation	++	++	+/-	+(+)	+/-	+/-
MHCII presentation	++	++	+/-	++	Follicular CD4+	++

B MOUSE

	splenic DC subsets		
			
	CD11b+ CD8α-	**CD11b- CD8α+**	**PDC**
Pattern Recognition Receptors	TLR 1-9, MDA5, RIG-I	TLR 1-4,6,8, 9, 12,13 MDA5, RIG-I	TLR 1, 2,4-9,12, MDA5, RIG-I
MHCI presentation	+/-	++	+/-
MHCII presentation	++	+/-	+/-

FIGURE 1 | (A) Human dendritic cell subsets have overlapping functions and phenotypes, but also show some degree of specialization. BDCA1$^+$ DCs and BDCA3$^+$ DCs both efficiently present antigen on MHCI and MHCII. pDCs can present antigen to CD4$^+$ and CD8$^+$ T cells, but likely their primary role in the immune response is the production of type I interferon during viral infection. LCs seem to be specialized for cross-presentation on MHCI, while CD14$^+$ dermal DCs prime naïve CD4$^+$ T cells to generate follicular helper T cells. Inflammatory DCs are monocyte-derived, and are present at sites of inflammation. There is also partial overlap between expression of PRRs among DC subsets. **(B)** A clear division of labor exists among mouse splenic dendritic cell subsets. CD11b$^-$ CD8α$^+$ DCs are far superior and essential at priming CD8$^+$ T-cell responses, while CD11b$^+$ DCs are specialized for presenting antigen on MHCII to stimulate helper T-cell immunity. pDCs can present antigen to CD4$^+$ and CD8$^+$ T cells, but likely their primary role in the immune response is the production of type I interferon during viral infection like their human counterparts. There is overlap between expression of PRRs among DC subsets, although CD11b$^-$ CD8α$^+$ DCs express much higher levels of TLR3 while CD11b$^+$ DCs uniquely express TLR5 and TLR7 (30, 35, 41, 58, 64, 147–151).

recently been shown to enhance the cross-presentation capacity of mouse CD11b$^-$ CD8α$^+$ DCs (67, 68).

Based on our current knowledge, there is no strong rational for the targeting of one DC subset over another to prime CD8$^+$ T-cell responses in humans. Further *in vivo* studies are needed to identify the DC subsets if any that are specialized in cross-priming of CD8$^+$ T cells. In this effort, it would be useful to better characterize DC subsets in non-human primates which appear to possess subpopulations of DCs that are similar to those present in humans (69) and therefore would be a more relevant model to humans than mice. Additionally, engagement of multiple DC subsets has been suggested to be important in generating a broad and potent T-cell response (70). For this reason, it may make sense to target a broad spectrum of DC subsets rather than a single DC subset.

ANTIGEN CROSS-PRESENTATION PATHWAYS

In the design of rational DC-targeted vaccines, there are important considerations related to the delivery of antigen to DCs and the downstream processing of antigen by DCs. Delivery of antigen to DCs is essential to generate strong and prolonged T-cell responses. DCs are able to non-specifically phagocytose and macropinocytose pathogen-associated antigen and can also uptake antigen more specifically via lectin receptors, Fcγ receptors, and scavenger receptors (5). It has been shown that antigens can be efficiently targeted to DCs using antibodies against these endocytic receptors (15, 71). This takes advantage of antibodies against DC-specific endocytic receptors either coupled or fused to antigen or attached to nanoparticles containing antigen. In mice, this delivery method is hundreds of times more efficient and potent

than untargeted antigens and offers options for antigen presentation on both MHCI and MHCII to $CD8^+$ and $CD4^+$ T cells, respectively (72). In addition, this strategy can also extend antigen cross-presentation to pDCs, which display poor phagocytosis and macropinocytosis capacity, and thus could potentially further promote T-cell responses *in vivo* (28–31). Another benefit of employing this strategy for antigen delivery is that it can allow for delivery to both immature and mature DCs. Unlike the non-specific phagocytosis and macropinocytosis, endocytic receptor-driven uptake continues even after DC maturation (73, 74). It would be best to selectively target DCs to reduce the dose of antigen required, while additionally limiting cross-presentation by other cell types. Indeed B cells and other non-hematopoietic cells can cross-present exogenous antigens, albeit with less efficiency than DCs, and induce peripheral tolerance under steady-state conditions and could potentially negatively impact vaccination efficacy (75–78). In addition, the binding of a target receptor by non-DCs may trigger a signaling pathway and thus may potentially have unwanted side effects.

DC subsets express different pattern of endocytic receptors and therefore the choice of receptor will determine which DC subsets are delivered antigen (**Table 1**). The choice of receptor also matters for other reasons. Some receptors can trigger DC maturation and induce immune responses of various natures as further discussed in the next section. In addition, they determine antigen intracellular trafficking that impacts antigen fate (28, 79). Some antibodies may also differentially alter antigen cross-presentation by modulating receptor trafficking (80). Antigen processing and loading on MHCI and MHCII happens in distinct intracellular compartments. For presentation on MHCII, antigen processing and loading occurs in the endosomal compartments, and peptide–MHCII complexes are transported to the plasma membrane (5).

Two main intracellular pathways for the cross-presentation of exogenous antigen on MHCI have been reported. They are referred to as the "cytosolic" and "vacuolar" pathways (**Figure 2**) (81, 82).

From extensive work with human and mouse DCs, the "cytosolic pathway" appears the most predominant pathway. It is proteasome-dependent, and therefore requires that internalized proteins escape the intracellular trafficking pathway and access the cytosol, where they are processed by the proteasome and transported into the ER and possibly in endocytic compartments by TAP1/2 transporters for loading onto MHCI (83–85). The molecular mechanism underlying transport of antigen from endocytic compartments to cytosol remains largely unknown. No specific

Table 1 | Expression, intracellular localization, and ability to deliver antigen to MHCI and MHCII pathways of selected endocytic receptors and antigen.

Receptors	Expression by DCs	Expression by other cells	Intracellular routing	DC activation	MHCI cross-presentation	MHCII presentation
CD11c	$BDCA1^+$, $BDCA3^+$, $CD14^+$, LC, inflam. DC	Mono/MØ, neutrophil	Early endosome	No	+++ (Peptide)	?
CD32	$BDCA1^+$, $BDCA3^+$, $CD14^+$, LC, inflam. DC, pDC	B, mono/MØ, NK, endothelial, neutrophil	Lysosome	Yes	+++ (Protein)	+++ (Protein)
CD40	$BDCA1^+$, $BDCA3^+$, $CD14^+$, LC, inflam. DC, pDC	B, mono/MØ, endothelial	Early endosome	Yes	+++ (Peptide) +++ (Protein)	+++ (Peptide) +++ (Protein)
CD205	$BDCA1^+$, $BDCA3^+$, $CD14^+$, LC, inflam. DC, pDC	B, mono/MØ, T, endothelial	Lysosome	No	± (Peptide) +++ (Protein)	± (Peptide) +++ (Protein)
CD206	$BDCA1^+$, $CD14^+$, inflam. DC	Mono/MØ, epithelial	Early endosome	No	+ (Peptide) +++ (Protein)	+++ (Protein)
CD207	LC	–	Birbeck granules	No	– (Virus)	+++ (Protein) +++ (Virus)
CD209	$CD14^+$, inflam. DC, pDC	Mono/MØ	Early endosome/lysosome	No	+++ (Protein)	+++ (Protein)
DNGR1	$BDCA3^+$	–	Early endosome	No	+++ (Peptide) +++ (Protein)	+++ (Protein)
Dectin-1	$BDCA1^+$, $CD14^+$	Mono/MØ	?	Yes	+++ (Protein)	?
DCIR	$BDCA1^+$, LC, $CD14^+$, pDC	B, mono/MØ	Early endosome/lysosome	No/suppressive?	+++ (Protein)	?

Receptor selection for targeting DCs depends on four criteria: (1) whether the receptor is widely expressed among DC subsets, (2) whether other subsets of cells express the receptor, (3) upon internalization, where the receptor is trafficked, and finally (4) whether binding of this receptor activates DCs (28, 79, 80, 103, 105, 148, 149, 152–154). ?, not tested.

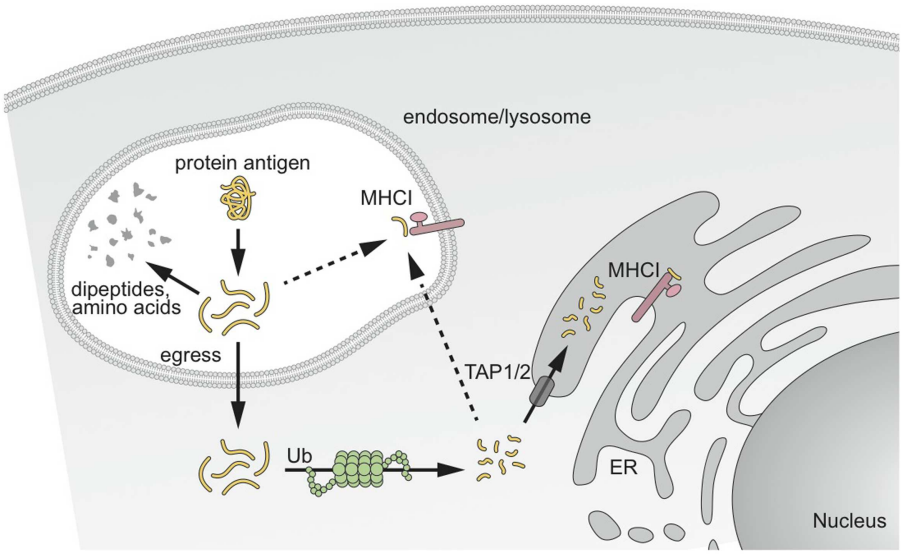

FIGURE 2 | MHCI cross-presentation pathways of captured antigens. Antigen captured by DCs has different potential fates. Antigens destined for cross-presentation on MHCI have two different intracellular routes. Antigen can be transported from the endocytic vesicles to the cytosol to access the classical MHCI pathway involving proteasomal degradation and transport into the ER or back into the endosomal compartment for loading onto MHCI. The second pathway results in degradation and loading directly in endosomal compartments before peptide–MHCI complexes are transported to the plasma membrane. Modified from Delamarre and Mellman (14).

transporter has been identified yet, despite substantial efforts from different laboratories. A role of the ER-associated degradation (ERAD) machinery has been suggested in antigen export to the cytosol (86, 87). Consistent with this finding, the recruitment of ER-resident proteins to the phagosomes, via the ER molecule Sec22b, is required for cross-presentation (88). Regardless of the exact mechanism, antigen transfer to the cytosol is rate-limiting to antigen access to the MHCI pathway. When the antigen actively gains access to the cytosol using listeriolysin O or a fusogenic virus, cross-presentation is 10-fold more efficient (28). ISCOMATRIX adjuvant, a saponin-based adjuvant, which disrupts lysosomal membranes and facilitates antigen translocation to the cytosol also enhances antigen cross-presentation (89).

The "vacuolar pathway" is dependent upon lysosomal proteolysis by cathepsins and IRAP (90, 91) and independent of the proteasome and TAP1/2 transporters. Exogenous antigens are degraded directly in endocytic compartments by lysosomal proteases and trimmed for loading onto MHCI.

The reason why certain antigens are cross-presented by one pathway rather than the other is unknown. The nature and the form of the antigen, and the ability of the proteolytic environment to generate MHCI epitopes are certainly contributing factors (90). Maybe counter intuitively, antigen intracellular targeting does not appear to influence the intracellular-processing pathway for cross-presentation in human blood DCs as cross-presentation of antigen required proteasomal processing independently of its intracellular targeting (79).

A feature essential to the ability of DCs to efficiently present antigens on MHCI and MHCII is their reduced ability for endosomal degradation. Although proteolysis is essential to the generation of MHC peptides, too much proteolytic activity leads to complete protein degradation into amino acids. Indeed, DCs are distinguished from other phagocytic cells (e.g., macrophages) by a remarkably low expression level of lysosomal proteases and a high lysosomal pH (92–94). The antigen susceptibility to degradation even by these reduced levels of proteases is a determinant factor to the efficiency at which MHCII–peptide complexes can be generated (95). Studies performed with murine DCs suggest that the MHCI pathway may be even more sensitive to lysosomal degradation. Indeed, inhibition of lysosomal proteases promotes antigen cross-presentation (96, 97). Murine CD11b$^-$ CD8α$^+$ DCs, which exhibit an increased ability for cross-presentation in comparison to the CD11b$^+$ CD8α$^-$ DCs, also generate high levels of reactive oxygen species in a NOX-2-dependent fashion so that their endocytic compartments stay at a more alkaline pH, thereby limiting antigen destruction (98). In addition, this phenomenon may also act to weaken or disrupt the vesicular membrane (99). As a result, antigen transport in the cytosol is increased. In addition, CD11b$^-$ CD8α$^+$ DCs also have higher levels of lysosomal inhibitors and lower levels of lysosomal proteases than CD11b$^+$ CD8α$^-$ DCs (46, 100). The constitutive activation of IRE-1α, a sensor of ER stress, is also a unique feature of CD11b$^-$ CD8α$^+$ DCs and appears essential to antigen cross-presentation (101). The precise mechanism by which activated IRE-1α promotes the MHCI cross-presentation pathway remains to be elucidated. At least, some of the features of the murine CD11b$^-$ CD8α$^+$ DCs are shared by human tonsil resident BDCA3$^+$ DCs but also BDCA1$^+$ DCs, both of which display similar cross-presentation capacity (51). Additionally, the three DC subsets efficiently export internalized proteins to the cytosol. However, another study found that blood BDCA3$^+$ DCs superior at cross-presenting antigen delivered to lysosomes (28). Furthermore, blood BDCA3$^+$ DCs express

lower levels of lysosomal proteases than BDCA1$^+$ DCs, suggesting that perhaps enhanced antigen release into the cytosol is favored by reduced lysosomal degradation. The lysosomal pH of blood DCs was not measured, and in the aforementioned study intracellular targeting of the antigen was not characterized. Further analysis will be needed to determine if different BDCA3$^+$ DC subsets display different properties.

Finally, recent studies from our group and others suggest that both early and late endosomal compartments are capable of serving as antigen portals for cytosolic entry and cross-presentation. However, early endosomal compartments appear to be far more efficient for some antigens. This is not dependent on internalization levels, but rather the low proteolytic activity of early endosomes (28, 79, 80, 97, 102). Surprisingly, there does not seem to be a direct correlation between the level of internalization and cross-presentation. CD40 and mannose receptor/CD206 both deliver antigen to early endosomes, but CD40, the receptor that is the least efficiently internalized, turns out to be the most efficient at promoting cross-presentation (79). Slow antigen internalization might preserve antigen and provide a continuous "time-release" pool of antigen that might be used over extended periods for the continuous formation of peptide–MHCI complexes. The importance of targeting antigen to compartments with low proteolytic activity most likely depends on the nature antigen and its stability. Chatterjee et al. used long peptides as antigen which are particularly susceptible to degradation and probably have reduced ability to survive long enough to escape into the cytosol. Protein antigens, however, may be inherently more resistant. This could explain why in some systems antigen delivered to lysosomes using DEC205 or FcγR, are efficiently cross-presented, with similar or better efficacy as antigen delivered to early endosomes via mannose receptor/CD206 (103–105).

Collectively, the data reviewed in this section indicate that targeting receptors for antigen delivery to DCs can promote CD8$^+$ T-cell responses by increasing the amount of antigen delivered to the desired DC subset(s). It can also enhance antigen presentation by controlling its intracellular routing and degradation, and extend antigen cross-presentation to DCs that might not be optimally equipped.

ADJUVANT

In absence of stimulation at steady-state DCs can induce tolerance. Antigen inoculation in absence of adjuvant leads to T-cell anergy or T-cell deletion (17, 72), and can induce regulatory T cells in the periphery (106–109). Hence, *in vivo* delivery of antigens to DCs in absence of adjuvant may also be a promising strategy to treat autoimmune disorders as reviewed elsewhere (110). But, to induce immunity rather than tolerance, it is essential to provide the DCs with an activation signal or "adjuvant" in addition to the vaccine antigen. Conserved components of microorganisms, or pathogen-associated molecular patterns (PAMPs) have been best characterized for their ability to activate DCs and their discovery offers the prospect of developing new vaccine adjuvants. PAMPs are recognized by pattern recognition receptors (PRRs) of the innate immune system. PRRs comprise a variety of receptors, including TLRs, cytosolic receptors [nucleotide-binding oligomerization domain-like (NOD-like) receptors (NLRs), RIG-I-like receptors (RLRs)], and C-type lectin receptors (111, 112). Activation of PPR signaling in DCs results in the enhancement of antigen presentation on MHCI and MHCII, cytokine production, and the upregulation of costimulatory molecules that are necessary for the induction of T-cell responses (5). Importantly, the nature of the adjuvant determines the type, the magnitude, the breadth, and the quality of the adaptive immune response. Differential patterns of expression of PRRs among DC subsets and different cytokine profiles induced by the triggering of distinct PRRs account for much of the diversity of phenotypes of the immune response (111, 113, 114) (**Figure 1**). Adding yet another level of complexity, adjuvants that trigger different pathways within a cell (115–117), or stimulate multiple cell types can cooperate to further enhance immune responses (70, 114, 118). In addition to PPRs, it was recently found that induction of stress response through sensing of amino acid starvation in DCs initiates autophagy and enhances MHCI cross-presentation (119). Stress sensors could therefore be possibly targeted to potentiate adjuvants.

The use of the mouse model to study and select adjuvants for human vaccine is limited because the pattern of expression of PRR can significantly differ between the two species. Because non-human primates express a similar repertoire of TLRs on immune cells to humans, they are a more relevant model to evaluate adjuvant effects (120, 121). While most adjuvants can induce antibody responses, generation of CD8$^+$ T-cell immunity has proved particularly difficult (122). Immunization studies in non-human primates showed that Poly ICLC which stimulate multiple PPRs (TLR3, RIG-I, and MDA-5) and TLR7/8 agonists are currently the most potent known adjuvants for induction of T helper 1 and CD8$^+$ T-cell responses (123–126). Poly ICLC and TLR7/8 agonist are the only TLR ligands capable of inducing both IL-12 and type I interferon, which are required for efficient cross-priming (53, 70, 114, 118). In mice, multiple cell types need to be stimulated for the production of IL-12 and type I interferon. IL-12 is produced by mDCs in response to Poly ICLC (through TLR3 triggering) and TLR7/8 agonist stimulation, whereas type I IFN is largely produced non-hematopoietic cells in response to Poly ICLC stimulation through MDA-5, and pDCs in response to TLR7/8 agonist, respectively. However, in mice reconstituted with a human immune system IL-12p70 and type I IFN production after TLR3 ligand stimulation resulted mainly from BDCA3$^+$ DCs (53). Even more surprising is that those BDCA3$^+$ DCs produce similar amounts of type I interferon as pDCs. These results are conflicting with those obtained after *in vitro* stimulation of BDCA3$^+$ DCs isolated from human blood and human tissues which produce only limited amount type I interferon (28, 41). Further studies will be needed to confirm this observation. Another potential benefit of those TLRs is that they appear broadly expressed on human mDC subsets (**Figure 1**), and therefore they can engage multiple DC subsets, which has been shown to improve T-cell responses (70). Multiple clinical studies have been initiated to evaluate Poly ICLC and TLR7/8 agonists as vaccine adjuvants which will help establish their potency in humans (www.clinicaltrials.gov).

The co-delivery of adjuvant and antigen to DCs is critical for the priming of the immune response. Co-delivery has been realized by coupling antigen to adjuvant (127–129), fusing antigen to protein adjuvant, or co-encapsulation in particles (130–132), and has lead

to significant increase in the magnitude of the immune responses and a better quality immune response (127). This enhanced T-cell priming may result from multiple effects: increased antigen uptake, altered intracellular routing, increased stability of the TLR agonist. The adjuvant effect may be even better achieved if the adjuvant and the antigen co-localize in the same endosomal compartments, as TLRs control MHCII presentation only in the compartments in which they are present (133, 134). Another benefit of coupled vaccines may be the local retention of the adjuvant at the site of injection, and thus the reduction of their toxicity. Indeed, free TLR agonists rapidly leave the site of injection and induce systemic innate responses resulting in high levels of serum cytokines (114). A more direct and controlled approach to reduce unwanted systemic effects of TLR agonists is to engineer their targeted delivery to DCs, although it might affect adjuvant effectiveness if activation of bystander cells contributes to the immune response (70, 118). Delivery of poly ICLC and TLR7/8 agonists through DEC205 or CD209 enhances DC activation and $CD8^+$ T-cell response in mice. Moreover, potent $CD8^+$ T-cell responses can be achieved with doses of adjuvant that do not induce toxic high serum cytokine levels (132).

Receptors other than TLRs have been shown to trigger DC activation. They are attractive due to their stimulatory capacity and their endocytic capacity that offer the potential of using a single molecule to deliver both antigen and activation signal to DCs. Dectin-1, a receptor involved in anti-fungal immunity, is a syk-coupled C-type lectin receptor that stimulate DC through its ITAM-like domain (112). Antigen delivery to human monocyte-derived DCs and $BDCA1^+$ DCs through Dectin-1 leads to enhanced MHCI cross-presentation and cell activation *in vitro* (135, 136). However, mouse immunization studies suggest that Dectin-1 may be more potent at priming $CD4^+$ T-cell responses than $CD8^+$ T-cell responses (137). A more promising receptor may be the CD40 receptor, which is expressed by all DC subsets. Not only does it efficiently deliver antigen to the MHC presentation pathways in DCs (28, 79), but its ligation induces DC stimulation and promotes cross-presentation (138, 139). Immunization studies confirmed that anti-CD40 agonistic antibody/Ag conjugates can prime $CD8^+$ T-cell responses in mice (140, 141). However, the use of agonist anti-CD40 antibodies in vaccine formulation may be limited by a narrow therapeutic window. CD40 is broadly expressed on B cells, monocytes, platelets, and endothelial cells, and CD40 ligation can induce high serum cytokine levels (142). It will be important to compare anti-CD40 antibodies with different agonistic function. Anti-CD40 with weaker agonistic function may be better tolerated and therefore allow higher antigen payload and vice versa for strong agonists. How this will impact the outcome of the immune response remains to be determined. CD32/FcγRII cross-linking also induces DC maturation and efficient antigen cross-presentation after immune complex internalization (73, 105, 143). Like CD40, it has the advantage of targeting most DCs, but could induce some toxicity because of its broad expression on other cells.

CONCLUSION

Recent advances in DC biology and the mechanisms controlling adaptive immune responses have offered new insights for the rational design of novel vaccines. Immunization studies in mice indicate that there is a clear benefit to the targeting of antigens to DCs. A major challenge, however, remains to translate this approach developed in mice to humans. The preliminary data obtained from the first clinical trials testing vaccines targeting DEC205 (CDX-1401, Celldex) and mannose receptor/CD206 (CDX-1307, Celldex) indicate that this strategy can elicit immune responses (18–20), but maybe not as strong as one could have expected based on the mouse data. One explanation is that immunologist's favorite model antigen for mouse studies is ovalbumin, which is exceptionally immunogenic, and may lead to overestimating vaccine efficacy. Mouse and human immune systems have also significant differences that make translation difficult (144). Although the intracellular mechanisms involved in antigen cross-presentation pathway and the DC lineage appear conserved between the two species, the specialization of the DC subsets may not be conserved. In addition, the pattern of expression of endocytic receptors for antigen delivery and TLRs for DC activation are different between mice and humans. Clearly, using a different model such as mice with a reconstituted immune system or non-human primates, which have a human immune system more similar to the human immune system is essential to optimize these vaccines. Additionally, analysis of the immune response to successful human viral vaccines that induce potent $CD8^+$ T-cell responses could help further determine the mechanisms that control immune responses to vaccination and identify predictors of vaccine efficacy (145).

Another challenge specific to the therapeutic treatment of cancer and maybe persistent viral infection is that they developed mechanisms to evade immune clearance by impairing T-cell function (146). The presence of these suppressive factors may limit vaccine efficacy, and combination of a vaccine with immunomodulatory molecules to neutralize inhibitory signals may be necessary to produce effective T-cell immune response.

In spite of these challenges, we view the present as an exciting time to study vaccine development and foresee that continuing to design DC-based therapies will allow us to prevent and treat many of the major illnesses for which no vaccine currently exists.

ACKNOWLEDGMENTS

The authors thank Allison Bruce (Genentech) for excellent assistance with artwork.

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Conflict of Interest Statement: Lélia Delamarre is an employee of Genentech, and hence declares a competing financial interest. Lillian Cohn declares no conflict of interest.

Received: 05 February 2014; paper pending published: 16 April 2014; accepted: 15 May 2014; published online: 30 May 2014.
*Citation: Cohn L and Delamarre L (2014) Dendritic cell-targeted vaccines. Front. Immunol. **5**:255. doi: 10.3389/fimmu.2014.00255*
This article was submitted to Antigen Presenting Cell Biology, a section of the journal Frontiers in Immunology.

frontiers in
IMMUNOLOGY

REVIEW ARTICLE
published: 16 April 2014
doi: 10.3389/fimmu.2014.00171

T cell responses to viral infections – opportunities for peptide vaccination

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An effective immune response against viral infections depends on the activation of cytotoxic T cells that can clear infection by killing virus-infected cells. Proper activation of these T cells depends on professional antigen-presenting cells, such as dendritic cells (DCs). In this review, we will discuss the potential of peptide-based vaccines for prevention and treatment of viral diseases. We will describe features of an effective response against both acute and chronic infections, such as an appropriate magnitude, breadth, and quality and discuss requirements for inducing such an effective antiviral immune response. We will address modifications that affect presentation of vaccine components by DCs, including choice of antigen, adjuvants, and formulation. Furthermore, we will describe differences in design between preventive and therapeutic peptide-based vaccines. The ultimate goal in the design of preventive vaccines is to develop a universal vaccine that cross-protects against multiple strains of the virus. For therapeutic vaccines, cross-protection is of less importance, but enhancing existing T cell responses is essential. Although peptide vaccination is successful in inducing responses in human papillomavirus (HPV) infected patients, there are still several challenges such as choosing the right target epitopes, choosing safe adjuvants that improve immunogenicity of these epitopes, and steering the immune response in the desired direction. We will conclude with an overview of the current status of peptide vaccination, hurdles to overcome, and prospects for the future.

Keywords: DC, peptides, vaccination, virus, infection, chronic, acute

INTRODUCTION

Viruses are small infectious agents that consist of nucleic acid that is coated in a simple protein shell or a cell-membrane-like protein casing, and need to infect host cells to replicate (1). Viruses can cause acute and chronic infections. In acute virus infections, such as a common cold, the virus is typically cleared from the body within a week. However, in some cases, an acute infection is followed by persistence of the virus in the host. Herpes simplex virus is an example of a virus causing a persistent infection, due to ability of the virus to hide in neurons. Often, these types of persistent infections do not cause any symptoms in healthy hosts (2). Chronic infections are a type of persistent infection often caused by an inefficient immune response of the host, leading to long-lasting symptoms. Especially, acute and chronic virus infections have a major general health impact. Annual influenza epidemics, for instance, result in about 3–5 million cases of severe illness and approximately 250,000–500,000 deaths worldwide (3). An example of a chronic infection causing major health impact is Human Immunodeficiency Virus (HIV). In 2012, more than 35 million people were living with an HIV infection and 1.6 million people died from an AIDS-related illness (4). Some persistent virus infections, such as Epstein–Barr virus (EBV) and Human Papillomavirus (HPV) can lead, under certain conditions, to the development of tumors (5, 6). Because viruses have such a major impact on health, strategies to limit or prevent virus infections are of major importance.

Mammals have developed a refined immune system to cope with all kinds of infections. Especially, the adaptive arm of the immune response is important in limiting and clearing viral infections. The humoral immune response consists of antibodies specific for the virus that can capture and neutralize virus particles before they enter the cell. However, if these antibodies are ineffective, viruses are able to infect host cells and can only be cleared by the cellular arm of the immune response. Once a virus infects a cell, the virus will use the protein-synthesis machinery of the host cell to synthesize its own proteins. During this process, some of the newly synthesized proteins will be degraded into peptide fragments and, if they have sufficient binding affinity, bind to MHC class I molecules. These MHC class I-peptide complexes will then be presented on the cell surface of an infected cell and activated $CD8^+$ T cells, specific for the peptide, can recognize the MHC class I-peptide complex and induce apoptosis of the infected cell by releasing cytotoxic granules. Activation of these $CD8^+$ T cells occurs in the draining lymph nodes, where antigen-presenting cells (APCs), such as dendritic cells (DCs), and naïve T cells encounter each other. In these lymph nodes, DCs and $CD4^+$ T cells provide the co-stimulation necessary for proper activation of $CD8^+$ T cells. This process is summarized in **Figure 1** and will be further discussed in the next paragraphs.

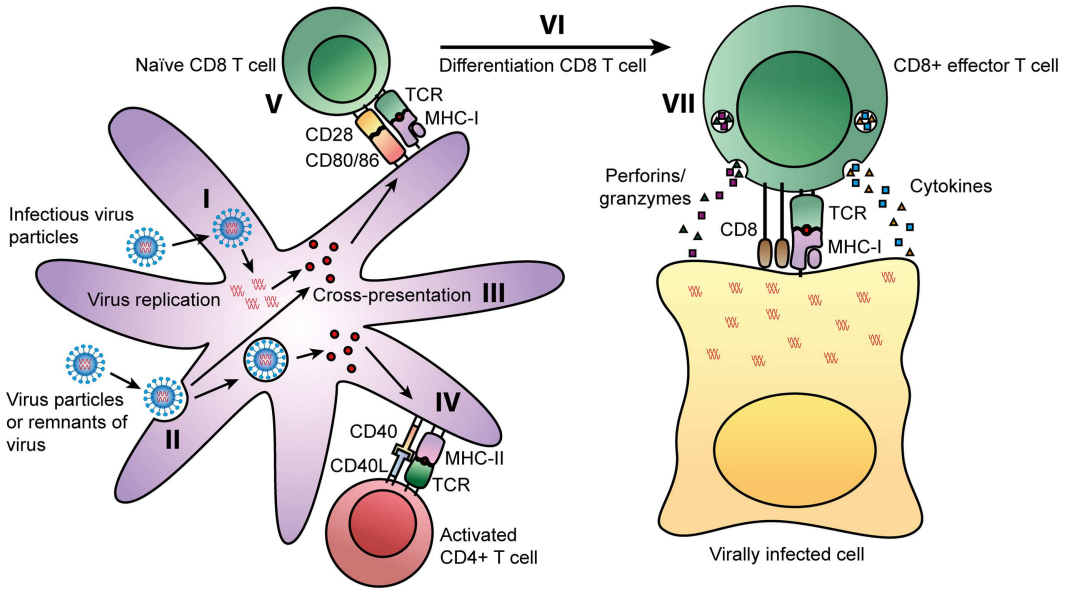

FIGURE 1 | Routes of presentation of viral peptides on DCs. Viruses can enter cells by two ways: some viruses can infect cells directly, leading to replication of virus inside the cells. During this process, some of the viral proteins will be degraded into peptide fragments, which will be presented on MHC class I molecules to $CD8^+$ T cells (I). APCs, such as DCs can also take up viral particles or remnants of virally infected cells (II). During processing by professional APCs, viral peptides can be presented on MHC class I molecules via the cross-presentation pathway (III). In parallel, these extracellular-derived peptides will be presented on MHC class II molecules. The TCR of virus-specific $CD4^+$ T can recognize MHC class II-peptide complexes on professional APCs. Next to the interaction of the MHC class II-peptide complex with the TCR, $CD4^+$ T cells can activate DCs by interaction of CD40 with CD40 ligand on the DC (IV). This interaction activates DCs and results in upregulation of maturation markers CD80/CD86. CD80 and CD86 interact with CD28 on naïve $CD8^+$ T cells (V). Together with the recognition of the MHC class I-peptide complex by the TCR, CD28 signaling will result in the activation of the $CD8^+$ T cell (VI). These activated $CD8^+$ T cells will differentiate into effector T cells that can recognize the MHC class I-peptide complex on virally infected cells. Binding of the TCR to the MHC class I-peptide complex leads to activation of the $CD8^+$ T cell and the release of cytotoxic granules containing perforins and granzymes, and the production of cytokines such as TNF-α and IFN-γ (VII).

During the initial phase of a viral infection, there is a significant increase in the number of $CD8^+$ T cells. Priming of these naïve T cells will not only occur through the classical pathway via infection of a cell, directly leading to presentation of peptides on MHC class I molecules, but also through cross-presentation. Cross-presentation enables the presentation of viral peptides, taken up from extracellular sources, on MHC class I molecules. Several different cell types have been demonstrated to cross-present antigens *in vivo*, including professional APCs such as macrophages and DCs (7). $CD8^+$ T cells, activated either through the classical or cross-presentation pathway, induce apoptosis of virus-infected cells by the release of cytotoxic granules and the production of TNF-α and IFN-γ as depicted in **Figure 1**. The cytotoxic granules contain perforins, granzymes, and granulysin. Perforins aid in delivering contents of granules into the cytoplasm of the target cell. Granzymes, such as granzyme B, and granulysin activate apoptosis of the target cell. TNF-α can interact with the TNFR-I receptor, which induces apoptosis of infected cells. IFN-γ is an important cytokine in the immune response to various viral infections, since it can induce an antiviral state in uninfected cells and enhance the cytotoxic function of $CD8^+$ T cells. By the classical antigen presentation pathway or by the cross-presentation pathway, any form of virus can be presented on MHC class I and MHC class II and thereby stimulate antiviral responses by both $CD8^+$ T cells and $CD4^+$ T cells, respectively, leading to a broad cellular response to infection (8). After infection, some of these activated T cells will develop into memory T cells. In the event that a secondary infection occurs, these cells can rapidly mature into effector cells and respond to infection.

Antigen-presenting cells that reside at the site of infection, can take up viral particles or remnants of virally infected cells from extracellular sources, and present them on MHC class II molecules. Subsequently, $CD4^+$ T cells recognizing peptides in the context of MHC class II will be activated. These activated $CD4^+$ T cells are capable of producing a wide range of cytokines and chemokines and can even exert cytotoxic functions themselves. Based on cytokine production, $CD4^+$ T cells can be divided into several subsets, the most classical being Th1, Th2, and Tregs. Th1 cells are generally characterized by the production of IFN-γ. Th2 cells, on the other hand, produce mainly IL-4, IL-5, and IL-13 and are important for providing an immune response against helminths by activating eosinophils, basophils, mast cells, and B cells. The third classical subset are the Treg cells, which are characterized by the production of IL-10 and TGF-b, and have mainly regulatory tasks such as dampening effector functions and limiting immunopathology (8, 9). In addition to their effector functions, activated $CD4^+$ T cells can provide help to $CD8^+$ T cells by CD40-CD40L interaction, which induces up regulation of ligands, such

as CD80 and CD86, on DCs. These ligands interact with CD28 on naïve T cells, providing a co-stimulatory signal to activate $CD8^+$ T cells (10). The mechanism by which $CD4^+$ T cells can provide help to $CD8^+$ T cells is shown in **Figure 1**.

In this review, we will discuss the value of T cell responses in both acute and chronic viral infections and how knowledge of these responses can help in designing effective vaccines.

Currently, antiviral drugs are the main treatment option to combat viral diseases. However, antiviral treatment is associated with side effects and resistance through viral escape. Making use of the hosts own immune defense system by vaccination would be another powerful approach to combat viral diseases. However, many vaccination strategies are based on antibody-mediated protection and are only partially successful. Antibodies can be very efficient in preventing virus infection, but due to the variability of many virus surface proteins, the virus can escape and infect host cells. Once a virus has entered a cell, infection can only be cleared by a cellular response. We will highlight the history of synthetic T cell based vaccines as an important strategy to induce T cell responses and discuss current developments in this field. Then, we will discuss how the design of these vaccines, such as choice of antigen and adjuvant, influences their efficacy. Finally, we will conclude with potential pitfalls and recommendations for the design of effective peptide vaccines against virus infections.

T CELL RESPONSES IN VIRAL INFECTIONS

There are many viruses for which T cells, both $CD8^+$ and $CD4^+$, have been shown to play a role in protection, such as measles virus, cytomegalovirus (CMV), hepatitis C virus (HCV), and HIV (11–14). In general, an efficient antiviral adaptive response is thought to be of the Th1 type (15). However, many viruses can inhibit this Th1 response by downregulating the production of interferons (16, 17). This type of manipulation of the immune response can greatly influence the outcome of the infection. In infections caused by hepatitis viruses, manipulation of the immune response by the virus can lead to a persistent infection, in which the host is incapable of clearing the virus from the body. In mice, lymphocytic choriomeningitis virus (LCMV) is used as a model to study the role of $CD8^+$ and $CD4^+$ T cells in both acute and chronic infections. $CD8^+$ T cell-deficient mice, which were infected with a LCMV-strain that normally causes acute virus infection, were not able to control infection and developed a persistent infection. In mice depleted of $CD4^+$ T cells, infection with murine LCMV led to chronic infection, even in the presence of $CD8^+$ T cells. This model shows that in acute infection, $CD8^+$ T cells are sufficient to clear infection, but the help of $CD4^+$ T cells is required (18).

The importance of T cell responses during acute viral infections in humans can be illustrated by research from Sridhar et al. describing that individuals with higher numbers of pre-existing $CD8^+$ T cells specific for conserved CD8 epitopes, developed less severe illness after infection with pandemic H1N1 influenza virus (19). That not only $CD8^+$ T cells mediate protection to influenza challenge, has been shown in a unique human challenge study by Wilkinson et al. In this study, healthy volunteers were challenged with influenza A virus, and antibody and T cell responses against influenza before and during infection were monitored. They showed that, in the absence of antibody responses, pre-existing $CD4^+$ T cells responding to influenza internal proteins were associated with less severe illness and lower virus shedding. Further characterization of these $CD4^+$ T cells showed that these cells had a cytotoxic function (20). These studies describe the importance of both $CD8^+$ and $CD4^+$ T cells in the immune response against influenza virus.

During chronic viral infections, when the host is not able to clear the virus, the main role of cytotoxic T cells is to limit disease severity and delay disease progression. This is exemplified by studies on HIV infection. Early during infection with HIV, there is a decline in viral replication as measured by the number of HIV RNA copies in plasma samples (21). In the first stages of HIV infection, it has been shown that patients with higher numbers of memory cytotoxic T cells show a much lower viral load in plasma than patients with a lower number of memory cytotoxic T cells, indicating that this decline is mediated by cytotoxic $CD4^+$ T cells (22). In addition, cytotoxic $CD4^+$ T cells are an immunological predictor of disease outcome. Patients that controlled HIV replication without antiretroviral therapy showed an increased number of $CD4^+$ T cells specific for HIV proteins (23). The importance of $CD8^+$ T cells in delaying HIV disease progression is shown in studies where a loss of $CD8^+$ T cells coincides with disease progression (24, 25). Findings that HIV escape mutations often occur at HLA-binding sites specific for CD8 epitopes, the strong association of certain HLA-alleles with protection from HIV disease progression, the temporal relationship between viral load decline and increase in specific $CD8^+$ T cells, and $CD8^+$ T cell depletion studies in simian models, underline the importance of $CD8^+$ T cell responses (11, 26–29). Knowledge on the mechanism of protection of T cell responses in immunity against viruses can be helpful in designing preventive and therapeutic therapies, such as vaccination.

HISTORY OF PEPTIDE VACCINATION

Many vaccines against virus infections are based on inducing antibody responses, consequently, these vaccines are often poor inducers of T cell responses (30). Since T cells are important in protection against many viral infections, there is a need for T cell inducing vaccines. By including small protein fragments (peptides), in a vaccine, which can be presented by MHC-molecules to $CD4^+$ and $CD8^+$ T cells, specific T cell responses can be induced. In **Table 1**, characteristics of two of the classical preventive vaccines for viral infections, i.e., protein vaccines and live attenuated vaccines, are compared to peptide vaccines. The main advantage of peptide vaccines over classical vaccines is that it is possible to specifically induce T cell responses and that the production process of these vaccines is relatively easy. The first synthetic peptide vaccine able to induce a T cell response in mice was published by Aichele et al. This vaccine contained a 15-mer peptide, derived from the NP protein of LCMV, suspended in incomplete Freund's adjuvant (IFA) (31). Further experiments showed that these peptide vaccines were able to render a certain amount of protection against challenge with virus (32, 33). These results were promising, but in later studies where mice were vaccinated with 15-mer CTL epitopes derived from adenovirus type 5 early region (Ad5E1) oncogenes in combination with IFA, an enhanced outgrowth of tumors was observed following

Table 1 | Comparison of classical protein vaccination, live attenuated vaccination, and peptide vaccination.

	Classical protein vaccine	Live attenuated vaccine	Peptide vaccine
Composition	Inactivated split virion or purified subunit	Attenuated virus, capable of replication	Synthetic, small protein fragments
Humoral response	Yes, induces humoral response	Yes, mimics natural infection	Possible, depends on peptides included
CD4 response	No	Yes	Yes
CD8 response	No	Yes	Yes
Preexisting response	Not important	Important, Ab can capture vaccine	Not important
Adjuvant	Required for cellular response	Not required	Required
Production	Biological	Biological	Synthetic
Safety	Risk of contamination with extraneous agents and proteins of the production substrate	Risk of contamination with extraneous agents and proteins of the production substrate	Well controlled and highly pure production process
Flexibility to match escape variants	Not easy	Not easy	Easy
Target conserved components	No, primarily strain-specific response	To some extent, limited cross-reactivity	Yes, capable of inducing a broad response

Protein vaccines are a form of inactivated vaccines that consist of purified subunit or subvirion products. Live attenuated vaccines are attenuated viruses, derived from disease-causing virus. These attenuated viruses still replicate in the host, but do not cause disease. Peptide vaccines are completely synthetic vaccines, comprised of small protein fragments.

vaccination (34). In hindsight, this observation might not be that surprising. Only peptides of 20 amino acids or longer will need to be degraded by proteolytic enzymes and are therefore presented exclusively by professional APCs, thereby ensuring sufficient co-stimulation. Shorter peptides can be directly loaded on any MHC molecule, also on non-professional APCs, which may lead to the induction of tolerance. Additional research showed that indeed the problem with the 15-mer adenovirus peptides was induction of tolerance, since they were presented by non-professional APCs lacking appropriate co-stimulation, resulting in suboptimal presentation of the peptide. When mice were vaccinated with peptide-loaded DCs, there was an anti-tumor response and no tolerance induction, showing that presentation of these peptides on professional APCs can be effective without induction of tolerance (35).

PEPTIDE LENGTH

Thus, the first advantage of peptides of 20 amino acids or longer, which are considered as long peptides, is that they require processing of these peptides by professional APCs, thereby reducing the chance of inducing tolerance by peptide vaccination (36). Furthermore, they may contain multiple epitopes specific for different MHC-molecules. Thereby, broadening the potential response in both the individual and at population level (37). Another advantage of using long peptides is that, next to CD8 epitopes, this type of peptide often contains CD4 epitopes. These CD4 epitopes provide co-stimulation during priming of $CD8^+$ T cells and promote memory $CD8^+$ T cells (38, 39). One year after the first successful immunization of mice with a free synthetic LCMV peptide, Fayolle et al. described that this 15-mer peptide not only contained a CD8 epitope but also a CD4 epitope (40). This discovery confirms the valuable contribution of co-stimulation in a vaccine. In addition to considering the importance of the length of the peptide, other characteristics are equally or even more important. Therefore, considerations for the choice of antigen will be discussed next.

CHOICE OF ANTIGEN

Aspects hampering the design of an effective preventive strategy for virus infections are that these viruses have, besides great genetic diversity, also developed multiple mechanisms to evade the host's immune response (41, 42). A promising approach is to direct the immune response to conserved parts of the virus, which do not allow for mutations. Virtually all viruses contain certain proteins or peptides that are highly conserved. Indeed, for HIV, the Gag protein appears to be a good candidate for use as a T cell vaccine component. The Gag protein is highly conserved, and although it is a late structural protein, Sacha et al. showed in a simian model that $CD8^+$ T cells recognize Gag-derived epitopes as early as 2 h post infection. This fast processing and presentation is thought to be necessary for early clearance of the virus (43). In an *ex vivo* study on PBMCs of HIV-infected individuals, vigorous $CD8^+$ T cell responses to Gag epitopes were observed and the breadth of the $CD8^+$ T cells specific for conserved Gag epitopes inversely correlated with viremia (44). A screening in patients with both acute and chronic HCV infection showed that specific T cell responses were found against conserved parts of the virus. Immunogenic regions were identified within core, NS3 and NS4 proteins (45). Influenza virus also contains good candidate proteins, such as nucleoprotein (NP), which is a major target of T cell responses (46). These

studies show that there are T cells available directed toward conserved parts of the virus. Knowledge of these parts can be used in the design of a T cell inducing vaccine.

Since the introduction of sequence analysis tools, it is relatively easy to determine whether a certain peptide sequence is conserved. However, a high level of conservation is not the only requirement for a peptide vaccine to be effective. The peptide will have to be processed by the proteasome and then bind to the MHC molecule. Bio-informatic tools can be helpful to predict, which sequences may be immunogenic for T cells. These tools can predict which sequences will bind to MHC, based on preferred amino acids of peptide anchor binding positions of these molecules. Furthermore, tools are available that predict which sequences will be processed by the proteasome and by TAP (transporter associated with antigen processing) transport (47). Together, these tools provide means of selecting a number of possible conserved T cell epitopes. Schellens et al. showed in PBMCs of HIV-infected individuals that indeed these bio-informatics tools are valuable for predicting novel T cell epitopes (48).

Another important requirement for inducing T cell responses is that there are T cells available that can recognize the peptide. Tan et al. described the importance of the availability of naïve epitope-specific $CD8^+$ T cells in the host prior to infection and showed that precursor frequencies are indeed a good predictor for responses observed after infection, since a higher number of epitope-specific $CD8^+$ T cells led to an increased T cell response after infection (49). Next to precursor frequencies, binding affinity of peptides to MHC is also a predictor of immunogenicity as has been shown in peripheral blood lymphocytes of acute HBV patients (50). Some groups have shown that it is possible to enhance peptides by increasing binding affinity of the peptide to the MHC molecule (51, 52). These enhanced peptides might induce a T cell response to conserved, but otherwise too low affinity epitopes. Another important consideration when vaccinating with short peptides is HLA-specificity. Since peptides of 8–11 amino acids long bind directly into the MHC class I binding groove the peptide has to match the HLA type of the vaccinated individual. To overcome the need for individualized vaccination, Tan et al. selected short epitopes with the capacity to bind to multiple HLA-alleles. HLA-A2 transgenic mice vaccinated with this multi-HLA peptide vaccine, showed a reduction of virus in the lungs and increased survival following influenza infection, compared with mock vaccinated mice, showing that vaccination with peptides can positively influence disease outcome (53).

Presentation of peptides by APCs greatly depends on the form in which they are offered to APCs. Zhang et al. compared intact proteins and long peptides in the cross-presentation pathway and showed that long peptides traffic to both the endosomes and the cytosol, whereas whole protein was found to traffic only to the endosomal compartments. Therefore, whole proteins could not be processed through the cross-presentation pathway. This difference in processing led to a $CD4^+$ T cell restricted response after immunization with protein, while immunization with peptides also led to a $CD8^+$ T cell response (54). Rosalia et al. compared whole protein processing to processing of long peptides, both in mouse and in human DCs. Soluble protein antigen ended up mostly in the endolysosomes, while long peptides seemed to be more efficiently internalized by DCs leading to a faster intracellular routing. Therefore, long peptide vaccination ultimately leads to enhanced $CD8^+$ T cell activation compared to whole protein (55). In line with these findings, recent research on peptide vaccination is mainly directed to improving antigen presentation of the peptides of choice, by choosing the right form in which the peptides are presented. Rosario et al. used an HIV-synthetic long peptide vaccine to boost HIV-specific T cell responses in a macaque model and showed that boosting with these synthetic long peptides primarily increased the breadth of the $CD4^+$ T cell responses (56).

FEATURES OF AN EFFECTIVE RESPONSE

To induce an effective response against viral infections, there are several requirements that should be met. One important requirement is that there is a sufficient number of T cells available to kill virus-infected cells. The need for an appropriate magnitude of T cells in order to clear virus was elegantly shown by Thimme et al. in a $CD8^+$ T cell depletion study in chimpanzees. Chimpanzees were depleted of $CD8^+$ T cells, and subsequently infected with HBV, complete depletion of $CD8^+$ T cells in the chimpanzees resulted in the inability to clear virus. When $CD8^+$ T cells reappeared in the animal, 98% of viral DNA was eliminated from the liver. However, while the number of $CD8^+$ T cells remained suppressed, the animal was not able to clear virus completely. Only when the number of $CD8^+$ T cells was able to expand further, the virus was completely eliminated (57). Furthermore, an increased breadth of T cell responses can be beneficial. Analysis of $CD8^+$ T cell responses in untreated HIV-infected individuals showed that an increasing breadth of Gag-specific responses is associated with decreased viremia (58). In parallel with these findings, vaccination of mice with a vaccine containing multiple epitopes, were more effective in generating a response to influenza infection than vaccination with single epitopes (49). These findings indicate that a broad response is more effective than a response dedicated to only one peptide. Another advantage of induction of a broad response is that small mutations of the virus will not lead to escape of the virus from the immune response. Next to a broad response, T cell responses of high avidity also contribute to an antiviral response. *Ex vivo* screening of T cell responses in HIV-infected patients showed that controllers reacted to lower antigen concentrations compared to non-controllers, indicating that controllers have T cell responses of higher functional avidity and that this higher avidity is advantageous (59).

A fourth requirement is that an effective antiviral response should be of proper functionality to enable control or clearance of the virus. $CD8^+$ T cells are the main cell type that is involved in clearance of viral infections. These cells are characterized by the production of Th1-cytokines such as IFN-γ and by the expression of degranulation marker CD107a (60). CD107a is an indicator of cytotoxic functions such as the production of granzymes and perforins. IFN-γ increases expression of both MHC class I and II molecules and enhances the antigen-presenting function of MHC class I by stimulating loading of peptides onto this molecule. Thereby, IFN-γ can induce the cytotoxic function of $CD8^+$ T cells and promote the production of other cytokines such as TNF-α, IL-2, and type I interferons. TNF-α induces apoptosis of virus-infected cells and IL-2 is an important growth factor for T cells.

Type I interferons, such as IFN-α and IFN-β, can induce resistance to viral infections in uninfected cells, increase MHC class I expression and antigen presentation and activate both DCs and macrophages (8, 61). Activated macrophages in their turn produce chemokines such as MIP-1β to attract more T cells. Together, these cytokines, chemokines, granzymes, and perforins enable control or clearance of the virus from the host. In HIV infection, a polyfunctional $CD8^+$ T cell response is observed in non-progressors, while progressors show a more limited response (62). As reviewed by Seder et al., a polyfunctional response, characterized by production of IFN-γ, TNF-α, and IL-2, was indeed shown to induce more robust T cell proliferation and protection against several viral infections (63).

However, elevated amounts of inflammatory cytokines can also lead to immunopathology as has been shown in H5N1 influenza A virus infection (64). The immune system normally has its own regulatory mechanisms, such as the production of anti-inflammatory cytokines including IL-10 and TGF-β. IL-10 is produced by a wide range of cells, including T cells, macrophages and neutrophils. The main function of IL-10 is to act as a negative feedback loop to suppress the production of IFN-γ and other pro-inflammatory cytokines (65). TGF-β acts by inducing apoptosis of $CD8^+$ T cells, which regulates T cell homeostasis and prevents immune inflammation (66). These feedback loops are a way of the immune system to regulate itself, however viral factors can negatively impact this balance as is illustrated in HCV infection. Patients with progressive liver injury showed upregulation of Th1-cytokines IFN-γ and IL-2 and down regulation of the regulatory cytokine IL-10 (67). Another regulatory mechanism is the upregulation of inhibitory receptors such as PD-1, LAG-3, and CTLA-4, which leads to decreased activation potential of T cells and the activation of inhibitory genes in T cells (68). However, upregulation of these receptors has also been shown to be responsible for the exhaustion of T cells and thereby a diminished response in chronic viral infections (69). Summarizing, an effective antiviral response consists of a broad variety of antigen-specific T cells of sufficient magnitude, affinity, and appropriate polyfunctionality. Furthermore, these T cells should be capable of performing cytotoxic functions, but should not induce immunopathology. Such a response greatly depends on the way antigen is presented to the T cells, emphasizing the important role APCs play in antiviral responses.

CO-STIMULATION AND PEPTIDE VACCINATION

In recent years, multiple strategies were developed to increase the quality of antigen presentation of peptides. One of the strategies, already described above, is the addition of CD4 help. Long peptides often contain CD4 epitopes that can provide co-stimulation for $CD8^+$ T cells. However, more general CD4 helper peptides are available. One example is the non-natural pan HLA-DR binding peptide (PADRE), which is engineered by introducing anchor residues for different DR motifs within a polyalanine backbone. This peptide binds with high or intermediate affinity to the most common HLA-DR types, and allows it to activate a wide range of $CD4^+$ T cells (70). The addition of PADRE epitopes is used, for example, in Dengue virus and HBV virus vaccine development, showing promising results *in vivo* (71, 72). Another group of universal T helper epitopes are natural tetanus sequences, which are very promiscuous in their capacity to bind to MHC class II, and thereby very efficient in acting as a co-stimulus (73). These universal T helper epitopes can be fused to $CD8^+$ T cell epitopes, eliciting good immunogenicity, as shown for CMV by La Rosa et al. (74). However, it remains under debate whether CD4 help should be antigen-specific or is otherwise not able to stimulate proper $CD8^+$ T cell responses. A study in which mice were vaccinated with either non-specific CD4 help or antigen-specific CD4 help, showed that memory $CD8^+$ T cells can only be efficiently activated by antigen-specific CD4 help, while effector $CD8^+$ T cells can be activated by non-specific CD4 help (75).

An important factor in $CD4^+$ T cell help in short peptide vaccination appears to be CD40-CD40 ligand interaction (76). Ligation of CD40 to CD40 ligand can trigger the production of high levels of IL-12 by DCs. IL-12 induces Th1-mediated immune responses and inhibits Th2-mediated responses (77). Furthermore, CD40 ligand stimulates up regulation of ICAM-1, CD80, and CD86 molecules on DCs. By these mechanisms, DCs can trigger proliferative responses and IFN-γ production by T cells (78). By adding CD40 ligand as a co-stimulatory molecule, DCs can be activated through CD40 and in their turn, DCs are able to activate $CD4^+$ T cells and $CD8^+$ T cells (79).

Another way to activate APCs is by targeting their Toll-like receptors (TLRs). TLRs are pathogen recognition receptors (PRR) that recognize molecules shared by pathogens, for example, double stranded RNA in certain viruses. Activation of these TLRs can then lead to the production of inflammatory cytokines. By covalently coupling TLR-activating lipids to the peptide, resulting in so-called lipopeptides, self-adjuvanting peptides are created. These lipopeptides can target the vaccine by activating the TLRs on the required APCs and the peptides can then be internalized and presented on MHC-molecules. Thereby, lipopeptides can signal through the TLRs to induce DC maturation, leading to enhanced antigen presentation. Jackson et al. designed a synthetic vaccine composed of a CD4 T helper epitope, a CD8 target epitope, and the lipid moiety Pam_2Cys that provided TLR2 targeting, which could induce DC maturation and antibody and CTL responses (80). Chua et al. used the TLR2 agonist Pam_2Cys to enhance the immunogenicity of their virus-like particles, containing HCV structural proteins. The addition of lipopeptide resulted in increased DC maturation at low doses of the vaccine (81). Indeed, lipopeptide vaccination can induce protective CTL responses, as shown by Day et al. in a mouse influenza virus challenge model (82).

ADJUVANTS IN PEPTIDE VACCINATION

To improve the effectiveness of peptide vaccines, there are several types of adjuvants available, with different effector mechanisms. Some adjuvants induce depot formation; others directly stimulate the immune response through additional signals. In earlier work on peptide vaccination, strong adjuvants were necessary for induction of immunogenicity. A commonly used adjuvant for peptide vaccination is IFA, which was applied in the first peptide vaccine, or the human equivalent Montanide. These water in oil formulations form a depot at the site of injection, leading to "leakage" of antigen into the body (37, 83). Research by den Boer et al. showed that the short Ad5E1 peptide still leaks from the IFA depot at day

200 (84). This depot of antigen and adjuvant can lead to chronic inflammation of the site of injection that may persist for a long time. Harris et al. showed that repeated vaccination can even lead to a site suggestive of a new lymphoid structure, including the association of mature DCs with proliferating T cells in perivascular dermal aggregates (85). However, the risk with such depots is that the peptide might be present for a long time after vaccination, but the adjuvant might not be, allowing presentation of the peptide without the necessary co-stimulation and with the risk of inducing tolerance (86). Furthermore, although effective in therapeutic vaccination, IFA does lead to the formation of lesions on the site of injection, making it less attractive for use in a preventive vaccine (87). Two clinical trials, one with HIV peptides and another with malaria surface proteins mixed in Montanide, have even been terminated because of these severe adverse events (88, 89).

An alternative for water in oil formulations could be the use of vesicular delivery systems. Depending on the nature of the delivery system, they provide the possibility to incorporate immune modulators to direct the immune response, protect against degradation of the peptide, directly target the antigen to the place of interest and, finally, actively transport the antigen across the target membrane. Currently, there are several delivery systems available for peptide vaccination, i.e., liposomes, virosomes, virus-like particles, ISCOMs, and nanoparticles (90). Liposomes consist of a lipid bilayer, in which antigens or other substances can be entrapped in the lumen or the lipid bilayer, depending on traits of the peptide. The lipid bilayer of liposomes can fuse with other bilayers, such as a cell membrane. Thereby, liposomes can deliver antigens to the cytosol of APCs (91). Liposomes, containing a short CD8 lipopeptide in combination with CpG, were able to induce protection in a murine influenza challenge model (92). However, liposomes cannot induce maturation of DCs without addition of an adjuvant and are therefore not sufficient to induce co-stimulation. To address this problem, several groups are developing modified liposomes to increase targeting to DCs by adding targets for C-type lectin receptors such as glycans or mannose, which are typically expressed on DCs (93). Virosomes, or influenza derived virus-like particles, have similar membrane-fusion capacities as live influenza virus, which allows them to actively fuse with cell membranes and thereby deliver antigens directly into the cytosol of APCs leading to cross-presentation of antigenic peptides (94). Furthermore, they have been shown to induce up regulation of maturation markers on bone marrow-derived DCs, in mouse models (95, 96). However, as of now, DC maturation capabilities of virosomes have not been shown in human systems. Thus, liposomes, virosomes, and other delivery systems can successfully be used to deliver antigens to the place of interest. In addition, they can provide the necessary co-stimulation for APCs, either due to their own properties or by adding other adjuvants to the formulation.

CURRENT PROGRESS IN PEPTIDE VACCINATION

The first and most successful, peptide-based vaccine that is currently licensed is a therapeutic vaccine against HPV. This vaccine contains long synthetic peptides directed against viral oncoproteins, mixed in Montanide, which induces vaccine-specific $CD4^+$ and $CD8^+$ T cell responses in all patients (87). Since the success of this therapeutic cancer vaccine, many groups are exploring peptide vaccination for other viral agents. Therapeutic vaccination, with synthetic peptides, of HCV patients not responding to standard treatment, resulted in a decrease in viral RNA as shown in two separate studies. Klade et al. performed a Phase II clinical study in HCV patients with their IC41 vaccine, consisting of five synthetic peptides formulated with a Th1 type adjuvant, poly-I-arginine. All patients that were vaccinated intradermally with TLR7 agonist imiquimod as adjuvant, showed a modest decline in viral titers (97). The study by El-Awady et al., in which HCV patients were vaccinated with a peptide vaccine consisting of three envelope proteins, showed that in two thirds of the patients both antibody and T cell responses were detectable resulting in decreased viral titers (98). However, although these studies provide a proof of concept for peptide vaccination for therapeutic use in HCV infection, the improvements are only minor.

For a preventive peptide vaccine, there are different necessities. First of all, it should target conserved sequences, which could lead to a universal vaccine. Possible target proteins have been identified for viruses such as HIV, HCV, and influenza (44–46). Especially in influenza vaccine development, the threat for a new pandemic to occur has boosted research on the development of such a universal vaccine. The research of Tan et al., in which they make use of lipopeptides directed to conserved components, is one of many examples of strategies that are currently developed and have proven themselves in mouse models but not yet in human systems (53). Other vaccination strategies, currently in development, include the use of virus-like particles in combination with an antibody-inducing influenza protein such as the relatively conserved M2e protein or lipopeptide in combination with liposomes (92, 99, 100). A recent advancement is that there are some peptide-based vaccines against influenza virus infection in Phase I clinical trials, that are able to induce vaccine-specific cellular immunity (101, 102).

CONSIDERATIONS FOR PEPTIDE VACCINE DEVELOPMENT

When designing peptide-based vaccines, there are several things to take into consideration, such as virus traits, side effects, location of the response, and traits of the host (see **Table 2** for an overview). First, the objective of vaccination should be taken into consideration. Vaccines can be largely divided into therapeutic and preventive. Preventive peptide-based vaccines should elicit a robust memory T cell response, since vaccine-induced T cells need to respond rapidly after infection to clear the virus before it causes illness or at least to limit disease burden. In the case of therapeutic vaccination to chronic infections, the response should be vigorous and elongated and a rapid response is of less importance. Both for therapeutic and preventive vaccines, eliciting this response at the required location is of great value. Peptide-based vaccines for respiratory viruses, for example, might be more effective when administered intranasally, since lung resident immune cells might then be primed more easily (103). However, changing the route of administration is not always sufficient and then adjuvants in the form of delivery vehicles might aid in transporting vaccine components to the right location in order to elicit an efficient T cell response.

Although inducing T cell responses is very important in protection against many pathogens, there are also indications that these

Table 2 | Design of a peptide-based vaccine for preventive or therapeutic use.

Factor	Preventive	Therapeutic
Route of immunization	Unimportant Time to develop response	Wanted Virus present on certain location
Existing response	Unimportant Inducing new response	Important Boost existing T cell response
Rapid effector response	Wanted Preventing or limiting disease	Unimportant Clearance in the end
Inducing memory	Wanted T cells available when infected	Unimportant Recall response not necessary
Side effects	Unwanted Reason to withdraw vaccine	Unimportant Accepted for certain diseases

There are several factors to take into consideration when designing peptide-based vaccines, such as location of the response, type of response to be induced, and side effects. The contribution of these factors in the design of preventive versus therapeutic vaccines are summarized in the table.

T cell responses cause harm. This is illustrated for influenza infection, in which a high number of virus-specific $CD4^+$ T cells in patients infected with pandemic influenza A virus from 2009, correlated with more severe illness (104). In the case of HCV infection, a broad and specific T cell response is able to control virus infection (105). However, during chronic viral infection, liver damage occurs, which is assumed to be immune-mediated. In a study by Maini et al., a high number of antigen-specific T cells in the blood did not correlate with the amount of liver damage as measured by alanine transaminase (ALT, indicative of liver damage). In contrast, Feuth et al. show a direct correlation between the number of differentiated $CD8^+$ T cells, which contain high perforin levels, and liver fibrosis measured by fibroscan elastography (106). Since a large number of T cells are detected in the liver of patients with liver damage, damage has been proposed to be caused by the recruitment of non-virus-specific T cells (107). Although in humans the mechanism by which immunopathology develops is not clear, it is important to bear in mind that an exaggerated T cell response to infection or vaccination may lead to unwanted immune-mediated damage. Therefore, vaccine-induced T cell responses should be effective against the virus, without eliciting major side effects.

Traits of the host also influence the effectiveness of a vaccine. Therefore, it is important to consider the target group for vaccination. During a human's lifetime, the immune system will change continuously. Vaccination in early childhood can have a major impact on the immune response in later years as described by Bodewes et al. in which it was shown that annual vaccination with a seasonal inactivated subunit influenza vaccination hampers the development of influenza-specific $CD8^+$ T cells (108). To underline this finding, Hoft et al. compared a live attenuated influenza vaccine (LAIV) with a trivalent inactivated influenza vaccine (TIV) in young children, and found that only LAIV induced diverse T cells responses (109). Both studies show that the type of vaccination is of crucial importance both for the induction of T cell responses directly after vaccination and to T cell responses to the pathogen later in life. That age of the target group should be an important factor in the design of a vaccine is further exemplified by a study on influenza vaccination in elderly. In this study, antibody titers did not predict who developed influenza related illness, while T cell responses did (110). This effect is supported by evidence that T cell responses wane in elderly individuals. Several studies have shown that T cells from elderly individuals have a more differentiated phenotype characterized by the lack of CD27 expression and upregulation of CD57. The presence of CD57 on $CD8^+$ T cells is associated with decreased proliferation of $CD8^+$ T cells. Lack of markers, such as CD28, leads to an increased Th1 skewed response, which may contribute to decreased antibody titers in elderly individuals (111–113). Not only T cell responses wane, but also antibody responses diminish (114). Therefore, age of the target group should be an important consideration for the development of vaccines.

PROSPECTS FOR PEPTIDE VACCINATION

Taken together, severity of side effects is an important factor in the consideration of vaccine application. The licensed HPV peptide-based vaccine contains Montanide, which is a strong adjuvant causing lesions at the site of infection (87). For the therapeutic HPV vaccine, these side effects were deemed acceptable; however, they were one of the reasons to abort studies with Montanide-containing vaccines for HIV and malaria (88, 89). Consequently, before this peptide-based vaccine concept can be widely implemented, Montanide has to be replaced by another adjuvant. However, to elicit a response to these long overlapping peptides, a strong adjuvant is necessary. Therefore, the challenge is to increase immunogenicity of conserved targets for which T cells are available (43–45). A promising self-adjuvanting approach, which induces a broad response, is using multiple antigenic peptide (MAP). This approach was implemented in HCV patients by El-Awady et al. and was capable of inducing both antibody and T cell responses in two thirds of the patients (98, 115).

The ultimate goal in protection against rapidly mutating viruses such as influenza, is to develop a universal vaccine, protecting against currently circulating influenza strains, but also able to cross-protect against newly emerging strains and thereby preventing future pandemics. These preventive peptide-based vaccines should elicit a robust memory T cell response, since vaccine-induced T cells need to respond rapidly after infection to clear the virus before it causes illness. To induce a pool of both memory $CD4^+$ T cells and memory $CD8^+$ T cells, efficient priming of naïve T cells is required. Professional APCs need to present the antigen to both $CD4^+$ and $CD8^+$ T cells. As most vaccines induce T cells via extracellular routing, cross priming is of specific significance since it enables the presentation of extracellular-derived particles on MHC class I molecules. Targeting the more conserved parts of the virus by designing peptide-based vaccines, is a promising concept in the design of these preventive vaccines. Especially in influenza vaccine development, there are several examples of pilot vaccines directed to more conserved parts of the virus that should

cross-protect to heterologous viruses. These vaccines often contain both antibody and T cell inducing components (116, 117).

Concluding, in addition to antibody responses, T cell responses are of major importance in limiting and clearing virus infections. Effective therapeutic and preventive vaccines should therefore be able to induce both antibody and T cell responses. Peptide-based vaccines can meet these demands and induce both antibody and T cell responses. Furthermore, because peptides are synthetic, they are safe and relatively easy to produce. Currently, several peptide-based vaccines, for viruses such as EBV, HBV, and influenza virus, are evaluated in clinical trials (101). Hurdles to overcome are choosing the right target epitopes and choosing adjuvants that improve immunogenicity of these epitopes and steer the immune response in the desired direction. Adjuvants for peptide-based vaccines should target antigen to DCs, or other APCs capable of cross-presentation, and provide stimuli to ensure efficient presentation of the antigen. In addition, an overstimulation resulting in immunopathology should be avoided. Providing, these criteria are met, the future of peptide-based vaccines is very promising.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 31 January 2014; paper pending published: 27 February 2014; accepted: 31 March 2014; published online: 16 April 2014.
*Citation: Rosendahl Huber S, van Beek J, de Jonge J, Luytjes W and van Baarle D (2014) T cell responses to viral infections – opportunities for peptide vaccination. Front. Immunol. **5**:171. doi: 10.3389/fimmu.2014.00171*
This article was submitted to Antigen Presenting Cell Biology, a section of the journal Frontiers in Immunology.

frontiers in
IMMUNOLOGY

REVIEW ARTICLE
published: 08 April 2014
doi: 10.3389/fimmu.2014.00149

Understanding the biology of antigen cross-presentation for the design of vaccines against cancer

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Antigen cross-presentation, the process in which exogenous antigens are presented on MHC class I molecules, is crucial for the generation of effector $CD8^+$ T cell responses. Although multiple cell types are being described to be able to cross-present antigens, *in vivo* this task is mainly carried out by certain subsets of dendritic cells (DCs). Aspects such as the internalization route, the pathway of endocytic trafficking, and the simultaneous activation through pattern-recognition receptors have a determining influence in how antigens are handled for cross-presentation by DCs. In this review, we will summarize new insights in factors that affect antigen cross-presentation of human DC subsets, and we will discuss the possibilities to exploit antigen cross-presentation for immunotherapy against cancer.

Keywords: cross-presentation, dendritic cells, antigen processing and presentation, anti-cancer vaccine, $CD8^+$ T cells

INTRODUCTION

For the induction of antigen-specific $CD8^+$ T cells, antigen needs to be presented in MHC class I molecules in order to be recognized by the TCR/CD3 complex on $CD8^+$ T cells. Peptides derived from endogenous proteins degraded in the cytosol, that are transported into the endoplasmic reticulum (ER), are loaded on MHC class I molecules, which will be transported to the plasma membrane as a stable peptide–MHC class I complex (1). The presentation of endogenous-derived peptides allows the immune system to detect cells that present altered self peptides or foreign peptides and is therefore an important defense mechanism against cancer or viruses (2). Although peptide–MHC class I complexes can be directly recognized by naïve $CD8^+$ T cells, these cells require adequate co-stimulation from antigen-presenting cells (APCs) in order to become potent effector $CD8^+$ T cells with cytotoxic potential. Besides, APCs can also encounter exogenous antigens, namely of microbial or tumor origin, which they internalize for processing and presentation in MHC class I molecules, a phenomenon known as antigen cross-presentation.

Although multiple APCs are able to cross-present antigens, dendritic cells (DCs) are the most efficient cells *in vivo* (3–5). The potential of DCs to cross-present antigen has initiated many research questions aimed at finding strategies to enhance cross-presentation of DCs in order to improve tumor- and viral-specific $CD8^+$ T cell responses for the treatment of cancer or infectious diseases. Several questions remain unanswered, such as the molecular basis for the differences in cross-presentation efficiency observed amongst different DC subsets, in steady-state or under inflammatory conditions. In addition, recent studies also suggest that the capacity to cross-present can be influenced by the type of antigen and the presence and timing of inflammatory signals (6). This would imply that antigen cross-presentation is not a functional specialization of certain DC subsets, but a process that can occur in many APCs under specific conditions. In this review, we will discuss the factors that have been described to influence cross-presentation of various human DC subsets, and their implication in the design of immunotherapies against cancer.

CELL BIOLOGY OF ANTIGEN CROSS-PRESENTATION

A defining aspect of the adaptive immune system is its capacity to elicit antigen-specific cellular immune responses by the instruction of antigen-specific $CD4^+$ and $CD8^+$ T cells. This property is entirely based on the presentation of antigen in MHC molecules (the peptide–MHC complex) and its recognition by the T cell receptor. The loading of extracellular antigen in MHC-II, recognized by $CD4^+$ T cells, occurs in a different intracellular compartment than the loading of antigen in MHC-I, recognized by $CD8^+$ T cells. In the case of MHC-II, after its synthesis in the ER, complexes are formed with CD74 (also known as the invariant chain) to allow proper folding, trafficking, and protection of the peptide-binding groove. CD74 helps guiding the CD74–MHC-II complex move on to the endolysosomal pathway, where late endosomal proteases such as cathepsin S and L degrade CD74 and leave MHC-II complexed to the peptide-binding groove part of CD74 (the CLIP peptide), which is later exchanged for an antigenic fragment with the help of the chaperone HLA-DM (7). Although the process leading to antigen presentation on MHC-I also involves six basic steps (8); namely, acquisition of antigens (1); tagging of the antigenic peptide for destruction (2), proteolysis (3), transport of peptides to the ER (4), loading of peptides to MHC-I molecules (5), and the display of peptide–MHC-I complexes on the cell surface (6); the variety of intracellular compartments and pathways involved in MHC-I antigen presentation is considerably more complex than that of MHC-II.

The acquisition of antigenic peptides for MHC-I presentation is a highly heterogeneous process and multiple pathways have been

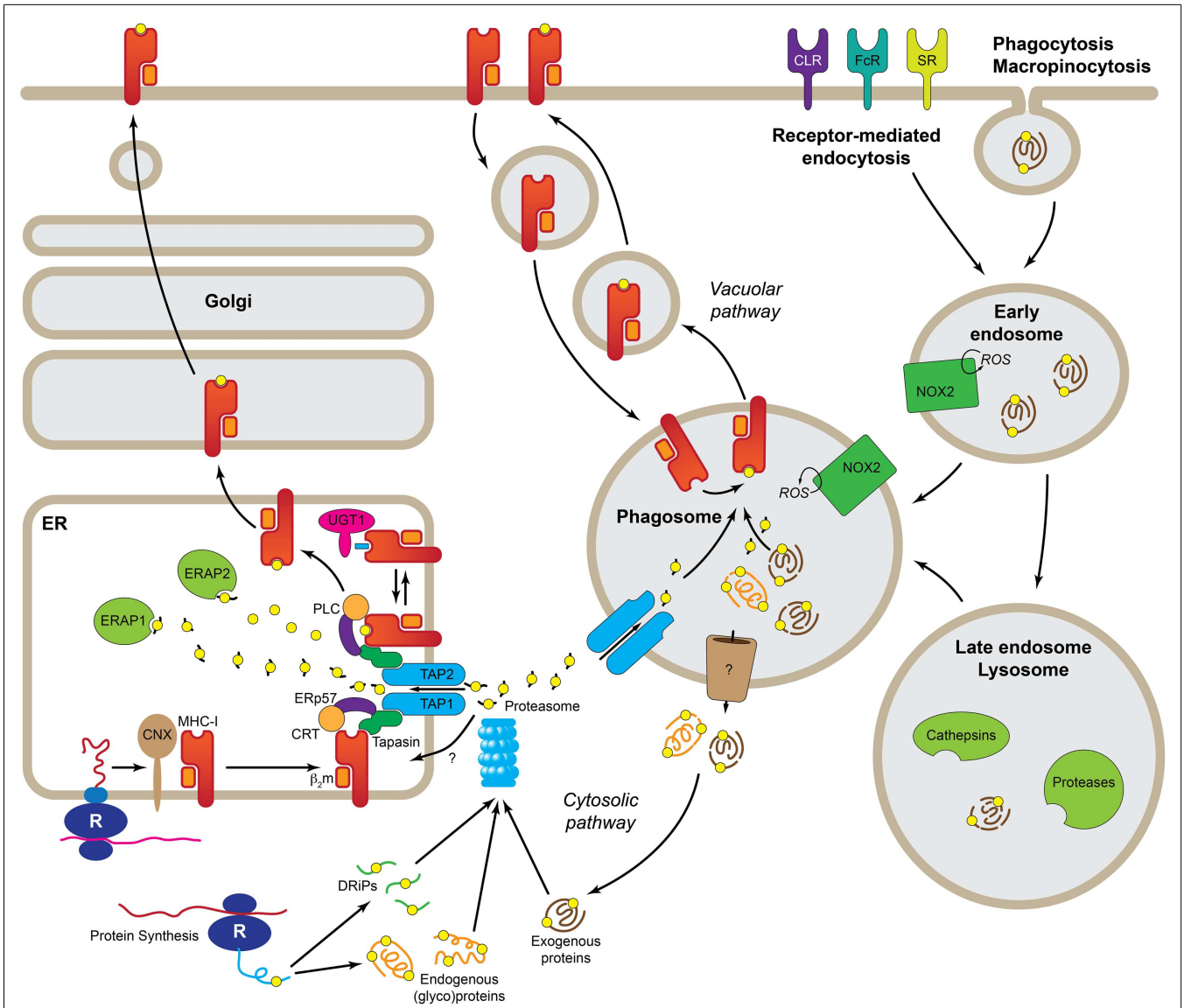

FIGURE 1 | Molecular pathways leading to cross-presentation in DCs. DCs take up Ag by three general mechanisms, receptor-mediated endocytosis, phagocytosis, or macropinocytosis. Once the Ag reaches the endolysosomal pathway, depending of the specific routing, it may be degraded by the concourse of the mild pH and different types of cathepsins and other proteases. At this point, properly degraded Ag can be directly loaded into recycling MHC-I in the phagosome (*Vacuolar* pathway). Ag that still needs further processing must be transported to the cytosol (*Cytosolic* pathway) where it is degraded, together with endogenous proteins and DRiPs, by the proteasome. The peptides generated by the proteasome are transported by TAP or a yet uncharacterized transporter into the ER where they are loaded into MHC-I with the help of the peptide-loading complex. Further trimming in the ER prior to loading, it is possible by the presence of ER-localized endopeptidases (ERAP1 and 2). R, ribosome; CNX, calnexin; CRT, calreticulin; b2m, b2microglobulin; UGT1, UDP-glucose:glycoprotein glucosyltransferase 1; ERAP1/2, ER-aminopeptidases 1/2; PLC, peptide-loading complex; ERp57, protein disulfide isomerase 3; TAP1/2, transporter associated with antigen-presenting 1/2; DRiPs, defective ribosomal products; ROS, reactive oxygen species; NOX2, NADPH oxidase 2; CLR, C-type lectins; FcR, Fc receptors; SR, scavenger receptors.

described so far. There are two main sources of antigens for MHC-I presentation, intracellular and extracellular (**Figure 1**). Antigenic peptides derived from cytosolic proteins, e.g., viral proteins, are the prime source of peptides for MHC-I (9), but other proteins carrying signal sequences targeting to the secretory pathway can also be presented on MHC-I, either from defective ribosomal products (or DriPs) (10) or from mature proteins (11). These mechanisms are at play on all cells expressing MHC-I. However, what makes DCs and, to a lesser extent also macrophages and B cells, best at cross-presentation is their capacity to use extracellular antigens as source of peptides for MHC-I presentation. The uptake of extracellular antigens by APCs is achieved by three main transport pathways, namely receptor-mediated endocytosis, phagocytosis, and macropinocytosis; although there are differences in the efficiency of each of these pathways amongst DCs, B cells, and macrophages. Thus, macrophages seem to be best at phagocytosis, whereas DCs

prefer receptor-mediated endocytosis. Amongst the many classes of receptors that mediate endocytosis of antigens are the B cell receptor (specific for B cells), Fc receptors, heat-shock protein receptors, scavenger receptors, and the C-type lectin receptors (CLRs). In general, these receptors mediate internalization of antigens to endosomes, however, the nature of the endosomes and their fate seems to vary for the different receptor types involved and, consequently, also their efficiency in inducing cross-presentation. Furthermore, many of the receptors involved in antigen uptake for cross-presentation are also able to mediate signaling and, in several cases, it has been demonstrated that signaling is necessary for cross-presentation. This was elegantly demonstrated in experiments where bacteria were opsonized with either antibodies or complement. Although both opsonization modalities lead to efficient phagocytosis, only the Fc receptor-mediated resulted in effective $CD8^+$ T cell responses (12). Signaling through other receptors, such as the C-type lectins, Dectin-1 (13) or DNGR-1 (also known as Clec9A) (14) also enhances cross-presentation.

Both endogenous and to a minor extent exogenous antigen can thus be loaded on MHC class I. A factor that conditions the access of peptides to MHC-I is the biosynthetic pathway of the MHC-I molecule. The MHC-I complex consists of a heavy chain, a transmembrane glycoprotein with a short cytoplasmic domain that, upon translation in the ER, assembles with β_2-microglobulin into a heterodimer. This process is integrated with the incorporation of the peptide into the peptide-binding groove of the heavy chain, and requires the participation of the peptide-loading complex, which consists of multiple components, including the ABC peptide transporter TAP that allows the transport of peptides from the cytosol into the ER (15). The key concept is that to this process, the MHC-I heterodimer is stabilized until a high-affinity peptide is incorporated into the peptide-binding groove. In most cases, cross-presentation is TAP- and proteasome-dependent (16), also called the *cytosolic* pathway. The proteasome is a self-compartmentalized, energy-dependent nanomachine that works as a protease to degrade misfolded, damaged, and inaccurately synthesized proteins (17). In the context of IFN-γ or DC maturation (18), the proteasome undergoes structural changes in its substrate-binding pockets that contribute to optimizing the quality and quantity of the generated peptides (19). Still, peptides generated by the proteasome may require further trimming by two ER-resident aminopeptidases (20). To make it more complex, proteasome-dependent, yet TAP-independent cross-presentation has been recently described, suggesting the existence of a still unidentified peptide transporter (**Figure 1**) (21).

A cross-presentation pathway referred to as *vacuolar* uses endolysosomal proteases to degrade internalized bacteria and other antigens, frequently particulated, in order to allow loading on MHC-I molecules recycled from the extracellular membrane (22). Also proteasome-derived peptides may enter the vacuolar pathway (23, 24). Data obtained from $TAP^{-/-}$ DCs, that are unable to incorporate peptides via TAP into the ER, indicates that cross-presentation is still possible, though to a lesser extent (25).

Several questions remain unsolved, such as the mechanism by which antigens are exported from endosomes into the cytosol for proteasomal degradation (9), whether hybrid organelles resulting from the recruitment of TAP and the peptide-loading complex to phagosomes and endosomes exist (26–28), and if interconnected ER-phagosomes are involved in cross-presentation (29). Regardless of this issue, evidence indicates that the accumulation of antigen in endosomes with low (but steady) proteolytic and relatively high pH conditions favors cross-presentation (30–32). In this respect, it has been proposed that limited antigen degradation correlates with efficient cross-presentation (30). Primarily decreased proteolysis is found in the endocytic compartments of DCs compared to other phagocytes, which is due to low levels of lysosomal proteases, or decreased protease activity. These can be regulated by high pH present, or high activity levels of the NADPH oxidase 2 (NOX2) in endosomal and phagosomal compartments of DCs.

HUMAN DC SUBSETS AND ANTIGEN CROSS-PRESENTATION

Two main subsets of human DCs have been described: plasmacytoid DCs (pDCs) and myeloid DCs (mDCs, also known as conventional DCs). The majority of pDCs are located in the blood and their main function is the production of type I IFN upon microbial infection (33). Recent data also show that human pDCs are capable to cross-present antigens either derived from apoptotic cells (34) or when antigens are encapsulated in nanoparticles and targeted to specific uptake receptors expressed by pDCs (35). Next to pDCs, two major populations of mDCs can be identified in blood: $BDCA1^+/CD1c^+$ DCs and $BDCA3^+/CD141^+$ DCs. The $BDCA3^+$ DCs are described as potent inducers of $CD8^+$ T cell responses *in vitro* and *in vivo* (36–39); however, it is not yet clear how this capacity relates to the other human DC subsets (39–41). A recent publication showed that blood $BDCA3^+$ DCs are more potent in cross-presentation compared to $BDCA1^+$ DCs when antigens of necrotic cells or soluble antigen were given that ended up in late endosomes and lysosomes (41, 42). In contrast, when antigens were targeted to early endosomes, using antigens conjugated to an anti-CD40 monoclonal antibody, $BDCA1^+$ DCs were as efficient at cross-presentation as $BDCA3^+$ blood DCs. These results suggest that the capacity of DC subsets to cross-present is not intrinsic, but might also be determined by the route of antigen uptake and subsequent accumulation of the antigen in early endocytic compartments.

Due to the small number of mDCs in tissues, studies on human mDCs have been hampered, with the exception of the human skin. Based on the expression of CD1a and CD14, the human skin contains at least three main subsets of DCs: $CD1a^+/CD1c^+$ dermal DCs (dDCS), $CD14^+$ dDCs, and $CD1a^{High}$ epidermal Langerhans cells (LCs), which all migrate to the skin-draining lymph nodes upon activation (43). LCs and $CD1a^+$ dDCs seem to be more efficient at cross-presentation, as compared to the $CD14^+$ dDCs (44, 45). In addition to the three main populations of skin DCs, a minor $BDCA3^{High}CD14^-CD111c^{low\text{-}int}$ subset of DCs is recently identified in human skin, lung, and liver. Parallel phenotypic analyses suggest that these cells are potentially related to blood $BDCA3^+$ DCs. The skin $BDCA3^{High}$ DCs have shown to be superior in cross-presentation of soluble antigens when compared to the other skin DC subsets, as well as compared to $BDCA3^+$ DCs, $BDCA1^+/CD1c^+$ DCs, and $CD14^+$ monocytes derived from blood (41). Care should be taken not to confuse the $BDCA3^{High}$ skin DCs described by Haniffa et al. with the

dermal BDCA3$^+$CD14$^+$ DCs described by Chu et al. (46). The latter are immunoregulatory tissue-resident DCs characterized by the constitutive secretion of IL-10 (46).

Altogether, findings on cross-presentation capacity of human DC subsets show that most subsets are capable to cross-present antigens. However, it becomes clear that other factors also influence the capacity to cross-present, like the antigen formulation, the mode of delivery, and the intracellular routing of the antigen, as well as the activation signals for the DCs.

FACTORS DETERMINING CROSS-PRESENTATION

The capacity of DC to cross-present antigens is not only dictated by characteristics of a given DC subset, but it starts to become clear that additional factors influence the cross-presentation capacity of these DC subsets as well. It must be mentioned however that most knowledge about human DC function is obtained from *in vitro* studies and thus may not fully reflect their behavior *in vivo*.

MODE OF ANTIGEN INTERNALIZATION

Antigens can be taken up by DCs via multiple mechanisms, including non-specific, receptor-independent processes, like pinocytosis and phagocytosis, or via specific, receptor-mediated processes such as uptake through CLRs, Fc receptors, and scavenger receptors. Blood BDCA3$^+$ DCs are reported to be able to cross-present untargeted pp65 recombinant protein to a lesser extent than blood BDCA1/CD1c$^+$ DCs *in vitro*. However, when the cells were stimulated with polyI:C, the BDCA3$^+$ blood DCs became more potent to cross-present the pp65 protein compared to CD1c$^+$ blood DCs (39). These results were confirmed by Mittag et al., who showed that CD1c$^+$ blood DCs are more potent in cross-presenting soluble influenza protein without TLR stimulation, but in the presence of polyI:C the BDCA3$^+$ blood DCs became more potent (47). Surprisingly, they also show that pDCs were able to cross-present soluble protein in the absence of polyI:C. Whether human blood-derived pDCs are capable to cross-present soluble proteins is questionable, since others provided evidence that pDCs were unable to cross-present soluble proteins in the presence and absence of TLR stimulation (48–50).

In addition, cross-presentation of NY-ESO-1 antigen administrated as antigen-antibody immune complexes (IC), allowing Fcγ receptor-mediated uptake, did not enhance antigen-specific CD8$^+$ T cell responses by pDCs (50). In comparison, BDCA1$^+$ blood DCs cross-presented the Fcγ receptor-targeting NY-ESO-1/IC more efficiently compared to the soluble protein formulation. Another study also showed that Fcγ receptor-mediated uptake of pp65-IC enhanced the cross-presentation capacity of both the BDCA1$^+$ and BDCA3$^+$ DCs compared to the uptake of HCMV pp65 protein (50, 51). These studies indicate that the mode of antigen internalization and antigen formulation have a profound impact on cross-presentation capacity.

Besides the uptake of antigen via Fcγ receptors, receptor-mediated uptake is also often studied using CLRs to stimulate antigen cross-presentation and CD8$^+$ T cell responses. CLRs are a family of pattern-recognition receptors expressed by DCs and recognize various carbohydrate structures. Upon recognition and binding to the receptor, most CLRs respond by internalization and processing of the antigen (52). Their specific expression on certain DC subsets and the capacity to internalize antigens, make CLRs interesting targets to induce cross-presentation.

Targeting of antigen to the CLR DCIR, which is expressed by all human DC subsets tested, including LCs and blood mDCs and pDCs, resulted in improved cross-presentation by all subsets (53). Again, the blood mDC subset induced the highest percentages of tetramer-positive CD8$^+$ T cells, indicative of a superior capacity to cross-present antigens, also when they are taken up in a receptor-mediated fashion. However, not all receptors show the same effects on antigen cross-presentation, as shown by Cohn et al. (42). Their study showed that BDCA3$^+$ DCs were superior in cross-presentation of antigens taken up via the CLR DEC-205, which routes antigen to late endosomes and lysosomes, compared to BDCA1$^+$ DCs and pDCs. However, when antigens were delivered to early endosomes through conjugation to CD40 or CD11c, BDCA1$^+$ DCs and pDCs were as efficient in antigen cross-presentation as the BDCA3$^+$ DCs (42). Furthermore, Chatterjee et al. have shown that targeting antigen to CD40 resulted in the most efficient cross-presentation in human moDCs and BDCA1$^+$ DCs, despite the fact that CD40 was least efficient in antigen internalization compared to DEC-205 or mannose receptor (MR) (54). These results indicated that routing of antigen to more degradative, late endosomes, via DEC-205- or MR-mediated uptake, may have a negative effect on cross-presentation compared to antigen routing to early endosomes. Altogether, the results demonstrate that the intracellular routing of antigens is of importance for antigen cross-presentation. Thus, all human DC subsets seem to have the capacity to cross-present antigens, provided that the antigen is given in a suitable formulation under appropriate conditions.

ANTIGEN FORMULATION

The antigen form and mode of delivery is crucial in determining the efficiency of cross-presentation. As DCs encounter antigens in many sizes and shapes, derived form various sources, multiple antigens might be differently handled by DCs, which might result in modification of the intracellular routing of antigen, thereby affecting the potency to cross-present. As described above, antigen can be soluble, as synthetic long peptides, protein, or it can be included in a pathogen/viral structure, as necrotic cells or as immune complex. Alternatively, antigens can be conjugated to antibodies specific for DC uptake receptors, or glycans that interact with CLRs. These different antigen formulations may affect the size of the antigen and receptor-targeting specificity, possibly affecting the type of DC that interacts with the antigen and the mode of uptake and intracellular routing.

To achieve and promote cross-presentation, different antigen formulations have been studied, such as nanoparticles, apoptotic cells or monoclonal antibodies, or glycans conjugated to antigens as discussed earlier. Targeting antigen to DNGR-1/CLEC9a, which expression in humans and mouse is restricted to CD8α$^+$-like DCs (55), using PLGA nanoparticles conjugated to CLEC9a Moabs increased cross-presentation compared to isotype-control PLGA nanoparticles, implying that antigen uptake via CLEC9a enhances routing of the antigen to the cross-presentation machinery (53). Our own results show that targeting antigen to the CLR DC-SIGN using glycan- or antibody-modified liposomes results in enhanced cross-presentation capacity of DCs *in vitro* and *in vivo*

(56). Furthermore, dendrimer technology has shown that a multivalent presentation of antigen, as well as particle size, enhances cross-presentation by DCs. Glycosylation of dendrimers enhances the DC-SIGN-mediated uptake of the particles, favoring enhanced $CD4^+$ and $CD8^+$ T cell responses (57).

There is evidence that also for LCs, the antigen formulation is crucial in order to allow cross-presentation by LCs. It has been shown that isolated human LCs were incapable to cross-present heat-inactivated measles virus, which is specifically taken up via Langerin (58). In contrast, others have shown that skin-derived human LCs were capable to cross-prime influenza-specific $CD8^+$ T cells after targeting with an influenza protein conjugated to anti-Langerin antibodies (48), demonstrating that there is an inconsistency whether human LCs can cross-present or not and under which circumstances. Altogether, these findings demonstrate that the formulation of antigen (either small peptides or bigger particles, like viral- or bacterial-antigens, necrotic cells, and nanoparticles) has proven to have an influence on the cross-presentation capacity of various DC subsets.

ADJUVANTS AND DC MATURATION STATUS

In general, DC maturation enhances the potency of DCs to cross-present antigen. A large set of TLR ligands are known that act as adjuvants and stimulate cross-presentation. Because each DC subset may express a specific set of TLR receptors, they may differently respond to TLR ligands, influencing the induction of cross-presentation. For example, isolated human LCs show increased cross-presentation of antigenic peptides in the presence of the TLR3 ligand polyI:C, whereas addition of the TLR4 adjuvant LPS or the TLR7/8 adjuvant R848 does not enhance the capacity to cross-present (Fehres et al., submitted). For instance the human skin, an attractive site for vaccination because it harbors many, easy-accessible DCs, is currently studied to identify suitable adjuvants to trigger and activate skin DCs for cross-priming. We and others have shown that intradermal administration of soluble TLR ligands does not induce DC maturation as observed with *in vitro* generated monocyte-derived DCs (59). The discrepancy between DC maturation after TLR activation *in vitro* and *in situ* might be caused by specific, local suppression within the skin microenvironment. Ideally, the adjuvant simultaneously stimulates several cell types, resulting in a mix of activated immune cells, cytokines, and chemokines at the vaccination site. Most promising into this respect seems Aldara, an FDA-approved immune response modifier skin cream, containing 5% of the TLR7 agonist imiquimod. Aldara is mostly used to treat non-melanoma skin tumors. Recently it was shown that application of Aldara cream results in inflammasome activation and IL-1 release by keratinocytes in naïve murine skin (60). This effect was mediated independent of TLR7 activation and attributed to isostearic acid, the major component of the vehicle. However, for induction of full inflammation, both imiquimod and the vehicle cream were shown to be required. Following topical application of Aldara skin cream to human skin explant, we observed enhanced migration and maturation of dermal DCs (Fehres et al., submitted). Combining the Aldara skin cream with Mart-1-peptide vaccination in human skin affected the migratory potential of $CD14^+$ skin DC, which was associated with up-regulation of co-stimulatory molecules and increased activation and IFN-γ secretion of Mart-1-specific $CD8^+$ T cells. Notably, the enhanced effects on DC and T cell activity were not observed when injecting soluble TLR7 and/or 8 ligands intradermally.

Besides being used as adjuvant in cancer vaccines, the aforementioned DC stimuli have also been used as stand-alone immunotherapeutics. It is anticipated that application of adjuvants at the tumor site reverses the immune-inhibitory phenotype of tumor-infiltrated DCs that ingest tumor antigens (TA), herewith restoring TA-specific T cell priming and anti-tumor immunity. An advantage of local delivery is a strong reduction in immune-related adverse events such as cytokine release syndrome and liver toxicity observed with systemic treatment. Indeed, topical application of the imiquimod containing cream led to residual tumors in 8% of patients in basal and squamous cell carcinoma patients (61). Furthermore, near tumor injection of low doses of agonistic anti-CD40 antibodies in a slow-release formulation was shown to activate TA-specific $CD8^+$ T cells, which were able to act systemically and eradicate distant tumors (62). In addition, intra-tumoral injection of a TLR2/6 agonist spectacularly prolonged survival of pancreatic cancer patients with 9 months (63). The beneficial effects of TLR2/6 treatment were attributed to emergence of a strong immune response. Increased NK cytotoxic activity as well as elevated levels of TNF and IL-6 were noted.

Although soluble TLR ligands do not evoke strong maturation of skin DCs when injected into the skin as adjuvant, the discovery that tumor cells express TLRs has evoked interest in application of TLR agonists as monotherapy at the tumor site (64). Administration of a TLR3 agonist in melanoma lesions limits cell proliferation directly. Additionally, combined with a protein synthesis inhibitor even tumor cell death was induced (65).

The use of intradermal injected cytokines as immunostimulators has been explored (66, 67). In particular, granulocyte-macrophage colony-stimulating factor (GM-CSF) enhanced recruitment of DCs to the vaccine administration site, which ensures presentation of the administered TA by professional DCs and consequently priming of TA-specific T cells (66, 68). Furthermore, clinical trials have been conducted and/or are ongoing in which patients receive irradiated tumor cells genetically engineered to over-express GM-CSF (69). A small number of responses were demonstrated in Phase I trials in renal cell carcinoma and melanoma patients (68). However, in subsequent studies, GVAX monotherapy did not result in clinical responses. Indeed, the efficacy of GVAX might be improved by combining with immune check-point inhibitors, which aim to prevent inhibition of effector T cells and/or to silence Tregs. In murine pre-clinical models, GVAX combined with anti-CTLA-4 treatment enhanced efficacy and tumor regression in the B16 melanoma model, along with the presence of certain toxicities, such as skin depigmentation (70). Recently, a phase I study was completed showing dose escalation and safety, warranting further investigation of treating patients with this combination. Alternatively, GVAX has been combined with chemotherapeutic agents such as cyclophosphamide, which is currently being tested in clinical trials in metastatic melanoma patients. However, chemotherapy has been associated with immunosuppressive effects at standard doses, rendering issues related to dosing and timing of application critical.

The effect of GM-CSF may be further enhanced by co-administration of IL-2. Adjuvant activity has also been attributed to IL-2, which has been widely used in clinical trials and usually is administered systemically. However, in murine tumor models GM-CSF and IL-2 were shown to act synergistically when applied intradermal in emulsion along with a peptide, leading to improved and long-lasting peptide-specific CTL responses (66).

However, care should be taken using IL-2 as it may negatively impact on anti-cancer responses (e.g., promoting the accumulation and/or activation of Tregs). Recently, attention has focused on another cytokine belonging to the common gamma chain family: IL-21. IL-21 can exert potent anti-tumor effects due to its ability to induce and expand $CD8^+$ CTLs and NK cells. Importantly, IL-21 suppresses FOXP3 expression and the expansion of regulatory T cells (Tregs). Recently, it has been shown that tumor-infiltrating lymphocytes expanded *ex vivo* with APCs engineered to secrete IL-21 performed better than those expanded in the presence of IL-2 (71). Moreover, the $CD8^+$ T cells expanded in the presence of IL-21 exhibited a less differentiated, "young" phenotype. To date, there are no studies describing inclusion of IL-21 in therapeutic vaccines. Yet, promising results have been obtained *in vitro*: mature DCs transfected with IL-21 were superior in priming naïve $CD8^+$ T cells than non-transfected DCs (72).

MICROMILIEU RENDERING T CELLS DYSFUNCTIONAL

Both, chronic antigen expression and suboptimal priming in the tumor-environment renders TA-specific T cells dysfunctional. Chronic exposure to TA leads to exhausted T cells while suboptimal priming due to poor antigen presentation at tumor sites drives T cells into anergy (73, 74). These different aspects of T cell function can be discerned by addressing expression of specific sets of inhibitory receptors on TA-specific T cells. TA-specific CTLs present in peripheral blood lymphocytes (PBL) or at tumor sites have been shown to up-regulate PD-1 expression, which regulates their expansion (75–77). Next to PD-1, also the inhibitory receptors Tim-3 and LAG-3 can be upregulated on tumor-infiltrating T cells and serve as markers for exhausted T cells. By contrast, anergic T cells are characterized by BTLA expression (78). Notably, BTLA has been detected on spontaneous Mart-1- and NY-ESO1-specific $CD8^+$ T cells in advanced melanoma patients (79, 80).

Expression levels of PD-1 on exhausted T cells correlate with inhibition of different aspects of CTL function (81). As blocking Abs display most affinity for PD-1^{high} expressing cells, functions inhibited due to low and/or intermediate PD-1 levels will not be regained (i.e., IL-2, TNF-α production and proliferation and cytotoxic activity, and IFN-γ production, respectively). The observation that PD-1 block does not alleviate the function of TA-specific CTLs on a per-cell basis argues in favor of combining this strategy with blocking other immune check-point inhibitors. Indeed, studies performed in patients and in mice with advanced melanoma showed that blockade of both PD-1 and Tim-3 acts synergistically to enhance TA-specific $CD8^+$ T cell numbers and functions, resulting in decreased tumor growth (82–84). Likewise, combining Lag-3 blockade with PD-1 blockade may enhance activity of PD-1 blockade.

It has been shown that TA-specific $CD8^+$ T cells exhibited variable levels of dysfunction, which correlated with a specific expression pattern of markers (80). BTLA blockade has been shown to act in concert with PD-1 and Tim-3 blockades to further enhance NY-ESO-1-specific $CD8^+$ T cell expansion and function (80). The specific combination of inhibitory and anergy-related molecules might indicate a hierarchical loss of T cell function in patients with advanced melanoma in context of chronic antigen stimulation. Moreover, BTLA expression is inversely correlated with CD8 T cell maturation and thus anergic $BTLA^+$ T cells are likely to represent young TA-specific CTLs. Recently, a positive association of $CD8^+$ T cells expressing BTLA with clinical response to adoptive T cell therapy in late-stage melanoma patients has been suggested by Haymaker (85).

Alternatively, these approaches may be even more active when combined with other agents that activate or inhibit key molecular regulators of T cell function, such as, for example, the tryptophan converting enzyme indoleamine-2,3-dioxygenase (IDO) and the membrane-bound CD39 and CD73 that breakdown arginase. IDO is highly expressed in both tumor cells and immune cells in the tumor-environment and implicated in inhibiting anti-tumor immunity by promoting the induction of anergic and/or regulatory T cells (86–89). Importantly, using pre-clinical animal melanoma models it was recently shown that IDO is responsible for mediating resistance to anti-CTLA-4 and anti-PD-1 therapy (90). Two IDO inhibitors have entered clinical trials: the tryptophan analog 1-methyl-tryptophan (1-MT) and the enzymatic inhibitor of IDO termed INCB024360. Both IDO inhibitors have been effective in pre-clinical models, attenuating tumor growth in wild type but not immuno-deficient mice (91, 92). INCB024360 has now entered Phase II trials, where it will be tested as a monotherapy in ovarian cancer and as a combination therapy with ipilimumab (anti-CTLA-4) for metastatic melanoma (**Figure 2**).

IMPLICATIONS FOR THERAPY DESIGN – FUTURE DIRECTIONS

Our understanding in the mechanism of cross-presentation is crucial in the design of vaccination strategies aimed to induce protective immunity in the field of infectious diseases and cancer, which depends on the induction of both effector $CD4^+$ and $CD8^+$ T cells. Enhanced immune protection was obtained by long synthetic peptides compared to short peptides, which required cross-presentation of DC, resulting in long-lasting T cell stimulation that leads to the eradication of tumors (93, 94). Studies on improving cross-presentation-based vaccinations have emerged as a promising tool for immune intervention, based on many human *in vitro* studies and murine *in vivo* work. Strong focus on DC-targeting receptors *in vivo* that mediate endocytosis show potential of efficient induction of $CD8^+$ T cell cross-priming, but can also lead to $CD8^+$ T cell cross-tolerance. This fine tuning between the induction of immunity or tolerance is dictated by the various parameters that affect cross-presentation as mentioned under the Sections "Human DC Subsets and Antigen Cross-Presentation and Factors Determining Cross-Presentation," the vaccine formulation, DC subset, receptor-targeting, endocytosis, and maturation stimuli. Many *in vivo* DC-targeting studies have been performed in mice that have demonstrated effective induction of tumor $CD8^+$ effector T cell responses through targeting of CLRs, such as CD205,

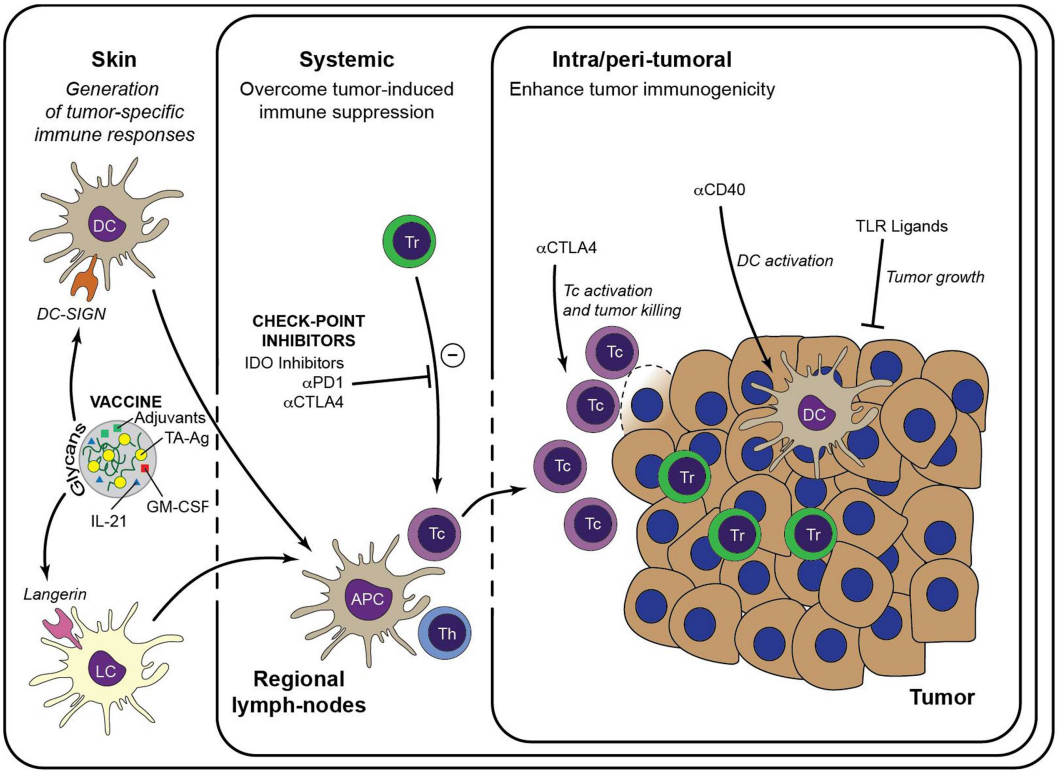

FIGURE 2 | Immunotherapeutic strategies to enhance anti-tumor immunity. Generation of a large pool of effector TA-specific T cells is induced by the intradermal injection of anti-tumor vaccines. Targeting of the vaccine to a particular skin DC subset is facilitated by modification with specific glycans that bind either to DC-SIGN or Langerin. Subsequent vaccine internalization induces presentation of TA-Ag and maturation of the DCs. Matured DCs migrate to draining lymph nodes to prime TA-specific $CD8^+$ T cells and $CD4^+$ T helper cells, leading to a large pool of cytotoxic effector T cells that are capable to infiltrate the tumor lesion and lyse tumor cells. Priming of TA-specific T cells may be enhanced by inclusion of immunostimulators such as GM-CSF and IL-21 in the DC-targeting vaccine. Systemic or intra-tumoral administered check-point inhibitors, such as anti-PD-1 and anti-CTLA-4, release the break on the anti-tumor immune response by limiting the activity of suppressive Treg and alleviate the priming and/or function of TA-specific CTLs. Similarly, anti-tumor immunity may be enhanced by manipulation of the local micromilieu via administration of DC activating antibodies (e.g., anti-CD40) or of TLR ligands that act directly on the tumor cells. It is anticipated that these strategies may enhance the efficacy of DC-targeted vaccination. Tc, cytotoxic $CD8^+$ T cell; Th, T helper cell.

MR, CD207 (Langerin), CD209 (DC-SIGN), CLEC9A or other cell-surface receptors like integrins, HSP receptors, and glycolipids. In contrast, only a few of these DC-targeting vaccines have been tested in human clinical trails. Easy translation from mouse models to humans is complicated by the different expression levels of DC-targeting receptors and DC restriction and usage of TLRs between mouse and human. Moreover, still little is known on the cross-presenting capacity of human DC *in situ*. This has hampered the development of novel-targeted vaccination strategies for clinical applications, and is a complicated task to fulfill in the coming years. Highly effective DC-targeting therapies should overcome the mechanism of immune escape dictated by the tumor microenvironment. Therefore, combined regimens consisting of strategies to improve DC-induced T cell responses, increasing the frequency of anti-tumor T cells, reversing T cell exhaustion that stimulate trafficking to the tumor site, along with a blockade of immune-inhibitory pathways, all may be necessary to achieve clinical benefit for cancer patients.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 29 January 2014; accepted: 21 March 2014; published online: 08 April 2014.
*Citation: Fehres CM, Unger WWJ, Garcia-Vallejo JJ and van Kooyk Y (2014) Understanding the biology of antigen cross-presentation for the design of vaccines against cancer. Front. Immunol. **5**:149. doi: 10.3389/fimmu.2014.00149*
This article was submitted to Antigen Presenting Cell Biology, a section of the journal Frontiers in Immunology.

frontiers in
IMMUNOLOGY

REVIEW ARTICLE
published: 01 April 2014
doi: 10.3389/fimmu.2014.00140

Antigen cross-presentation of immune complexes

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The ability of dendritic cells (DCs) to cross-present tumor antigens has long been a focus of interest to physicians, as well as basic scientists, that aim to establish efficient cell-based cancer immune therapy. A prerequisite for exploiting this pathway for therapeutic purposes is a better understanding of the mechanisms that underlie the induction of tumor-specific cytotoxic T-lymphocyte (CTL) responses when initiated by DCs via cross-presentation. The ability of humans DC to perform cross-presentation is of utmost interest, as this cell type is a main target for cell-based immunotherapy in humans. The outcome of a cross-presentation event is guided by the nature of the antigen, the form of antigen uptake, and the subpopulation of DCs that performs presentation. Generally, CD8α$^+$ DCs are considered to be the most potent cross-presenting DCs. This paradigm, however, only applies to soluble antigens. During adaptive immune responses, immune complexes form when antibodies interact with their specific epitopes on soluble antigens. Immunoglobulin G (IgG) immune complexes target Fc-gamma receptors on DCs to shuttle exogenous antigens efficiently into the cross-presentation pathway. This receptor-mediated cross-presentation pathway is a well-described route for the induction of strong CD8$^+$ T cell responses. IgG-mediated cross-presentation is intriguing because it permits the CD8$^-$ DCs, which are commonly considered to be weak cross-presenters, to efficiently cross-present. Engaging multiple DC subtypes for cross-presentation might be a superior strategy to boost CTL responses *in vivo*. We here summarize our current understanding of how DCs use IgG-complexed antigens for the efficient induction of CTL responses. Because of its importance for human cell therapy, we also review the recent advances in the characterization of cross-presentation properties of human DC subsets.

Keywords: anti-tumor immune responses, DC subset functions, cell type-specific cross-presentation, IgG-complexed antigens, Fc receptor-mediated antigen uptake, CD8$^+$ T cell priming

INTRODUCTION

The mechanism of cross-presentation allows exogenous antigens to access the processing and presentation machinery of a cell so that exogenous antigenic peptides are displayed on MHC class I molecules for T cell recognition, which consequently leads to the priming of CD8$^+$ T cell responses. As such, the cross-presentation pathway is essential for inducing cytotoxic T-lymphocyte (CTL) responses against viruses as well as intracellular bacteria, which do not infect the APC (1–4). Additionally, cross-presentation is thought to be crucial in mounting immune responses against tumor antigens. Indeed, cross-priming of tumor reactive cytotoxic CD8$^+$ T cells through cell-based tumor vaccines is a major goal in cancer immunotherapy (5, 6). Induction, the so called priming, of tumor-specific CD8$^+$ T cells is an appealing therapeutic strategy because the generated CTLs not only mediate antigen-specific killing of the targeted tumor via cell–cell contacts, but also provide the host with long-lasting memory responses that may prevent cancer recurrence.

Dendritic cells (DCs) have been proven to be superior in routing exogenous protein antigen toward cross-presentation; however, they comprise a heterogeneous cell population, and significant differences in the cross-presentation capacity of different DC subsets have been reported (4). Importantly, cross-presentation of antigen does not result solely in the priming of CTLs but can also lead to the induction of cross-tolerance (7). The latter immunological outcome should by all means be avoided during cancer therapy. Thus, to take full advantage of the therapeutic potential of antigen cross-presentation by DCs, significant effort was made to delineate precisely how cross-presentation is initiated and regulated. By now, many mechanistic details of antigen cross-presentation have been discovered whereas others still remain enigmatic. In contrast to MHC class II-restricted antigen presentation, the default pathway for the display of exogenous antigens for immune recognition and the induction of CD4$^+$ T cell responses, cross-presentation *in vivo* is thought to be controlled rather strictly by the type of DCs used as antigen-presenting cells. In this review, we summarize the current knowledge on how immune complexes facilitate antigen cross-presentation and expand the cross-presentation capacity of specific DC subsets. We also discuss the therapeutic potential of this cross-presentation pathway.

IgG IMMUNE-COMPLEXED ANTIGENS ENTER THE CROSS-PRESENTATION PATHWAY THROUGH Fc RECEPTORS

Our immune system has to respond to a variety of different forms of antigens and thus has developed an array of mechanisms to

deal with antigenic diversity. Antigens can be small soluble molecules, which are taken up by fluid phase mechanisms, or larger particles, such as bacteria, which are phagocytosed. To facilitate antigen uptake and processing, DCs also use an assortment of endocytic receptors (**Figure 1**). Several of these endocytic receptors belong to the C-type lectin family. For example, DEC-205, the mannose receptor, and Clec9a have been shown to efficiently shuttle antigen for cross-presentation. Several recent reviews give detailed insight into the functional differences of these endocytic receptors, and they are therefore only briefly mentioned here (8–10). Importantly, monoclonal antibodies against these endocytic receptors have been employed to target antigen to DCs for cross-presentation, and using this strategy, encouraging anti-tumor immunity was initiated in mice (11–13). Thus, strong emphasis is continuously put on targeting of cross-presenting DCs to elicit anti-tumor responses, as exhibited in several ongoing clinical trials (11, 14–16). A so far therapeutically less exploited but remarkably effective way for DCs to internalize antigen for cross-presentation is via Fc receptors (**Figure 1**). Antigens, especially under inflammatory conditions, can be found already bound to antigen-specific antibodies, and these antigen–antibody complexes (referred to as immune complexes or immune-complexed antigen) can be recognized by Fc receptors through the Fc region of the antibodies. Binding of the immune complexes typically triggers crosslinking of the Fc receptors, their internalization together with the antigen, and shuttling of the immune complexes toward antigen presentation compartments (17, 18).

Before the crucial role of Fc receptors in antigen cross-presentation was identified, their value in enhancing antibody-dependent cellular cytotoxicity (ADCC) by inflammatory cells, including neutrophils and macrophages, was already recognized (19). Enhancement of T cell proliferation via antigen-specific antibodies that bind Fc receptors became evident in the mid-1980s (20–22). Studies using Fcγ receptor knockout mice revealed the general requirement of Fcγ receptor engagement for the effectiveness of anti-tumor immune responses *in vivo*. The finding that anti-tumor antibodies require the induction of CTL responses to be effective suggested early on that Fcγ receptors contribute to anti-tumor immunity in addition to mediating ADCC (23). Shortly after, it was compellingly demonstrated that endocytosis of immune complexes via Fcγ receptor allows MHC class I-restricted antigen presentation and the priming of CTLs (24, 25). The finding that DCs use immunoglobulin G (IgG)-immune complexes to efficiently prime specific $CD8^+$ CTL responses was shortly thereafter confirmed *in vivo* (26). Furthermore, it was shown that only antigen targeting to FcγR on DCs, but not antigen targeting to surface immunoglobulins on B cells, induces efficient cross-presentation, despite the fact that both targeting strategies allow these cell types to present antigen via MHC class II with equal efficiency (27).

The therapeutic potential of Fc receptor-mediated antigen uptake for anti-tumor immunotherapy became evident early on. Studies with human cells demonstrated that coating human myeloma cells with monoclonal antibodies promotes cross-presentation of myeloma-associated antigens by human DCs. The enhanced cross-presentation of tumor antigen was preventable by pretreatment of the DCs using Fcγ receptor blocking antibodies (28). Notably, this study did not observe that Fcγ receptor-mediated antigen uptake induces significant phenotypic maturation of human DCs, as it has been described for murine DCs (24, 26, 27). The possible absence of maturation induction in human DCs through immune complexes is important to keep in mind with regard to a clinical applicability of Fc receptor targeting. DC maturation in the context of antigen uptake is considered to be a crucial attribute that must be achieved to induce efficient CTL responses by cross-presentation receptors because otherwise cross-tolerance may be induced (7). Overall, although there is substantial evidence suggesting that cross-presentation of immune-complexed antigen via Fcγ receptors is a promising tool to develop DC-based vaccination strategies, there are several factors, which we will discuss below, that have so far hampered the applicability.

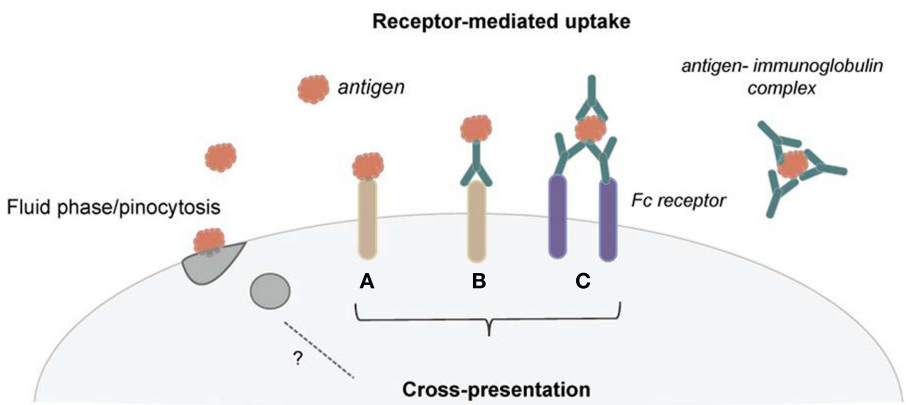

FIGURE 1 | Dendritic cells use several mechanisms of antigen uptake for cross-presentation. **(A)** Several receptors have been shown to efficiently shuttle exogenous antigen into the cross-presentation pathway. **(B)** These receptors are now employed to target DCs *in vivo* for cancer immunotherapy using receptor-specific antibodies coupled with antigen. **(C)** Immunoglobulins can bind to antigen and form immune complexes. These immune complexes can then be taken up via Fc receptors and deliver antigen for cross-presentation. Pinocytosis seems not to be an effective mechanism for routing antigen toward cross-presentation.

CROSS-PRESENTATION OF IMMUNE COMPLEXES AND THE DIVERSITY OF Fc RECEPTORS

A major difficulty for studying and determining the therapeutic applicability of cross-presentation of immune complexes is the complexity of the Fcγ receptor family [**Table 1**; Ref. (29)]. Several types of Fc receptors have been found in addition to species-dependent differences. In mice, four different classes of Fcγ receptors comprising FcγRI, FcγRIIB, FcγRIII, and FcγRIV have been described. The activating Fc receptors FcγRI, FcγRIII, and FcγRIV consist of an immunoglobulin binding α-chain and a signal transducing γ-chain, which carries an immunoreceptor tyrosine-based activation motif (ITAM). In contrast, FcγRIIB is a single chain inhibitory receptor with an immunoreceptor tyrosine-based inhibitory motif (ITIM). The human FcγR system seems to be far more complex as exemplified by the presence of gene families for FcγRI and FcγRII, as well as the presence of several allelic forms for FcγRIIIA, FcγRIIIB, and FcγRIIB. Mouse FcγRIV is most closely related to human FcγRIIIA whereas mouse FcγRIII is most similar to human FcγRIIA. FcγRIIIB is unique for the human system, but both species have the inhibitory function of FcγRIIB in common.

Dendritic cells simultaneously express activating and inhibitory Fc receptors [reviewed in Ref. (18)]. The conserved expression of an inhibitory Fc receptor along with activating Fc receptors among species suggests that Fc receptor-mediated cross-presentation is tightly regulated *in vivo*. The requirement of strictly controlling Fc receptor-mediated cross-presentation was demonstrated by studies that show that antibody-mediated cross-presentation of self-antigens contributes to autoimmune disease (34, 35). The authors looked at the development of autoimmune diabetes in RIP-OVA mice. In this model, the transfer of OVA-specific naïve $CD8^+$ T cells induces peripheral tolerance. Importantly, the co-administration of anti-OVA IgG leads to $CD8^+$ T cell-driven diabetes through the activating Fcγ receptors on DCs. The disease pathogenesis in this model was further augmented in FcγRIIB knockout mice, suggesting a tolerogenic function of FcγRIIB *in vivo*. In line with a tolerogenic function of this receptor, it was shown that DCs from FcγRIIB knockout mice generate overall stronger immune responses and that blocking immune complex binding to FcγRIIB promotes DC maturation, which is considered one of the most important factors for efficient priming of CTL responses (36–39). This suggests that expression of inhibitory FcγRIIB, which restricts DC maturation under non-inflammatory conditions and thus probably prevents autoimmunity, may hamper immunotherapeutic approaches against tumors and microbial infections (29, 40). Hence, it is important to be aware of the expression patterns and ratios of activating versus inhibitory Fc receptors on murine and human DCs when studying the effects of immune complexes.

Additionally, IgG subclass composition of immune complexes has been shown to influence binding affinity resulting in different binding properties to individual Fc receptors (41). For example, immune complexes composed of human IgG1 bind with relatively high affinities to all Fc receptors, whereas IgG2 immune complexes seem to bind primarily to human FcγRIIA and FcγRIIIA (42). Furthermore, disparities in the binding affinities of immunoglobulin isotypes for specific Fcγ receptors exist between mice and humans. Thus, predictions of immune complex functions drawn from wild-type mouse models might be inadequate. A prominent example of the failure of previous studies in accurately recapitulating the specificity and diversity of Fcγ receptor interactions is the outcome of a clinical trial using a CD28-specific superagonistic antibody; this led to severe side effects including severe pain and extreme swelling, as well as one individual suffering from heart, liver, and kidney failure (43). To address this problem, an FcγR humanized mouse strain was recently generated through transgenic expression of the entire human FcγR family under the control of their human regulatory elements on a genetic background lacking all mouse FcγRs (44). The animals demonstrate normal lymphoid tissue development and generate normal immune responses. Thus, this mouse strain offers a greatly improved model to study immune complex-mediated cross-presentation, although it addresses only the species-specific differences regarding Fcγ receptors. Humans and mice also display differences in the expression patterns of Fc receptors for IgE and IgA, which might contribute to cross-presentation of immune-complexed antigen *in vivo* (45–48).

Increasing evidence suggests that allelic isoforms and polymorphisms of Fc receptors are shaping immune responses in humans. FcγRIIA (CD32A), the major phagocytic FcγR in humans, exhibits a polymorphism in the ligand-binding domain (49). Individuals homozygous for the R allelic form of CD32A (CD32AR allele) have been described as more susceptible to bacterial infections and autoimmune diseases compared to individuals homozygous for the H allelic form of CD32A (CD32AH) and CD32AR/H heterozygous individuals (50, 51). A binding study using two-dimensional affinity measurements also demonstrated

Table 1 | Overview of human and murine Fcγ receptors.

Human/mouse	IgG receptor	CD	Function	Affinity	Structure
Human (30–33)	FcγRIIA	CD32A	Activation	Low to medium	α-Chain with ITAM
	FcγRIIC	CD32C	Activation	Low to medium	α-Chain with ITAM
	FcγRIIIA	CD16A	Activation	Low to medium	α-Chain and γ_2-chains with ITAM
	FcγRIIIB	CD16B	Activation	Low to medium	GPI-linked α-chain
Human and mouse (30–33)	FcγRI	CD64	Activation	High	α-Chain and γ_2-chains with ITAM
	FcγRIIB	CD32B	Inhibition	Low to medium	α-Chain with ITIM
Mouse (30–33)	FcγRIII	CD16	Activation	Low to medium	α-Chain and γ_2-chains with ITAM
	FcγRIV		Activation	Low to medium	α-Chain and γ_2-chains with ITAM

that compared to CD32AH, CD32AR has significantly lower affinity toward IgG2, as well as to IgG1 and IgG3, suggesting that the lower binding of CD32AR to IgGs might be responsible for the lack of immune complex clearance, which leads to increased susceptibility to bacterial infections and autoimmune diseases (52). Genetic variations in Fc receptors have also been linked to cancer susceptibility (53–55). However, less efficient immune complex binding might also be reflected in less efficient antigen uptake and presentation via this receptor, and thus consequences for immune complexes cross-presentation should be expected. Of note, glycosylations in the IgG–Fc region can also affect Fc receptor-binding properties as discussed in detail in a recent review (56). How antigen cross-presentation of immune complexes and T cell priming is altered by differences in IgG subclass composition, IgG–Fc glycosylation, and Fc receptor polymorphisms is currently unknown, but is important to address. In conclusion, the complexity of interactions of IgG with the Fc receptor system in addition to concerns about species specificity presents a major hurdle that needs to be overcome for successful therapeutic applications.

CROSS-PRESENTATION OF IMMUNE COMPLEXES AND THE DIVERSITY OF DC SUBPOPULATIONS

Whether it would be beneficial to target a specific DC subset that displays a superior capacity to cross-present antigen for therapeutic approaches is currently a field of extensive investigation (4, 57). We will first focus on what we know so far about the cross-presentation capacity of DC subsets in general and then discuss our current understanding of cross-presentation of immune complexes in regard to DC subsets. DCs are a heterogeneous cell population, and substantial effort was made to characterize different subsets in mice and identify their human counterparts [reviewed in Ref. (58–60)]. In principal, murine and human DCs can be divided into two major subsets, classical/conventional DCs (cDCs) and plasmacytoid DCs (pDCs). In mice, cDCs comprise $CD8\alpha^+$ and $CD8\alpha^-$ lineages, which have been found to differ in their ontogeny and display functional specializations. Since the expression of surface markers on human and murine DCs is not conserved, only recently has gene expression profiling allowed for the identification of human $CD141^+$ DCs as functional equivalents of the mouse $CD8\alpha^+$ DCs, while human $CD1c^+$ DCs appear to be comparable to mouse $CD8^-$ DCs (61, 62).

In mice, the $CD8\alpha^+$ DC subset is considered to be more efficient at antigen cross-presentation than other DC subsets (63–66). The corresponding human subset, $CD141^+$ DCs, is also potent at inducing $CD8^+$ T cell responses *in vitro*, although their superiority to other human DC subsets is uncertain (67–73). Several groups have now reported that all human DC subsets can efficiently cross-present several forms of antigen [reviewed by Ref. (57)]. Initially, $CD141^+$ DCs isolated from human blood were described to better cross-present CMV protein pp65 in comparison to $CD1c^+$ DCs and pDCs from the same donor (67). It is important to note, however, that cross-presentation *in vivo* occurs rather in secondary lymphoid organs. A recent study has overcome the difficulties in isolating sufficient amounts of human DCs from lymphoid tissue and characterized in detail the cross-presentation properties of tonsil-resident DCs (73). An important finding of this study was that all tonsillar DC subsets (i.e., pDCs and the two populations of cDCs, $CD1c^+$ DCs and $CD141^+$ DCs) displayed comparable capacities to cross-present soluble antigens in contrast to macrophages, which lacked this ability. Interestingly, necrotic cells were phagocytosed and cross-presented by $CD1c^+$ DCs and $CD141^+$ DCs with similar efficiency, while pDCs were poor at taking up necrotic particles, consequently resulting in inefficient cross-presentation. Tonsillar macrophages were found to be the most efficient at taking up dead cells, but despite this fact they completely failed to cross-present necrotic cells. Collectively, the ability to efficiently cross-present in humans seems less restricted to a specific DC subpopulation than as observed in mice. Along these lines, it has been shown that the cross-presentation properties of human DCs depend on the antigen uptake pathway and the ability of the pathway to route the antigen into an early endosomal compartment rather than on a specific DC subset (74, 75). $CD141^+$ DCs are superior cross-presenters compared to $CD1c^+$ DCs only when the antigen is delivered via CD205, a receptor that preferentially targets antigens to late endo/lysosomal compartments. If antigen is targeted through CD40, $CD1c^+$ DCs are as efficient as $CD141^+$ DCs. These findings argue that targeting one specific DC subset for the design of DC-based vaccines may not offer the presumed advantage.

The cross-presentation studies discussed above focused primarily on soluble antigen uptake and targeting antigen via several endocytic receptors. How does cross-presentation of immune complexes fit into this picture? Targeting DCs through IgG immune complexes has been proven to be superior to soluble immune complexes for inducing $CD8^+$ T cell responses and as anti-tumor vaccines by utilizing murine bone marrow-derived DCs (76, 77). In addition, circulating specific antibodies have been shown to enhance systemic cross-priming by delivering immune-complexed antigen to murine DCs *in vivo* (78). Notably in mice, immune-complexed antigen allows the $CD8\alpha^-$ DC subset, which has been proven to be very poor at presenting soluble antigen, to become potent cross-presenting cells (79). Interestingly, cross-presentation by $CD8\alpha^-$ DCs depends on activating Fcγ receptors. Lack of the signal transducing γ-chain specifically abolishes presentation of immune-complexed antigen on MHC class I molecules but not on MHC class II molecules (79). Another remarkable feature regarding cross-presentation of immune complexes is their reliance on FcRn, an IgG binding receptor that is primarily located intracellularly and binds IgG independently from their Fcγ receptor interaction sites (80). How FcRn promotes cross-presentation of immune complex is discussed later in more detail.

Our knowledge regarding cross-presentation of immune-complexed antigen by human DC subsets is still very limited. The effects of Fcγ receptor antigen targeting on the efficiency of cross-presentation in human DCs were recently investigated using human cytomegalovirus (HCMV) pp65 as a protein antigen (81). In line with the data obtained from murine models, immune-complexed antigen is more efficiently cross-presented than comparable amounts of soluble antigen by human DCs. The enhanced cross-presentation capacity observed was not mediated by increased antigen uptake or induction of DC maturation through the immune-complexed antigen. The authors also demonstrated that both of the two major intracellular cross-presentation pathways (4), the cytosolic and

the vacuolar/endosomal pathway, are involved in Fcγ receptor-mediated uptake of immune complexes and their processing. Notably, monocyte-derived DCs as well as CD141$^+$ DCs required antigen processing by both intracellular pathways. The finding that CD141$^+$ DCs, which are the human equivalent to CD8α$^+$ DCs, use both processing pathways for immune complexes points to unique features of human DCs. Murine CD8α$^+$ DCs mainly use the cytosolic pathway to process antigen for cross-presentation, including the processing of immune complexes (82). Another difference to murine DCs is that the CD141$^+$ DC subset proved to be superior to CD1c$^+$ DCs in cross-presenting pp65 immune complexes (81). These findings point to obvious differences between murine and human DC subsets regarding immune complex-mediated cross-presentation. Since the human DCs were isolated from blood (81) and the murine DCs were isolated from the spleen (79, 80), it is possible that DCs from blood and lymphoid tissue generally differ in their cross-presentation capacities of immune complexes, which have similarly been observed for human DC subsets in response to soluble antigen as described above. In any case, the study by Flinsenberg et al. found that Fcγ receptor targeting increases cross-presentation of HCMV antigen by human blood and tonsillar CD141$^+$ DCs, which suggest that targeting of this DC subset with immune complexes might improve DC-based vaccination strategies. Another very important aspect of this study is the detailed characterization of Fcγ receptor expression on human DC subsets. Although CD1c$^+$ DCs expressed overall higher levels of FcγRII, CD141$^+$ DCs seem to express higher levels of the activating FcγRIIA relative to the inhibitory FcγRIIB. Thus, this study clearly demonstrates that the overall expression level of one specific Fcγ receptor does not determine the functional outcome, and that we need to consider the diversity of Fcγ receptor expression by distinct DC subsets to evaluate the therapeutic potential of immune complex-mediated cross-presentation.

A further difference between mice and humans seems to be the cross-presentation capacity of pDCs. Several studies have reported that murine pDCs do not possess the ability to cross-present (83–86) or that their capacity is insignificant when compared to cDCs (87). In contrast to mouse pDCs, human pDCs can efficiently cross-present antigen and induce CD8$^+$ T cell responses (88–90). Human pDCs also express FcγRIIA, and this receptor has been shown to mediate internalization of immunoglobulins bound to chromatin (91), Coxsackie virus (92), the model antigen KLH (93), and the tumor antigen NY-ESO-1 (94). In addition, the group of de Vries described that pDCs can use several receptor-targeted antigen uptake pathways, including the activating FcγRIIA receptor, to target antibody-coated nanoparticles for cross-presentation. Although this study did not use classical immune complexes, together with a vaccination study in which pDCs significantly prolonged overall survival in melanoma patients (95), it supports the notion that pDCs are interesting targets for DC-based immunotherapeutic strategies.

Collectively, we should keep in mind that some of the observed differences between human and murine DC subsets regarding cross-presentation of immune complexes most likely stem from differences in their Fc receptor expression and from different binding affinities for IgG isotypes. Recently, various published and publicly available microarray data were compiled, and this mRNA collection provides an excellent overview of mouse and human Fcγ receptor expression by DC subsets, monocytes, and macrophages (18). Overall, the Fcγ receptor expression levels obtained by mRNA analysis correspond well with the surface expression levels acquired by flow cytometric analysis (FACS) (**Table 2**). For the future, it will be important to determine whether the Fcγ receptor expression of human DC subsets isolated from blood also matches the expression on tissue-resident DCs from different organs.

REGULATION OF Fcγ RECEPTOR EXPRESSION IMPACTS CROSS-PRESENTATION OF IMMUNE COMPLEXES

Efficient cross-presentation for inducing protective immune responses against tumors or viruses is strongly governed by the ratio of activating versus inhibitory Fcγ receptors expressed on DCs. In addition to the DC subset, the maturation/activation state of DCs likely impacts their Fcγ receptors expression pattern. The maturation/activation state of DCs is in general strongly influenced by the cytokine milieu of the microenvironment, and a considerable number of cytokines have been shown to regulate Fcγ receptor expression *in vitro*. TGF-β1 down-regulates surface expression FcγRI and FcγRIII on monocytes (99). IL-4, a cytokine associated with Th2-type immune responses, increases the expression of inhibitory FcγRIIB. In contrast, the Th1-cytokine IFN-γ increases expression of activating Fc receptors on monocytes (100). Monocytes also have been shown to respond to IFN-γ and TNF-α treatment with enhanced immune complex binding via FcγRI, even when saturated with pre-bound monomeric IgG (101). Cytokine-induced changes in Fcγ receptor expression were also found using monocyte-derived DCs (96). Immature DCs generated with GM-CSF and IL-4 from monocytes express high amounts of inhibitory FcγRIIB, which is down-regulated upon DC maturation induced by TNF-α. The authors also showed that blood DCs activated with a cytokine cocktail containing TNF-α, IL-1β, IL-6, and PGE2 induce more influenza-specific CD8$^+$ T memory cells via targeting of FcγRI and FcγRIIA. Interestingly, crosslinking of inhibitory FcγRIIB only reduced the cross-presentation ability of immature DCs but not of mature DCs. Treatment of mature blood DCs with IL-10, or a combination of IL-10 and IL13, was found to increase expression of FcγRIIA and FcγRIIB (96). To sum up, although we know that cytokines can modulate Fcγ receptor expression, and that tumors create cytokine-rich microenvironments that involve the production of immunosuppressive as well as inflammatory cytokines to drive tumor progression (102, 103), our knowledge is very limited as to how cytokines from the tumor microenvironment affect cross-presentation of immune complexes by DCs. Thus, regarding anti-tumor therapy, this gap in knowledge might explain why the long-term therapeutic outcomes of immune complex-based strategies were not more successful, although efficient cross-presentation is induced by IgG-complexed antigens. One explanation could be that the tumor microenvironment promotes the induction of cross-tolerance by keeping the DCs in an immature state, which is associated with high expression levels of inhibitory FcγRIIB. Another possible scenario would be that immune complex-mediated cross-presentation via activating Fcγ receptors, which is known to result in inflammatory cytokine

Table 2 | Fcγ receptor expression by murine and human DC subsets.

Human DCs				Mouse DCs			
	Expression High: +++; low: +				Expression High: +++; low: +		
DC subset	Receptor	FACS[a]	mRNA[b]	DC subset	Receptor	FACS (79, 80, 89)	mRNA[b]
CD141$^+$ (BDCA3$^+$, XCR1$^+$)	FcγRI	–	–/+	CD8$^+$	FcγRI	–/+	+
	FcγRIIA	+	–/+		na		
	FcγRIIB	+	+		FcγRIIB	+++	++
	FcγRIIIA	–	+		FcγRIII	+++	+
	na				FcγRIV	–/+	+
CD1c$^+$ (BDCA1$^+$, SIRPα$^+$)	FcγRI	+[c]	+	CD8$^-$	FcγRI	–/+	+
	FcγRIIA	++	+++		na		
	FcγRIIB	+++	+++		FcγRIIB	++	++
	FcγRIIIA	–/+	+		FcγRIII	++	+
	na				FcγRIV	–/+	+
pDCs	FcγRI	–	–/+	pDCs	FcγRI	–	+
	FcγRIIA	++	+		na		
	FcγRIIB	+	+		FcγRIIB	+	++
	FcγRIIIA	nd	+		FcγRIII	–	+
	na				FcγRIV	–	+
Monocyte-derived DCs	FcγRI	+	+	Bone marrow-derived DCs	FcγRI	–/+	++
	FcγRIIA	++	+++		na		
	FcγRIIB	+++	+++		FcγRIIB	++	–/+
	FcγRIIIA	–/+	+		FcγRIII	++	++
	na				FcγRIV	–/+	++
Slan DCs (CD16$^+$)	FcγRI	++	nd	na			
	FcγRIIA	++					
	FcγRIIB	+					
	FcγRIIIA	+++					

[a] *Published surface expression determined by flow cytometric analysis (FACS) (81, 96–98).*

[b] *mRNA data from compiled microarrays (18).*

[c] *CD1c$^+$ DCs isolated from blood; tonsillar CD1c$^+$: DC –/+.*

nd: not determined.

na: not applicable.

production by the DCs, actually contributes to an inflammatory tumor microenvironment, which fosters tumor progression by supporting, for example, angiogenesis. Therefore, future studies are needed that not only address which activating and inhibitory Fcγ receptors are expressed by DC subsets, but also define how their expression patterns are regulated and which cytokines are induced by DC subsets after immune complex-mediated activation *in vivo*.

FcRn – AN INTRACELLULAR RELAY RECEPTOR THAT GUIDES CROSS-PRESENTATION OF IgG-CONTAINING IMMUNE COMPLEXES

In general, little is known about the intracellular mechanisms that are involved in processing of immune-complexed antigen for cross-presentation. Substantial evidence exists for an important role of FcRn in the cross-presentation of IgG-containing immune complexes. FcRn, which is an MHC class I-like molecule, was initially described only in intestinal epithelial cells of neonatal rodents, but it has since been shown to be expressed throughout life in several cell types, including human and rodent DCs (104–106). If CD8α$^-$ DCs do not express FcRn because of genetic alterations, the cell loses its ability to efficiently cross-present and fails to elicit CD8$^+$ T cell responses (80). Elegant studies showed that FcRn regulates the intracellular sorting of IgG immune complexes in CD8α$^-$ DCs. In contrast to CD8α$^+$ DCs where the endosomes are buffered around the neutral pH of 7.0 that prevents antigen degradation and promotes cross-presentation, Fcγ receptors in CD8α$^-$ DCs traffic antigens into acidic compartments (pH 6.0). The acidic environment is, by itself, not favorable for cross-presentation; however, it favors the binding of IgG to FcRn, and thus the model proposes that FcRn traps immune-complexed antigen and protects it from degradation

within an acidic loading compartment. The study also showed that in parallel to antigen entry into the FcRn-positive compartment, key components of the phagosome-to-cytosol cross-presentation machinery are rapidly recruited to the endo/lysosome. Vesicles that contained IgG-opsonized particles or IgG immune complexes rapidly acquired greater quantities of vacuolar ATPase (V-ATPase), gp91phox, and Rab27a than those that resulted from internalization of IgG mutants that cannot interact with FcRn. Consistent with this concept, it was described that the presence of FcRn also affects the oxidation state as well as the acidification of vesicles. Inhibitor studies demonstrated that FcRn-mediated cross-presentation depends on the proteasome as well as Sec61α, which is indicative for the cytosolic cross-presentation pathway. Since insulin-regulated amino peptidase (IRAP) enrichment was not depicted in FcRn-positive IgG immune complex-containing vesicles, and cathepsin inhibitors did not abrogate IgG immune complex cross-presentation, the authors concluded that the alternative vacuolar pathway was not involved. In summary, this study suggests that FcRn binding of IgG immune complexes enables a slower and more controlled antigenic degradation in CD8α$^-$ DCs, thereby permitting this population of DCs to become efficient cross-presenting cells.

The most compelling evidence for the exceptional importance of FcRn for cross-presentation of IgG immune complexes and IgG-opsonized particles is derived from *in vivo* studies that analyzed the effects of FcRn-deficiency on chronic intestinal inflammation and colonic cancer (107, 108). In a chemically induced chronic colitis model, which is associated with generating high levels of anti-bacterial antibodies that enter the host as IgG immune complexes, Baker et al. demonstrated that FcRn-dependent cross-presentation is carried out by CD8α$^-$ DCs *in vivo*, leading to greater levels of cytotoxic T cell activation during the course of colitis. In a recent study, the same group focused on the impact of FcRn on tumor development, clearly demonstrating the importance of this molecule for anti-tumor immune surveillance (108). The authors found that the DC-specific deletion of FcRn leads to increased tumor burden in experimental models of colon cancer and lung metastasis. Strikingly, this study also demonstrated that colon cancer patients with higher numbers of FcRn-positive DCs in the adjacent tumor tissue had significantly better prognoses, confirming the crucial role of FcRn and demonstrating the vital role of cross-presentation of IgG immune complexes in anti-tumor immunity in general. It will now be of utmost importance to elucidate the details of the intracellular mechanism of this process to evaluate whether the pathway can be employed for cancer immunotherapy.

CONCLUSION

Although ample evidence suggests that Fcγ receptor targeting through immune complexes allows for more efficient cross-presentation compared to soluble antigen, it still needs to be proven which advantages it may have over targeting of other endocytic receptors on DCs, especially *in vivo*. In this respect, it is very important to continue developing better murine models which more accurately reflect the human immune system. The recently published humanized FcγR mouse strain is here a promising step in the right direction. For therapeutic manipulations, we also need to better understand how Fcγ receptor expression by DCs is regulated. Can we use cytokines and/or TLR ligands to modulate the ratio of inhibitory versus activating Fcγ receptors expressed by DC subsets to improve therapeutic strategies? TLR-2 ligands, for example, have been shown to increase expression of inhibitory FcγRIIB in macrophages (109), a consequence not desirable in the context of viral or tumor vaccine development. Furthermore, how does the size of immune complexes influence cross-presentation? How does the antibody to antigen ratio in immune complexes influence cross-presentation? Indeed, it has been shown that immune complex size and glycosylation on IgG impact the binding to human Fcγ receptors (110). In summary, it is fair to conclude that many important questions remain open and need to be addressed. Irrespectively, cross-presentation of immune complexes represents an exciting potential pathway to improve DC-based vaccination strategies for anti-viral as well as anti-tumor therapy.

ACKNOWLEDGMENTS

This work was supported by grants from the National Institutes of Health: K01DK093597 (to Barbara Platzer) and AI075037 (to Edda Fiebiger). This work was also supported by the Harvard Digestive Diseases Center Grant P30DK034854.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 01 February 2014; paper pending published: 03 March 2014; accepted: 19 March 2014; published online: 01 April 2014.
*Citation: Platzer B, Stout M and Fiebiger E (2014) Antigen cross-presentation of immune complexes. Front. Immunol. **5**:140. doi: 10.3389/fimmu.2014.00140*
This article was submitted to Antigen Presenting Cell Biology, a section of the journal Frontiers in Immunology.

frontiers in
IMMUNOLOGY

REVIEW ARTICLE
published: 04 February 2014
doi: 10.3389/fimmu.2014.00013

Theories and quantification of thymic selection

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The peripheral T cell repertoire is sculpted from prototypic T cells in the thymus bearing randomly generated T cell receptors (TCR) and by a series of developmental and selection steps that remove cells that are unresponsive or overly reactive to self-peptide–MHC complexes. The challenge of understanding how the kinetics of T cell development and the statistics of the selection processes combine to provide a diverse but self-tolerant T cell repertoire has invited quantitative modeling approaches, which are reviewed here.

Keywords: thymic selection, T cells, mathematical modeling, repertoire selection, theoretical biology

INTRODUCTION

Conventional ($CD4^+$ and $CD8^+$) T cells are an integral part of adaptive immune systems in vertebrates. A key stage in their development is the creation of the T cell receptor (TCR) through a stochastic process of gene rearrangement. The resulting pre-selection TCR repertoire has the potential to recognize a very large array of peptides derived both from self and from foreign organisms, presented on Major Histocompatibility Complex (MHC) molecules on the surfaces of other cells. Much of T cell development occurs in a specialized organ in the chest called the thymus, within which this diverse potential repertoire of TCR is vetted. A process referred to as positive selection removes cells with TCR conformations that are generally non-responsive to self-peptide–MHC ligands (self-pMHC), and negative selection removes cells that are overly reactive to self-pMHC and pose a threat of autoimmune responses. The post-selection repertoire exported from the thymus comprises T cells that are largely non-responsive to self, yet capable of responding with remarkable specificity to foreign peptides.

There is a very extensive literature relating to thymic development and selection [for reviews, see for example Ref. (1–3)], but here we summarize the key ideas briefly (**Figure 1**). Conventional T cells begin life as lymphoid progenitors, which migrate from the bone marrow to the inner, cortical region of the thymus and begin a process of proliferation and maturation. Early in development in the cortex thymocytes are referred to as double negative (DN), lacking expression of the CD4 and CD8 co-receptors that are involved in TCR signaling. The TCR comprises two chains and is formed by a multi-step gene rearrangement process that first generates the TCRβ, γ, and δ chains (a small proportion of cells diverge at this stage to seed the $\gamma\delta$ T cell lineage) and then the TCRα chain at around the transition from the DN to $CD4^+CD8^+$ (double positive, DP) stage. TCR$\alpha\beta$ cells then migrate among cortical thymic epithelial cells and dendritic cells, auditioning for the ability to recognize self-pMHC. There is evidence that DP cells with non-functional TCR can undergo repeated TCRα rearrangements (4) to re-audition. Positively-selected cortical thymocytes begin negative selection and eventually move to the outer capsule of the thymus, the medulla. There they complete negative selection through interactions with medullary thymic epithelial cells and dendritic cells. TCR$\alpha\beta$ thymocytes, which recognize self-peptides presented on MHC class I or class II below an acceptable threshold of reactivity develop into the CD8 SP (single-positive, $CD4^-CD8^+$) or CD4 SP ($CD4^+CD8^-$) lineages, respectively, and are eventually exported into the peripheral circulation as naive T cells.

The topic of thymic selection has received substantial attention from the immunological modeling community, perhaps for two main reasons. First, selection has widely been viewed as a well-delineated optimization problem – how to craft a TCR repertoire that covers the space of possible pMHC ligands as widely as possible, while preserving sufficient specificity to discriminate between self and foreign (and between different foreign) peptides? This question naturally invites quantitative arguments. Second, the biology is well-characterized – a relatively small number of cell types and modes of interaction appear to be involved, and large amounts of experimental data are available. These simplify and constrain the construction of models.

Modeling studies have focused on many aspects of thymic selection but many questions and uncertainties remain. What are the rates and efficiencies of passage through the different phases of development and selection, and in what thymic microenvironments do each take place? How do thymocytes integrate signals received from interactions with pMHC to make fate decisions?

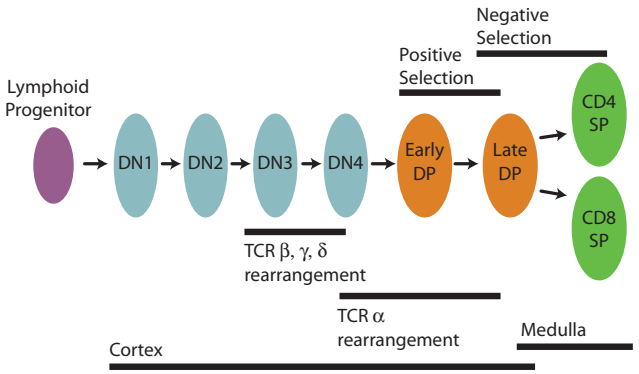

FIGURE 1 | Stages in the development of CD4 and CD8 T cells in the thymus.

What are the relative contributions of the MHC itself and its associated peptide to TCR signaling and fate determination? What influence do each of these have on the post-selection repertoire's diversity and coverage of the pMHC universe, and its ability to discriminate between self and foreign? How complete is the removal of potentially self-reactive clones? How many TCR interactions contribute to a thymocyte's fate decisions? What evolutionary pressures have determined the typical number of MHC alleles we possess? There have been many different theoretical approaches to these questions – from mean-field population dynamic models of progression through developmental stages, to probabilistic models of selection, to explicitly spatial models of migration within the thymus.

This review groups studies of these topics into broadly labeled categories, but in some cases the grouping is arbitrary – many of these questions are related and have been addressed either alone or in combination. The review has a bottom-up structure, beginning with an overview of experimental quantification of selection and modeling of thymocyte population dynamics. It then moves to studies of higher-level properties of the T cell repertoire, such as TCR cross-reactivity, and concludes with the problem of optimal within-individual MHC diversity.

THE POPULATION DYNAMICS OF THYMOCYTES

Basic elements of a quantitative understanding of thymic development are the steady-state population sizes of different developmental stages, the mean times to transit between them and the proportion surviving at each stage, which we refer to as the efficiencies of selection. While some quantities can be experimentally determined, mathematical models have helped us develop a more complete description of the kinetics of selection, both for the thymocyte population as a whole and for the CD4 and CD8 lineages in isolation.

To estimate the parameters of a dynamical system usually involves observing its response to perturbations. One method is to follow cohorts of cells as they progress through development using intra-thymic injection of a dye or radioisotope label (5–8). Arguably this method is less disruptive than cell transfers, but the uptake of marker can be heterogeneous (5, 7) and measurements of death rates using injected dyes rather than congenic markers may be confounded by loss of label (9). More recently, methods have included using GFP (green fluorescent protein) expressed during TCR rearrangement, its decaying intensity then a marker for time spent in development (10); inducible TCR signaling can be used to arrest, release, and follow cohorts of cells from the early DP stage (11); and small numbers of labeled thymocytes isolated at different developmental states can be followed after intra-thymic injection (11, 12). The population dynamics have also been exposed by transiently depleting thymocytes and observing the system's return to equilibrium (13). Various experimental systems, with or without associated dynamical models, are in general agreement over several quantitative aspects of thymic development but inconsistencies and uncertainties remain.

SELECTION EFFICIENCIES AND CELL FLUXES

Thymocytes begin to select against self-pMHC ligands at the DP stage following TCR rearrangement and so we focus on survival, proliferation, and differentiation from this stage onward. The proportion of DP cells that reach maturity (that is, survive both positive and negative selection) is widely agreed to be 5% or less (6, 11, 13–16). Within this pruning process, the general view is that positive selection is the most stringent, with 75–80% of cells failing to progress from the earliest DP stage, suggesting the majority of TCR generated are unable to recognize peptides in conjunction with MHC class I or II to any useful degree (11, 13, 15, 17, 18). Many studies have estimated that between 20 and 50% of positively-selected thymocytes then survive negative selection (11, 17, 19–22), although Itano and Robey (8) estimated a selection efficiency as high as 90% for DP cells into the CD4 SP lineage.

The rate of production of mature CD4 and CD8 cells in the thymi of young adult mice is roughly 1% of total thymocytes or $1 - 3 \times 10^6$ cells/day, a figure arrived at by a variety of labeling methods (5–7). Egerton et al. (6) estimated this to be just over 3% of the rate of entry into the DP population, meaning that fueling this trickle of output requires that roughly 30% of all thymocytes enter the DP stage each day. This again illustrates the extent of the filtering of the pre-selection repertoire that appears to be required to produce a functional and self-tolerant population of naive T cells. The thymus gradually involutes and its rate of output declines with age in both mice (23) and in humans (24), indicating that the bulk of the peripheral T cell repertoire is probably generated early in life.

THE MAJORITY OF THYMOCYTE DIVISION LIKELY OCCURS PRE-SELECTION

Labeled nucleotide uptake assays have revealed that substantial proliferation of thymocytes occurs before selection on self-pMHC ligands begins, stopping at or around the time of TCR rearrangement at the late DN/early DP stage (6, 25, 26). However, it is proliferation following TCR rearrangement that is most relevant for understanding how repertoire diversity is generated. Division during selection means a smaller proportion of TCR clonotypes may pass selection than measures of percentage survival suggest (27). The extent of division early in selection is unclear – estimates of the proportion of newly generated DP cells that are dividing have ranged from 11 to 68% (6, 25, 28), and CFSE labeling in

in vitro thymic organ cultures showed up to 5 divisions from DP onward (29). However, the DP population comprises cells pre- and post-TCR rearrangement, and there appears to be very little proliferation within the more mature DP population (6, 11, 15). There is a low level of proliferation during or just before the SP stage (11, 13, 25, 30), with CD8 SP more prone to division than CD4 SP (10).

Perhaps the most reliable experimental measure of average levels of proliferation during selection uses T cell receptor excision circles (TRECs). TRECs are circular DNA fragments that are stable remnants of the recombination events that generate the TCR and are shared randomly between daughter cells on division. The mean TREC content per cell is a rough measure of the mean number of divisions that have taken place since the TCR was generated. One caveat is that TREC studies are used most commonly in humans and much of what we discuss here derive from studies in mice. Another is that standard TREC measurements contain no information about the variance of the division number, and may gloss over even quite extreme heterogeneity in division patterns. Nevertheless a study of human infants observed 1–2 divisions on average between TCR rearrangement at the $CD3^{low}$ $CD4^+CD8^+$ stage and mature CD4 or CD8 SP; once shortly after TCR rearrangement, and another at the CD8 (but not CD4) SP stage (31). The high TREC content they observed at the early DP stage may reflect multiple rearrangements taking place in order to generate a functional TCRα-chain. In line with these results, the TREC content of naive $CD31^+CD4^+$ recent thymic emigrants in human infants is ~0.1–0.9/cell (32), suggesting that up to three divisions take place on average between TCR rearrangement and export to the periphery, although this may include some post-thymic proliferation and so is an upper limit on the extent of intra-thymic division.

TURNOVER RATES AND TRANSIT TIMES

Experimental estimates of the times taken to transit different developmental stages (immature DP → mature DP → SP → Export) are variable, particularly within the SP population (6, 10, 12, 25). Possible reasons for these discrepancies include different labeling protocols, different gating strategies defining thymic subpopulations, heterogeneity of cell populations, and differences in the kinetics of MHC class I-restricted and class II-restricted lineages. It has also been unclear whether selection is a "conveyor belt," first-in first-out, or has a more stochastic "lucky dip" nature (25). From a modeling perspective these are two points on a continuum. If an experimentally identifiable developmental stage comprises several shorter, sequential differentiation steps, the variance in the transit time through that stage is lowered with respect to a single-step model of transit. The more obligate steps, the more conveyor-belt-like the system appears.

There is general agreement that the transition from non-dividing mature DP to SP takes on average 3–4 days (6, 12, 15, 28), although it has been argued that it takes significantly longer to reach CD8 SP than CD4 SP (33). This transition is dependent on TCR signaling (15, 34). Observing a well-defined delay in the appearance of labeled SP cells, Egerton et al. (6) argued for a first-in-first-out kinetic in the DP population. This suggests DP cells must transit through a number of obligate steps. Subsequent experimental and modeling studies have addressed this, and are discussed below. The same study estimated a mean SP residence time of ~12 days, comparable to other estimates of the medullary residence time (6, 28). McCaughtry et al. (10) argued that this is an overestimate of the time mature conventional SP T cells take to develop, because the SP population is heterogeneous, also containing Treg, NKT, and $\gamma\delta$ T cells, which turn over more slowly. They estimated SP CD4/CD8 residence times to be 4.4/4.6 days. Saini et al. (33) arrived at similar estimates. As for DP cells, there are may be several developmental stages within the SP population and so it seems unlikely that SP residence times are exponentially distributed.

Stritesky et al. (12) estimated the total rate (cells per unit time) at which cells are negatively selected to be almost six times greater than the rate of positive selection, and found that both processes occur predominantly at the DP stage. Converting these figures into the relative efficiencies of positive and negative selection requires knowledge of how long cells spend in each selecting phase. If indeed positive selection is the more stringent, their result indicates that negative selection must take place over a relatively short timescale within the DP compartment. This is supported by a recent study observing negative selection of DP thymocytes taking place over ~12 h (35).

Interpreting data on transit or residence times can be problematic when both death and differentiation are taking place, as they clearly are at the DP stage(s) of development. If death and differentiation are modeled as independent processes, then at equilibrium transit rates through a compartment are not necessarily the same as turnover rates. If cells are maturing at rate μ and dying at rate δ, the population turns over at rate $\mu + \delta$ and the expected time a cell spends in that compartment is $1/(\mu + \delta)$. However, the mean time that successfully differentiating cells spend in each compartment is shorter because it is conditioned on survival, and is $\mu/(\mu + \delta)^2$ (if cells are capable of maturing but are simultaneously at risk of dying, those that successfully mature tend to do so early). This difference can be quite substantial, as we see below.

KINETIC MODELS OF THYMIC DEVELOPMENT

Data from these experimental studies and others have invited the use of population dynamic models to infer the kinetics of development. In the first studies to model thymic development, Mehr and collaborators utilized ordinary differential equation (ODE) models of the flow from DN → early DP → late DP → CD4/CD8 SP (36, 37). They utilized measures of steady-state population sizes and parameters either inferred from data or explored systematically to ask questions about the underlying dynamics. Mehr et al. (36) argued that positive selection likely involves triggering of proliferation as well as rescue from death, and while they were unable to use the steady-state data to make strong statements about the timing of positive versus negative selection, they inferred that most death at the DP stage is due to failure to positively select, consistent with many experimental and subsequent modeling studies.

There is evidence from fetal thymic organ cultures that populations of mature $CD4^+$ T cells resident in the thymus may enrich for the CD4 lineage while reducing thymic output. Mehr et al. (37) used a similar model with these data to propose that the mature resident cells increase survival of developing single-positive CD4 T cells while reducing proliferation or increasing the rate of

differentiation of DP cells. They suggest that mature CD4 T cells exert their influence by restricting the number of available pMHC ligands in the thymus, which could simultaneously reduce proliferation of DP cells (lowering thymic output) and decrease the stringency of negative selection (increasing the efficiency of maturation into the mature SP state). Again, these conclusions were reached using data from the thymus at steady-state.

Mehr and collaborators also studied the seeding of the cortical stroma with bone marrow-derived progenitor cells using a combination of modeling and experiment. They showed how migration between niche sites explained the competitive advantage of younger progenitors over older (38, 39), and that reconstitution of the progenitor population following irradiation is limited by damage to stromal niches and incumbent, surviving cells (40).

Thomas-Vaslin et al. (13) studied naive T cell homeostasis from the thymus through to the periphery. They induced systemic depletion of T cells for 7 days through expression of a suicide gene in dividing cells, and followed the kinetics of reconstitution. To interpret these data they developed a multi-compartment ODE model of T cell development, with a finer-grained treatment of transit through the DN, DP, and SP stages. In their model extensive proliferation occurs through the DN to early DP, with the latter population dividing 5 times. Their best-fitting model assumes all cell death (positive and negative selection) takes place at the late DP stage. They estimated 5% of total thymocytes (DN, DP, and SP) or $\sim 3 \times 10^6$ are exported as naive SP cells per day, and that 93% of DP thymocytes are lost, in line with existing estimates, and again suggesting that the bulk of negative selection occurs at DP. The mean times spent overall in the early DP (dividing), late DP (selecting), and SP compartments were estimated to be 1.2, 2.7, and 5.8 days respectively.

Sinclair et al. (11) used a different experimental system, with controllable TCR signaling that allowed arrest and release of cells at the early DP stage, and used a multi-compartment ODE model to quantify transit dynamics and selection efficiencies. Rather than simply early or late, they broke the DP stage into a branched developmental progression defined by the expression levels of CD5 and the TCR (33). In their schema, DP1 thymocytes are pre-selection; progression to DP2 requires a positively-selecting TCR signal; DP2 thymocytes consist of class I- and class II-restricted thymocytes in the first 12–48 h of development; and DP3 thymocytes are predominantly MHC class I-restricted cells that can select into CD8SP only. Thus cells destined for CD4SP transit DP1-DP2 only, and CD8SP transit through DP1, DP2, and DP3.

Sinclair et al. (11) estimated that ~75% in DP1 fail to progress to DP2, reflecting failure to positively select and dying of neglect. Overall, 5% of DP cells become CD4SP and ~2% become CD8SP, and so ~94% of DP cells are lost. They also saw relatively low levels of cell death in the SP compartment. These results suggest again that the bulk of negative selection occurs before cells transition to SP. They saw very little proliferation in their system, using a variety of methods, and so did not model cell division. Mean residence times in DP1 and DP2 were 3.5 and 1.4 days, respectively, with the smaller CD8 lineage spending an additional 7 days in DP3. They estimated 23% of all thymocytes at DP and SP enter the DP compartment per day. These selection efficiencies and the net flux agree with other estimates. Accounting for the selection bias on maturing cells, the model predicts that successful thymocytes spend on average 1.3 days in DP1 + DP2, 4.5 days in DP3. SP4 and SP8 residence times were 5 and 3.7 days, respectively, with very little cell death occurring. Their analysis therefore suggests that CD4SP/CD8SP cells take ~6.3/9.5 days from entry into DP1 to export.

MIGRATION WITHIN THE THYMUS AND THE TIMING OF POSITIVE AND NEGATIVE SELECTION

From the perspective of modelers attempting to connect models of thymocyte dynamics to data, it is important to understand when and where the different phases of development and selection occur. Selection begins in the thymic cortex, where the majority of thymocytes perform undirected random walks (41) encountering pMHC on cortical thymic epithelial cells. Sensitivity to medullary chemokine receptor signals begins to increase immediately following receipt of a positive selection signal and positively-selected cortical thymocytes eventually display rapid, directed motion toward the medulla (41), where they encounter pMHC on medullary thymic epithelial cells and dendritic cells. Negative selection takes place in the medulla (35, 42–44) but also late in migration through the cortex (45) and possibly even throughout development (46). The mapping between these migratory and selecting processes to developmental stages is not clearly defined. Cells undergoing negative selection in the medulla include DP populations (35), indicating that maturation from DP to SP does not coincide precisely with the cortical–medullary transition but further supporting the conclusion that the extensive cell loss at the DP stage comes from failure of both positive and negative selection. Further, antigen-presenting cells in the cortex and medulla appear to differ in their ability to provide positive or negative selection signals, either through differences in pMHC expression or diversity, or levels of co-stimulation (47–51). It seems therefore that negative selection at the DP stage takes place in at least two distinct spatial and TCR-stimulatory environments.

MODELS OF SELECTION WITHIN THE CORTEX AND MEDULLA

Motivated by this, Faro et al. (52) took a different perspective; rather than partitioning selecting thymocytes into developmental stages, they used a probabilistic model to describe selection within the cortex and the medulla. They aimed to quantify the number of selecting events, the number of selecting APC encounters and pMHC engagements, and the efficiencies of positive and negative selection in each region. Using the experimental estimates of overall selection efficiencies, and one experimental estimate of the efficiency of negative selection in the medulla, they inferred that most thymocyte death occurs by failure to positive select in the cortex, and cells are ~10 times more likely to be deleted (negatively selected) in the medulla than in the cortex. With these efficiencies, through a parameter search, they were able to infer the number of ligands each thymocyte selects on in each spatial compartment. They came to the striking conclusion that for each cortical thymocyte selection takes places on <60 pMHC ligand interactions, likely in order to achieve in their model the required high level of failure to positively select. However, this needs to be reconciled with the ~3-day mean lifetime of cells at DP1, which suggests cells have far more opportunities to positively

select, either through repeated encounters with APC or through repeated rearrangements of the TCRα chain [see Ref. (53) and refs therein], before dying of neglect.

IDENTIFYING THE SOURCE OF THE CD4:CD8 LINEAGE BIAS IN THYMUS

CD4 SP outnumber CD8 SP by roughly 4:1 in the thymi of many species. Using time courses of development in control mice and those lacking MHC class I or class II, Sinclair et al. (11) estimated the CD4 and CD8 lineage-specific selection efficiencies. In control animals, the highest death rate was at the positively-selected DP2 stage, and was substantially greater for MHC class I-restricted cells. MHC class I- and class II-restricted cells are indistinguishable at DP1 and DP2, but they were able to back-calculate the rates of production of precursors of the two lineages after TCR rearrangement, and found they were comparable. This suggests that the CD4:CD8 asymmetry in the thymus derives in large part from more stringent selection acting on MHC class I-restricted cells and not from any significant asymmetry in the predisposition of randomly generated TCR to recognize MHC class I or class II. Theirs is a model of CD4/CD8 lineage commitment in which the ability of a DP thymocyte to recognize MHC class I or class II dictates whether it will progress to the CD8 or CD4 lineages, respectively (8, 54). This is contrast to a less efficient, selective process in which a thymocyte's decision to downregulate either CD4 or CD8 expression is stochastic and decoupled from MHC preference, such that potentially viable TCR may fail positive selection [see, for example Ref. (55, 56); and Ref. (57) for a discussion of a hybrid mechanism]. Mehr et al. (36) proposed a purely instructive model of selection, in which pre-selection thymocytes are in principle able to recognize both MHC class I or II, and concluded that the most likely explanation of the CD4 bias is a difference in the *per capita* rates of maturation from DP into the two lineages, rather than differences in death rates.

The majority of models discussed here assume that thymocytes undergo screening independently. Mehr et al. (36, 37) implicitly allowed for competition with density-dependent proliferation rates at each developmental stage. However, there is some evidence that the probabilities of maturation can be impacted by competition between thymocytes, both globally and in lineage-specific ways. The efficiency of selection of transgenic TCRs varies with their abundance and with the availability of cognate pMHC (15, 58–60), and the selection of polyclonal MHC class I-restricted thymocytes is more efficient in the absence of MHC class II and vice versa (11). These observations suggest that selection efficiencies may be limited by competition both within and between lineages for access to pMHC or other resources needed for selection, and so may impact on the CD4:CD8 ratio emerging from the thymus. Two studies have used explicitly spatial, agent-based models of thymocyte migration and development to investigate this issue. Souza-e Silva et al. (61) modeled the movement of DN, DP, and CD4 SP and CD8 SP populations and their interactions with thymic epithelial cells (TEC) and chemokine gradients, using a 2D model. The structure of the epithelial networks was derived from histological samples from both mice and infant humans. Parameters were chosen to give agreement with published data regarding the repopulation of the thymus after sublethal irradiation, although a sensitivity analysis was not performed. In their model the CD4:CD8 ratio emerges as a result of competition for access to TEC and stochastic variation in the duration of signaling, which has been associated with CD4/CD8 lineage commitment (62). Their simulations also reproduce an observed variation in the CD4:CD8 ratio as irradiated thymi reconstitute and, in their model, the degree of competition increases. Efroni et al. (63) also took an agent-based approach and concluded that MHC class I and class II ligands on TECs are limiting. If continued access to pMHC stimulation is required for survival, and class I restricted cells stay conjugated to MHC for longer than MHC class II-restricted cells, exclusion of competitors leads to a higher death rate of cells developing into the CD8 lineage and a skewing of the CD4:CD8 ratio. Such a competitive model is an experimentally testable explanation of the differential death rates observed by Sinclair et al. (11).

CHARACTERISTICS OF THE TCR REPERTOIRE

Various summary statistics can be used to describe T cell populations pre- or post-selection. The *diversity* (or the *repertoire*) usually denotes the total number of distinct TCR sequences or clonotypes. The *cross-reactivity* measures a TCR's capacity for discrimination, and is quoted as either the average number or the proportion of different pMHC that one TCR responds to above some defined functional threshold. *Specificity* is inversely related to cross-reactivity. A mirror quantity is the *precursor frequency*, also referred to as the response frequency – the average proportion of all TCR capable of recognizing one pMHC. Further, selection operates in the context of an individual's own MHC alleles. *MHC restriction* measures the degree to which a given TCR is limited to recognizing peptides presented by one or more self-MHC; and *alloreactivity* is the proportion of TCR that respond to a foreign MHC, which is relevant for transplantation of tissues from one individual to another. In the sections that follow we describe how theoretical models have been used to understand how these quantities are linked and constrained by thymic selection.

TCR CROSS-REACTIVITY

A diverse TCR repertoire seems to be a requirement for coverage of pMHC shape space. However, the number of theoretically possible pMHC complexes appears to be far greater than any individual's capacity for unique TCR clonotypes (64–66); a simple calculation for just one MHC class I variant, assuming it presents 2% of all possible 9-residue peptides, yields $20^9 \times 0.02 \simeq 10^{10}$ possible pMHC, compared with the roughly 5×10^7 naive CD8 T cells in a mouse. To minimize the probability that any given foreign pMHC will escape detection by the immune system, some degree of TCR cross-reactivity therefore seems beneficial. Mason (64) used a variety of methods and data sources to estimate that one MHC class I-restricted T cell responds to between 10^6 and 10^7 nonamer peptides, or one in 10^3 to 10^4 pMHC using the theoretical estimate of the potential pMHC diversity; and Ishizuka et al. (65) used peptide libraries to estimate more directly that one CD8 T cell clone responds to roughly 1 in 3×10^4 peptide–MHC class I ligands. On the other hand, the average degree of cross-reactivity seems necessarily constrained from above, to avoid excessive deletion of the repertoire and to preserve specificity for self/non-self discrimination. It therefore seems plausible that evolutionary pressures

might have optimized this trade-off and determined the degree to which TCR can respond to multiple pMHC.

OPTIMAL LEVELS OF TCR CROSS-REACTIVITY – PROBABILISTIC ARGUMENTS

Several variants of essentially the same argument predict that the diversity of self-peptides involved in selection is the strongest influence on the optimum level of TCR cross-reactivity (64, 67–70). One version of the argument is as follows. The proportion of the positively-selected T cell repertoire R_0 that avoids deletion, f, decreases with both the number of self antigens N_s and the cross-reactivity r, $f = (1-r)^{N_s}$ which is approximately $\exp(-rN_s)$ for $r \lesssim 1/N_s$. A pathogen escapes immune recognition if all fR_0 surviving unique clonotypes fail to recognize (cross-react with) all x epitopes it generates, with probability

$$P_E = (1-r)^{fR_0 x} \simeq \exp(-rfR_0 x) \quad (1)$$

where again the approximation holds if $r \lesssim 1/(fR_0 x)$. This ignores MHC restriction, but including this refinement yields similar conclusions (67). Using the expression for f,

$$R_0 \simeq -\log(P_E)\frac{\exp(rN_s)}{rx}. \quad (2)$$

This equation connects the repertoire before negative selection R_0, the probability of immune escape P_E and the pre-selection cross-reactivity r. R_0 is relatively insensitive to P_E but very sensitive to the diversity of self, N_s. In this model, then, the strongest determinant of the size of the pre-selection repertoire is the diversity of self antigens, N_s, and not the requirement for minimizing the probability that a pathogen escapes detection (67).

The three-way relation expressed by equation (2) can then be used to estimate the optimal cross-reactivity under different evolutionary constraints. Suppose the potential repertoire size R_0 is relatively conserved and evolution has selected for the smallest P_E by tuning TCR cross-reactivity; in this case, the optimal cross-reactivity is simply the inverse of the number of distinct self-pMHC involved in selection, $r = 1/N_s$. The same value of r arises if evolution is assumed to minimize the required repertoire size R_0, whatever the value of P_E (67). Thus the more diverse the self-peptides involved in thymic selection, the more specific (less cross-reactive) the TCR needs to be. The same result can be derived in a very general way using extreme-value theory (70), requiring only the assumption that the negative selection threshold in the thymus is equal to the activation threshold in the periphery.

The induction of tolerance in the thymus is likely incomplete and there may be mature lymphocytes that are able to recognize self-peptides not involved in thymic selection. Borghans and De Boer (71) argued that to minimize the probability of these cells mounting a cross-reactive autoimmune response to this "ignored self" while responding to a pathogen demands higher levels of specificity than predicted by the simplest models. In this model, optimal cross-reactivity is then modulated by the potential diversity of the repertoire; the greater the number of possible T cell clonotypes, the lower cross-reactivity is required.

Percus et al. (72) took a different approach to studying optimal cross-reactivity, prompted by the observation that the sizes of the binding sites of the TCR and the B cell receptor (antibodies) are similar, at roughly 15 amino acids. They concluded that this size is large enough to provide discriminatory power but small enough that there is sufficient cross-reactivity for coverage of foreign antigen shape space. Interestingly this result does not arise from the demand for self–non-self discrimination, but rather from the constraint of the observation that the B and T cell repertoires comprise ~10^7 different receptors. However, this diversity itself may be derived from the self-tolerance arguments described above (64, 67–69). It has since been established that substantially fewer peptide residues are involved in TCR recognition. Burroughs et al. (73) analyzed the proteomes of humans and several microorganisms and showed that even the seven exposed (non-anchor) residues of the nine-mer peptides bound to one MHC class I allele may promote self/non-self discrimination, with <0.5% overlap in these sequences between humans and different microorganisms.

CONVERGENT ESTIMATES OF LEVELS OF NEGATIVE SELECTION

Several of these studies concluded that at the optimal level of cross-reactivity the probability of negative selection is roughly 63%, making various assumptions regarding the magnitude of parameters and maximizing the probability that the post-selection repertoire mounts a response to a foreign pMHC. However, the probability of negative selection can be derived without any assumptions regarding parameter values. From above, the fraction of the positively-selected repertoire with cross-reactivity r that survives deletion on N_s self-peptides is $f = (1-r)^{N_s}$. The probability that the post-selection repertoire $R = fR_0$ fails to recognize one given foreign pMHC is given by equation (1) with $x = 1$,

$$P_E = (1-r)^{fR_0} = (1-r)^{R_0(1-r)^{N_s}}. \quad (3)$$

This is minimized with respect to r at $r = 1 - \exp(-1/N_s)$, exactly (the optimal cross-reactivity $r \simeq 1/N_s$ then obtains if $N_s \gg 1$). So if evolution acts on cross-reactivity to minimize the probability of foreign pMHC escaping detection, the fraction of the positively-selected repertoire that survives negative selection is then simply $f = (1-r)^{N_s} = \exp(-1) \simeq 0.37$, or $\simeq 63\%$ of positively-selected thymocytes are deleted.

Mason (64) arrived at the same result assuming heuristically that the quantity to be maximized is the "reactivity" of the repertoire, proportional to the number of peptides each T cell can recognize multiplied by the proportion surviving negative selection;

$$\text{Reactivity} \sim \text{Cross-reactivity} \times \text{P(survive negative selection)} \sim r \times (1-r)^{N_s}.$$

Maximizing this reactivity is equivalent to minimizing the probability of escape in equation (3) when r is assumed to be small. There, using the Taylor expansion gives $P_E \simeq 1 - rR_0(1-r)^{N_s}$, and so the probability of responding $(1-P_E)$ is $\sim rR_0(1-r)^{N_s}$, or Mason's reactivity. Since r is small, the probability of negative selection is $(1-r)^{N_s} \simeq \exp(-rN_s)$ and so the reactivity is proportional to $r\exp(-rN_s)$, which is maximal with respect to r when argument of the exponential is -1. Thus again $f \simeq 0.37$ and the optimal cross-reactivity $r \simeq 1/N_s$.

An essentially identical argument applies to negative selection of B cells (67, 69). This estimate of f is remarkably consistent with estimates of levels of negative selection in the thymus from several experimental and population dynamic modeling studies (11, 17, 19–22).

ALTERNATIVE TREATMENTS OF CROSS-REACTIVITY

These models assume a universal cross-reactivity parameter r, but T cells may have the capacity to modulate their activation thresholds in response to their signaling environment (74, 75). Motivated by this, Scherer et al. (76) developed a model in which T cells tune their activation thresholds (and thus their cross-reactivity) to the level of their strongest interaction with self-pMHC during selection. If combined with a deletion mechanism that removes cells with activation thresholds so high as to be judged functionally inert, this model appears to be a more efficient mechanism of thymic selection than the standard clonal deletion model. Scherer et al. showed that the tuning model increases the probability of mounting an immune response to a given pathogen epitope, given a pre-selection repertoire size R_0, and the number of self-pMHC ligands involved in selection, N_s. The improvement offered by the tuning model is most striking for small pre-selection repertoires, $R_0 \ll N_s$, but disappears for $R_0 \gg N_s$. The latter inequality likely holds for mice and humans; the potential number of unique TCR sequences exceeds the estimated 10^3–10^5 self-peptides able to be presented by a given MHC allele (73, 77, 78). Further, equation (1) predicts that at the optimal cross-reactivity $r = 1/N_s$, the probability of one epitope ($x = 1$) escaping recognition is $P_E = \exp(-R_0/eN_s)$ where e is the base of the natural logarithm. For $P_E < 0.05$, expected in humans and mice, requires $R_0 \gtrsim 10N_s$. Despite this, Scherer et al. (76) argue that the tuning model is a more parsimonious mechanism of self-tolerance in the thymus than the standard model of deletion based on evolutionarily optimized cross-reactivity.

Finally, many of these arguments assumed thymic selection alone optimizes cross-reactivity, but the requirement for memory T cells to discriminate between different pathogens may impose a further constraint of its own (79, 80).

EXPLORING CROSS-REACTIVITY WITH SEQUENCE-BASED MODELS OF THYMIC SELECTION

A series of related papers by Detours, Perelson, and Mehr (27, 81–83) used a model of TCR–pMHC interactions to understand at a more mechanistic level how cross-reactivity, alloreactivity, and MHC restriction emerge in the post-selection repertoire. Here we focus on their treatment of TCR cross-reactivity, and return to alloreactivity and MHC restriction in the next section. Their starting point was an established model of protein binding (81, 84). They described the interaction between the variable region of the TCR and its pMHC ligand with strings of digits, and binding strengths between each digit pair were determined by the degree of complementarity between their binary representations (81). MHC and peptide contributed additively to the affinity of the interaction, the quantity assumed to drive selection. Given the number of digits ascribed to the polymorphic MHC residues in contact with the TCR, and the number of digits representing the peptide, selection could be performed on a randomly generated TCR repertoire using randomly generated peptide–MHC complexes. Affinity thresholds were then adjusted to give stringencies of positive and negative selection similar to those observed experimentally.

To circumvent the computational costs of selection using realistic numbers of peptides and unique pre-selection TCRs, they derived expressions for the mean-field predictions of the model for given parameter sets. This has the advantage of yielding population-level statements, which average over all possible TCR, MHC, and peptide sequences.

Detours and Perelson (82) estimated the precursor frequency, the proportion of naive T cells able to respond to a particular foreign pMHC. Experimental estimates of this quantity lie in the range 10^{-6}–10^{-4} (85–89). They term this the response frequency, R, and found it to be strongly and inversely related to the number of selecting self-pMHC ligands. Since precursor frequency is positively correlated with cross-reactivity (64), this result is in keeping with the theoretical studies discussed above (64, 67–70). It is also consistent with observations that repertoires selected on a restricted range of peptides exhibit higher cross-reactivity than normal (90–92). For R to lie in the observed range constrains the number of distinct peptides each MHC can present to be of the order 10^3–10^5, in line with estimates for murine MHC class I (77), MHC class II (78), and human MHC class I (73).

To explore the effect of thymic selection on specificity in more detail, Chao et al. (93) revisited the complementary digit-string model. Again peptide and MHC were assumed to contribute additively to an antigenic distance from the TCR, which was inversely related to affinity or the strength of a selecting signal. They confirmed that negative selection reduced the coverage of peptide space, defined as the proportion of peptides that are recognized on the selecting MHC. This was equivalent to a reduction in the cross-reactivity of the repertoire; it reduced the mean antigenic distance to foreign pMHC complexes.

Chao et al. (93) then used the model to address the question of why the number of pMHC that one T cell is able to respond to varies widely across TCR (94). Their simulations suggested that the degree of cross-reactivity to a foreign peptide was inversely related to the peptide's similarity to self, which can be understood with the following argument. In their model, in the pre-selection repertoire a TCR's affinity for the MHC and peptide portions of its ligand are uncorrelated. Selection introduces an inverse correlation between a TCR's affinity for its selecting MHC and its strongest affinity for self-peptide; to select, a TCR's strongest interaction with self must lie between the positive and negative selecting thresholds. (The narrower the range of affinities defining the selecting region, the stronger this correlation will be.) Selected T cells with high affinity for MHC then have a relatively low affinity for the self-peptide component and require only weak binding to foreign peptide to be activated (activation in their model is defined to be an interaction above the negative selection threshold). These cells are therefore cross-reactive to foreign peptides. Conversely, selected TCR that bind relatively weakly to MHC have higher affinity to self and require strong binding to foreign peptide for activation, and therefore have more specificity for foreign antigen. Thus it emerges from their model that a TCR's specificity to foreign peptide is positively correlated to its affinity for self-peptide; or

equivalently, a TCR's cross-reactivity is positively correlated with its affinity for MHC.

The effect of negative selection on cross-reactivity can be understood with a similar argument. A TCR with high affinity for MHC will survive negative selection only if it has low affinity to all self-peptides, which is unlikely. Negative selection therefore enriches for cells with lower affinity for MHC, which from the argument above tend to be less cross-reactive. This reduction in coverage means specificity to foreign peptide must be increased.

Kosmrlj et al. (95) used a more physical, mechanistic approach to understanding how negative selection increases specificity, with the aim of characterizing the properties of the amino acid sequences of specific and cross-reactive TCR. Using the Miyazawa–Jernigan matrix (96) to quantify the interaction energies of pairs of amino acids, they extended the digit-string model to calculate the binding affinities between the peptide and the CDR3 region of the TCR, with a constant contribution from the MHC. (The variable peptide element of the pMHC ligand can be assumed to include the polymorphic MHC residues; thus their model may allow for MHC restriction, although this was not discussed.) Košmrlj et al. (97) presents an analytical treatment of the model.

They observed that TCRs selected against multiple peptides on the same MHC had peptide contact residues enriched in weakly interacting amino acids. In their model this arises by a sort of buffering mechanism – such sequences are able to withstand multiple substitutions in the peptide sequence to which they bind most strongly, and so are more resistant to negative selection than those TCR with strongly binding residues. For these TCR to survive selection requires that the invariant MHC contribution to the binding energy is of moderate strength – contributing sufficiently to favor positive selection but well below the negative selection threshold.

Kosmrlj et al. (95) argue that it is this enrichment for weakly binding TCR driven by negative selection that underlies antigen specificity. Antigen recognition is assumed to occur when a TCR signal exceeds the negative selection threshold made up by several interactions. This requires the peptide to contain several amino acids capable of binding the most strongly to the generally weakly binding TCR contact residues. Each contributes significantly to the total binding energy, and so any mutation to the peptide sequence has a high probability of abrogating recognition. Thus there is a restricted peptide signature or "barcode" required to trigger the TCR. In their model, TCR selected against a single pMHC were enriched slightly for strongly interacting amino acids. For these TCR, they argue, fewer amino acids contribute on average to the binding energy, triggering is more robust to mutations in the peptide sequence, and so the TCR is more cross-reactive. Thus again the argument emerges that the cross-reactivity is inversely related to the diversity of self driving selection. Kosmrlj et al. (98) employed this idea to put forward an explanation of why the population of elite-controllers of HIV infection is enriched for the HLA-B57 allele. Using a predictive peptide binding algorithm they argued that HLA-B*5701 binds a lower diversity of self-peptides than average. Cytotoxic T cells restricted to this allele are then expected to be more cross-reactive than average and so are more resistant to virus mutations that might otherwise escape CTL control.

Chao et al. (93) and Kosmrlj et al. (95) took different approaches to the problem of how negative selection increases specificity. They came to the common conclusion that the most specific TCR are those with low to intermediate affinity to MHC – high enough to have a reasonable probability of passing positive selection, but low enough to avoid negative selection by allowing headroom for the additional contribution from the peptide component. The greater this headroom, the smaller the proportion of peptides that can trigger activation and so the greater the specificity.

THE EMERGENCE OF SPECIFICITY IN AVIDITY-BASED MODELS OF SELECTION

Van den Berg et al. (99) developed a statistical framework to study the question of how specificity and self-tolerance can derive from a pre-selection repertoire of relatively promiscuous TCR. In their formalism, T cell activation is avidity-based and related to the rate of TCR triggering. Their starting point is that TCRs are degenerate and low affinity, binding weakly to many pMHC. TCR perceive an average signal derived from endogenous self-pMHC, and are triggered only by pMHC with sufficiently high prevalence and affinity to be visible above this background. The authors introduce the concept of an antigen presentation profile (APP), characterizing the abundances of different pMHC on antigen-presenting cells (APC). Positively-selected cells are selected against a given number of APC each with distinct APPs. In their framework, negative selection acts only on ubiquitous peptides presented on all APCs, and decisions are made on the basis of the entire APP of one APC. TCR that are triggered by this constitutive self-background are deleted. This filtering acts to sharpen the boundary between triggering rates, which give low and high activation probabilities, and so specificity can emerge even from a highly degenerate TCR. Interestingly they predict that negative selection does not have to be particularly stringent to generate an acceptably self-tolerant repertoire. Nevertheless in this model the selected repertoire may still be reactive to self-peptides expressed heterogeneously in the thymus, and in particular to peptides expressed at high levels only on certain cell types. Van den Berg and Rand (100) review avidity-based models of ligand discrimination.

ALLOREACTIVITY AND MHC RESTRICTION

A high proportion (1–24%) of peripheral T cells are reactive to peptides presented on a foreign MHC allele (101–103), reflected clinically by acute T cell mediated rejection of grafts from MHC-mismatched donors. These promiscuous "allogenic" responses contrast with the low precursor frequency (10^{-6}–10^{-4}) in normal immune responses to peptides presented by an individual's own MHC. Allogenic responses are also apparently counter to the notion of MHC restriction. Reconciling these results may tell us great deal about the relative contributions of peptide and MHC binding motifs to the TCR signals driving selection, and how this breakdown influences the coverage and cross-reactivity of the T cell repertoire.

Detours and Perelson (82) used their digit-string model of TCR–pMHC interactions, described above, to show how the probabilities of responsiveness to self and foreign MHC emerge.

Mean alloreactivities of 1–2% arose naturally, at the lower end of the range of experimental estimates, and they showed that the alloreactivities of the pre- and post-selection repertoires are similar, as observed experimentally (17, 22). In essence, the modeling supports the hypothesis that the greater degree of alloreactivity than response frequency arises simply because many more pMHC ligands can be generated from one MHC than can be generated from one peptide (104). In other words, each TCR is triggered by ligands in a subset of pMHC shape space; one particular MHC along with its associated diversity of peptides will cover a far greater region of shape space than covered by one peptide and all the self-MHC alleles capable of presenting it; a given MHC will then stimulate far more of the T cell repertoire than will a given peptide.

They found that alloreactivity correlates with the extent of negative selection and inversely to the degree of MHC restriction. It can be seen intuitively how this emerges from their model. If negative selection is weak, positive selection must be correspondingly stringent in order to yield the selection efficiencies observed experimentally (3–5%). Stringent positive selection imposes an imprint of self-MHC on the repertoire – only those TCRs that bind strongly to self-MHC residues survive. The strength of binding to a randomly generated MHC not involved in selection (i.e., a foreign MHC) is then on average lower to that of self-MHC in the post-selection repertoire. This difference increases, and thus alloreactivity decreases, as the required strength of binding to self-MHC increases.

This trade-off between alloreactivity and restriction might be expected as they appear to be in conflict. However, experimental estimates of these two quantities are variable. The conclusions described above were derived analytically from a model capturing the mean-field behavior of the digit-string selection process, but did not deal with the variance in these measures of the repertoire outputs across specific simulations or experimental systems. The final study of the series (83) took a simulation-based approach, explicitly performing repertoire selection on random TCR and pMHC populations. This confirmed the inverse correlation between alloreactivity and MHC restriction and yielded sufficient variability to account for restriction ranging from absolute to partial in different settings.

Overall the digit-string model explored by Detours and colleagues yields remarkable agreement with many observations. Their model of TCR–pMHC binding is highly abstracted, but appears to be a powerful one. In part this might be because the relevant quantities for selection in their model are the minimum and maximum binding affinities that each TCR experiences during exposure to large samples of randomly generated pMHC strings. These two quantities will be drawn from extreme-value distributions, which should be insensitive to the distribution of binding strengths of randomly chosen TCR–pMHC pairs (70, 105). The additivity of the MHC and peptide contributions to the fate-determining signal is likely the most questionable assumption, as the authors point out. Fate decisions may be based on the sum of several TCR interactions (which means for example that positive selection may occur though proximal binding of multiple low-affinity ligands) and so an avidity-based model may be more appropriate. Another caveat is that the population-average model assumes that positive selection takes place on at most one MHC allele, which we will also return to.

INSIGHTS INTO FATE DETERMINATION MECHANISMS FROM STOCHASTICITY IN SELECTION

Regulatory T cells (Treg) are a distinct lineage of CD4SP cells thought to lie at the higher end of the spectrum of acceptable self-reactivity and play a crucial role in the control of autoimmunity and tolerance to innocuous antigens. Many experimental studies of Treg development have shown that cells with the same TCR can develop into conventional and regulatory T cells within the same selecting environment [see, for example, Ref. (58, 106)], illustrating again, as represented in so many models, the stochastic nature of selection. There are at least two possible sources of this stochasticity. In a purely selective model precursors with identical TCR might be predisposed to the conventional or Treg fates through natural variation in expression of factors involved in lineage commitment. In a purely instructive model, cells within a clone are uncommitted, and intra-clonal heterogeneity in fate may derive from variation in the experience of each thymocyte during selection – most likely because each encounters a different random sample of self-peptides.

Bains et al. (107) used a probabilistic, instructive model that reflects this view of fate determination driven entirely by antigenic experience during selection, in conjunction with data from Ref. (58) to infer the number of pMHC binding events involved in fate determination. In that study, the numbers of conventional and Treg cells with a transgenically expressed TCR were measured for varying abundances of that TCR's agonist peptide on thymic epithelial cells. Conventional cell numbers declined monotonically with agonist abundance, while Treg increased and then decreased. Thus as agonist abundance increased, it appeared that T cells were initially diverted into the Treg lineage, before the risk of deletion through exposure to agonist dominated. Using this information and a simple graphical argument they were able to infer that fate decisions could not be affinity-driven (that is, made on the basis of a single pMHC interaction) unless TCR sensitivity varies during development, for which there is evidence [see Ref. (107) and references therein]. This model also explains apparently paradoxical observations regarding the effect of partial and full TCR agonists on the efficiency of Treg production (108).

THE LIMITS OF NEGATIVE SELECTION

The potentially very large number of unique self-pMHC prompts the question of whether it is possible to tolerize thymocytes to all self-peptides within the timescale of thymic development. Müller and Bonhoeffer (109) studied this problem. Using constraints from the mouse proteome and the efficiencies of peptide production and binding to MHC, they estimated an upper limit of approximately 5×10^6 possible self-pMHC class I complexes. Notably, this diversity of self is several orders of magnitude lower than figures derived from the simple combinatoric arguments (64, 66) and is more closely aligned with an estimate that $\sim 10^5$ different nine-mers derived from the human proteome are expected to bind to one human MHC class I allele (73). The key quantity in Müller and Bonhoeffer's calculation is the probability P that a given self-pMHC is presented by any given APC in sufficient numbers for

negative selection to occur. The probability that a thymocyte specific for this (and only this) self-pMHC escapes negative selection is P_E in their notation – distinct from the probability of immune escape discussed above – and $P_E = (1 - P)^n$, where n is the number of unique APC encountered during selection. In this model, P_E is extremely sensitive to the number of copies h of a given self-pMHC that an APC needs to present in order to cause deletion – varying h between 15 and 1500 gives values of P_E between 10^{-11} and 0.8. Favoring the higher estimates of h, Müller and Bonhoeffer (109) concluded that negative selection on the potential diversity of self is likely to be very leaky. Instead, they suggest thymic selection operates on a restricted subset of self-pMHC, a constraint imposed by the number of APCs encountered during selection. This requires that further tolerogenic mechanisms operate in the periphery to prevent autoimmune response to self antigens not encountered in the thymus (53, 70).

To support their argument, Müller and Bonhoeffer (109) reverted to the older model of cross-reactivity and selection to generate another estimate of the number of selecting ligands using the observed efficiency of negative selection. Recall that the probability of thymocyte with cross-reactivity r escaping negative selection on N_s unique selecting ligands is $P = (1 - r)^{N_s} \simeq e^{-rN_s}$. Using the estimate of $r = 2 \times 10^{-5}$ (88), and $P \simeq 0.33$, they obtain $N_s \simeq 10^5$ unique selecting self-pMHC, or ~4% of the putative total number of self-pMHC. This estimate is consistent with those of Detours et al. (27). Both studies assume that this cross-reactivity r of thymocytes with self-pMHC is equal to the cross-reactivity of mature naive T cells to foreign pMHC. Since negative selection likely acts as a filter to reduce cross-reactivity in the post-selection repertoire (see above), this assumption is moot. But the need to meet the empirical constraint $e^{-rN_s} \simeq 0.33$ implies that higher values of r would reduce the number of unique selecting ligands N_s even further.

A subsequent exchange (110, 111) discussed the assumption that each TCR negatively selects only on a single self-pMHC ligand. Müller and Bonhoeffer (111) argued that in the Bernoulli trial model of cross-reactivity and selection, a 33% probability of survival implies that another third of all thymocytes were reactive to one self-pMHC only, giving some quantitative support to their original model. The discussion also addressed whether N_s is constrained by the residence time in the thymus or is a result of restricted presentation of self antigens. Müller and Bonhoeffer (111) favored the latter, presuming that evolution has optimized the thymic residence time for the purposes of efficient selection on a subset of self-peptides. More recently it has been argued that incomplete depletion of self-reactive cells in the thymus may be sufficient for robust self/non-self discrimination in the periphery, if interactions facilitating consensus between T cells are required for the initiation or suppression of immune responses (70).

OPTIMALITY OF INDIVIDUAL MHC DIVERSITY – CONSTRAINTS ARISING FROM THYMIC SELECTION

The polymorphism of the MHC is huge, with hundreds of alleles identified at the HLA-A, HLA-B, and HLA-DR loci in humans (MHC is referred to as HLA in humans but hereon the term MHC is generally used, for simplicity). This diversification is thought not to have occurred by genetic drift but by two non-exclusive mechanisms. Heterozygote advantage (112, 113) suggests that individuals expressing more unique MHC alleles gain fitness by being able to present a larger array of pathogen peptides. Overall the evidence for heterozygote advantage in experimental models of infection is equivocal, though, and it has been argued with a quantitative model that this mechanism alone is insufficient to explain the extent of allelic diversity (114). Another theory is that MHC polymorphism is maintained by frequency-dependent selection under pathogen pressure, in which rare alleles confer protection against pathogen subversion of peptide presentation by commonly expressed alleles (115).

Intriguingly, individuals possess only a small proportion of all MHC alleles. Heterozygous humans possess six at the major HLA-A, HLA-B, and HLA-C loci, which code for MHC class I molecules that present peptides to $CD8^+$ T cells, and six to eight at the HLA-DP, HLA-DQ, and HLA-DR MHC class II loci, which present to $CD4^+$ T cells. A common explanation for this restricted within-individual diversity is that it derives from the need to generate a broad, functional, and self-tolerant TCR repertoire in the thymus without excessive negative selection (116, 117). The qualitative argument is as follows. If n is the number of MHC alleles per person, then increasing n both increases the diversity of pathogen-derived peptides that can be presented and increases the probability that a thymocyte will be able to obtain positively-selecting signals. On the other hand, higher n will also increase the range of self-peptides that can be presented. This will increase the stringency of negative selection, leading to inefficient generation of T cells in the thymus and potential gaps in the repertoire's coverage of peptide space. The observed number of different MHC molecules per individual may result from a trade-off between these demands.

The nature of MHC restriction needs to be considered carefully in these arguments. If restriction is absolute and each TCR recognizes only one MHC allele, increasing the number of alleles per person simply increases the size and diversity of the T cell repertoire with no cost because selection operates on each MHC-restricted subset of the pre-selection repertoire independently. In this case an upper limit to within-host MHC diversity might derive only from the need for APC to display sufficient numbers of peptides in conjunction with each MHC molecule to reliably mediate selection or immune activation. The trade-off evident in the qualitative argument above arises when MHC restriction is not absolute and thymocytes are capable of being positively and/or negatively selected on more than one allele.

Woelfing et al. (118) provide an excellent review of theoretical approaches to understanding intra-individual MHC diversity, but we outline the key results here. Nowak et al. (119) were the first to assess the qualitative trade-off argument using a mathematical model. In their analysis they defined h and f to be the proportions of T cells capable of being positively and negatively selected, respectively, by a given MHC allele. If an individual expresses n distinct MHC alleles, they argue that the proportion of the T cell repertoire surviving selection is

$$(1 - (1 - h)^n)(1 - f)^n.$$

The first term represents positive selection; $(1 - h)^n$ is the probability that a TCR fails to be selected by any MHC. The second term

represents negative selection; $(1-f)^n$ is the probability that a TCR is not negatively selected by any MHC. The proportion of the repertoire surviving is maximized at $n=(1/h)\log(1+h/f)$. They argue that $h \leq f$, supported by the experimental and modeling consensus is that positive selection is more stringent than negative selection. This gives $n \sim 1/f$. However, using only the assumptions that $hn \ll 1$, or that it is rare for a TCR to be positively selected on more than one MHC allele, and that the proportion of all peptides that can bind to a given MHC is $\ll 1$, they calculate that $n=2/f$ maximizes the probability of a response to a randomly chosen foreign pMHC.

Borghans et al. (120) pointed out that this model contains an inconsistency, which allows for cells that fail to be positively selected on one MHC to be negatively selected by the same MHC. They denoted p and n to be the unconditional probabilities that one TCR is positively and negatively selected by a given MHC molecule. Then $n < p$, because the number of cells that fail negative selection on one MHC is necessarily smaller than the number that audition for it following positive selection on that same MHC. The proportion of the original repertoire that survives is then

$$(1-n)^M - (1-p)^M. \tag{4}$$

This model effectively lowers the stringency of negative selection expressed in Nowak et al. (119) and so reduces the cost of increasing the number of MHC alleles. They estimated the probabilities p and n were 0.01 and 0.005 respectively, using the known efficiencies of positive and negative selection in mice with known numbers of MHC alleles. The optimal value of M for these parameter values is far larger than observed allele numbers; conversely, asking what values of p and n correspond to the observed ranges of M being optimal leads to unrealistic levels of positive and negative selection. Their analysis therefore questions the trade-off hypothesis as an explanation of limited MHC diversity.

They suggest alternatives. They estimate that existing typical numbers of MHC alleles together with TCR cross-reactivity may be "good enough" for maximizing the probability of responding to a foreign peptide on self-MHC – in this case the selective pressure for increasing MHC alleles is weak or absent. Alternatively, increased numbers of MHC alleles may increase the risk of autoimmunity through cross-reactivity of T cells responding to antigen that have not been fully tolerized to self. Finally, limited numbers of MHC alleles may allow for sufficient densities of pMHC on the surface of antigen-presenting cells to be able to efficiently select and activate MHC-restricted T cells.

MHC restriction is not absolute in the models described above, although it holds approximately for positive selection when the per-allele positive selection probability p is small. However, there is evidence to suggest that MHC restriction is not manifest strongly at the positive selection stage. Zerrahn et al. (22) observed that a relatively large proportion of TCR still positively select when a single type of pMHC was expressed in the thymus. In that study, pre-selection TCRs had approximately a 5% chance of responding to a given class II MHC, independently for different alleles, validating one of the assumptions of these simple probabilistic selection models. On similar lines, Huseby et al. (92) found that the positively-selected repertoire contains TCR with a high degree of cross-reactivity across MHC alleles, and suggested that MHC restriction emerges as a result of negative selection. Finally, the high degree of alloreactivity suggests that positive selection is only weakly MHC-restricted, and that failure to positive select reflects a generic inability to bind to MHC.

Motivated by this possibility, Woelfing et al. (118) revisited these probabilistic models. They assumed positive selection is highly degenerate with respect to MHC and that even very weak cross-reactivity with any allele is sufficient. Under this assumption, one of the presumed advantages of high MHC diversity is removed. Maximizing the probability of mounting an immune response, they estimated the optimal MHC diversity to be in a physiological range of 3–25.

Van den Berg and Rand (121) used a very different and sophisticated approach to the same optimality problem using a mechanistic, stochastic model of TCR triggering rather than the probabilistic repertoire-based models described above. Considering negative selection only, they concluded that limited individual MHC diversity is beneficial for self–non-self discrimination. The essence of their mathematical argument is that restricting the "diversity of foreign" is the key to increasing the signal-to-noise ratio for aTCR attempting to discriminate a foreign peptide from the background of self. This is achieved with a combination of limiting the number of MHC alleles each TCR can recognize (MHC restriction) and limiting the number of peptides that can be presented from one protein on one MHC allele ("peptide selectivity") to be roughly one. However, the need to ensure that every foreign protein is represented requires multiple MHC alleles, placing a theoretical lower bound on their number. An upper bound comes from the requirement that the density of relevant pMHC ligands must not fall too low on the surface of an APC, similar to the suggestion in Borghans et al. (120) – if a given pMHC is diluted by too many MHC, the relevant TCR will experience fluctuations in signaling that may reduce its discriminatory power. They conclude that of the order 10 MHC alleles is optimal. Notably, as in Ref. (118), this estimate arises without any constraints from positive selection.

SUMMARY

This review has outlined how several relatively simple descriptions of single TCR–pMHC interactions have been used to understand aspects of TCR repertoire development. However, the discussion is necessarily incomplete. In particular, there is an extensive literature exploring the molecular mechanisms by which individual or collections of TCR discriminate between ligands of different affinities [see, for example, Ref. (100, 122–126)], which has direct relevance to thymic selection. It remains unclear how proximal TCR signals derived from multiple and diverse pMHC ligands can drive the emergence of specificity and MHC restriction in the post-selection repertoire, although the models of selection on ensembles of ligands have made steps in this direction (99, 121). Are repeated super-threshold contacts required for negative selection, or is a single encounter with a high affinity ligand sufficient to cause deletion?

Many of the models discussed here assume that a single interaction above a minimum signaling threshold is sufficient for positive selection. However, there is evidence that repeated or sustained TCR signaling is required during the DP stage for positive selection

to occur [see, for example, Ref. (17, 127)]. This may explain findings that positive and negative selection take place concurrently (46).

Overall it is remarkable how much insight into the quantitative aspects of thymic selection has emerged from highly abstracted models. However, there remain a lot of open areas for research, and many of the questions raised in the introduction are still unresolved. Regulatory T cell development in particular has received very little attention from modelers, and already it appears that the simplest extension to the simple probabilistic fixed-threshold model to include a fixed range of affinity or avidity for Treg selection is not sufficient to explain many experimental observations (107). The task of synthesizing and reconciling the huge diversity of experimental data related to thymic development is a daunting one, but the information available is perhaps currently underexploited by theorists.

ACKNOWLEDGMENTS

The author thanks Charles Sinclair and the reviewers for helpful comments. This work was supported by the NIH (R01AI093870).

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Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 10 September 2013; paper pending published: 23 October 2013; accepted: 09 January 2014; published online: 04 February 2014.
Citation: Yates AJ (2014) Theories and quantification of thymic selection. Front. Immunol. ***5****:13. doi: 10.3389/fimmu.2014.00013*
This article was submitted to T Cell Biology, a section of the journal Frontiers in Immunology.

frontiers in
IMMUNOLOGY

OPINION ARTICLE
published: 22 May 2014
doi: 10.3389/fimmu.2014.00239

Harnessing human cross-presenting CLEC9A$^+$XCR1$^+$ dendritic cells for immunotherapy

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Edited by:
Marianne Boes, University Medical Centre Utrecht, Netherlands

Reviewed by:
Natalio Garbi, University of Bonn, Germany
Marianne Boes, University Medical Centre Utrecht, Netherlands

Keywords: dendritic cells, immunotherapy, cross-presentation, DC targeting, Clec9A, XCR1

INTRODUCTION

Dendritic cells (DC) are professional antigen presenting cells (APCs) that play a pivotal role in the induction and regulation of immune responses, including the induction of cytotoxic T lymphocyte (CTL) responses. They are an important focus for the development of vaccines against cancers and many pathogens, including HIV and malaria, where CTL responses are required for protection and disease eradication. DC loaded *ex vivo* with tumor antigen (Ag) have been administered as vaccines to cancer patients for over 15 years. They are well-tolerated and induce immune responses, including some clinical regressions, but there is clearly room for improvement (1). The DC network in both mice and humans is heterogeneous, with specialized DC subsets driving specific immune functions (2). New developments in our understanding of DC biology have identified a subset of DC characterized by the expression of novel markers CLEC9A (DNGR-1) (3, 4) and XCR1 (5, 6) as being important for the induction of CTL responses (7). Vaccine strategies that deliver Ag and activators directly to CLEC9A$^+$XCR1$^+$ DC *in vivo* promise to overcome many of the logistical issues associated with *in vitro*-derived vaccines, allowing precision and specificity of the desired immune response (8). Here, we discuss the biological properties of CLEC9A$^+$XCR1$^+$ DC that make them such attractive targets for CTL vaccines and new vaccine approaches to target them *in vivo*.

CLEC9A$^+$XCR1$^+$ DC ARE ESSENTIAL FOR CTL INDUCTION

The emerging complexity of the DC network and the optimal DC subset to target is the first important consideration for the design of new vaccines that target DC *in vivo*. In human and mouse, multiple DC subsets exist that vary in location, phenotype, and specialized function (2). They can be broadly classified as (i) inflammatory monocyte-derived (Mo) DC that develop from monocytes and are rapidly recruited to sites of inflammation; (ii) plasmacytoid DC (pDC) that are major producers of type I interferons (IFN) in response to TLR 7/9 ligation and are key for anti-viral immunity; and (iii) conventional DC (cDC) that can be further divided based on location into "lymphoid-resident" and "migratory" DC (2). The lymphoid-resident DC capture Ag directly in lymphoid tissues, whereas the migratory DC reside in the peripheral organs (e.g. lung, skin, and gut) where they capture Ag then migrate to lymphoid tissues to share their Ag with other lymphoid-resident DC, or present Ag directly to T cells. In both locations, cDC can be further segregated into subsets with specialized functions. Increasing evidence points to a role for the mouse CD11b$^+$ cDC subset in the induction of CD4$^+$ T cell responses although a similar role for the equivalent human CD1c$^+$ DC subset has not yet been established (2, 9). However, it is the subset defined by expression of the C-type lectin-like receptor, CLEC9A, and the chemokine receptor, XCR1, that is crucial for the induction of CTL responses against cancers, viruses, and other pathogenic infections (2, 7).

CLEC9A$^+$XCR1$^+$ DC were originally identified in mice by expression of the markers CD8α on lymphoid-resident DC or CD103 on migratory DC and are commonly referred to as CD8α$^+$ lymphoid and CD103$^+$ migratory DC. In humans, CLEC9A$^+$XCR1$^+$ DC, commonly referred to as CD141$^+$ DC, are found in both lymphoid and non-lymphoid tissues, including skin, gut, liver, and lungs (6, 10–13). CLEC9A and XCR1 are exclusively expressed by this unique DC subset in lymphoid and non-lymphoid tissues of both species, with the exception of low levels of expression of Clec9A by mouse pDC. As these markers combined are currently the most specific means of defining these DC in both species, we hereafter refer to them as CLEC9A$^+$XCR1$^+$ DC. In addition to CLEC9A and XCR1, these DC share expression of the nectin-like protein, Necl2 (14) and TLR3, and are major producers of IFN-λ after TLR3 ligation (15). Importantly, they excel at cross-presentation, the mechanism that allows exogenous Ag, such as that captured from tumors and virally infected cells to be processed and presented on MHC I for recognition by CTLs (16).

WHAT MAKES CLEC9A$^+$XCR1$^+$ DC SO EFFECTIVE AT CTL PRIMING?

Although other cell types, including macrophages, B cells, and other DC subsets, can cross-present under particular

circumstances *in vitro* (17–20), there is substantial evidence to demonstrate that CLEC9A$^+$XCR1$^+$ DC are inherently more efficient at this process *in vitro* and *in vivo* (6, 7, 10, 11, 16). The precise molecular mechanisms are not understood but extensive efforts have yet to reveal specialized cross-presentation machinery unique to CLEC9A$^+$XCR1$^+$ DC (16). However, there are several features of these DC that collectively explain their superior cross-priming ability despite a similar Ag uptake capacity compared with other DC subsets. Firstly, CLEC9A$^+$XCR1$^+$ DC maintain a less acidic pH in endosomes and phagosomes, favoring cross-presentation from early endocytic vesicles (21), and facilitating cross-presentation of Ag targeted to late endosomes/lysosomes (20, 22). Secondly, CLEC9A$^+$XCR1$^+$ DC are more efficient at translocation of Ag from endosomes/phagosomes into the cytosol for access to the classical MHC I processing pathway (23). Thirdly, CLEC9A, a receptor for actin filaments exposed on dead cells, plays a key role in delivering Ag captured from dead cell for cross-priming (24–27). Fourthly, CLEC9A$^+$XCR1$^+$ DC express high levels of TLR3, a known enhancer of cross-priming (28). Finally, constitutive activation of unfolded-protein-response sensor, IRE-1α, and the transcription factor XBP-1 was recently shown to regulate cross-presentation specifically by CLEC9A$^+$XCR1$^+$ DC (29). There is also evidence that XCR1 and Necl2 are involved in CTL activation, although not directly via augmenting the cross-presentation pathway (5, 6, 14). These features provide a strong rationale to develop technologies that specifically deliver Ag to the cross-presentation pathway of CLEC9A$^+$XCR1$^+$DC *in vivo*.

TARGETING CLEC9A$^+$XCR1$^+$DC *IN VIVO*

Antibodies (Ab) specific for DC surface receptors, particularly Ag uptake receptors, can be harnessed to deliver Ag directly to DC *in vivo* (30). The choice of receptor depends on its specificity for the DC subset to be targeted in addition to the Ag processing and presentation pathway used by the receptor following internalization. A variety of C-type lectin receptors (CLR) have been exploited for this purpose, and this is reviewed elsewhere (1, 30) but for delivering Ag to CLEC9A$^+$XCR1$^+$ DC in mice, DEC-205 has been a major focus. Delivery of Ag via DEC-205 Ab induces both CD4$^+$ and CD8$^+$ T cell responses in the presence of adjuvant and is superior to *ex vivo* loaded DC vaccines at preventing tumor growth [reviewed elsewhere (31)]. Phase I/II clinical trials targeting NY-ESO-1 Ag for treatment of multiple solid malignancies expressing this Ag are in progress utilizing CDX-1401, a fully humanized Ab against DEC-205 (CellDex Therapeutics). In humans, DEC-205 is widely expressed on all DC, in addition to B cells, T cells, and NK cells. Although CLEC9A$^+$XCR1$^+$ DC, CD1c$^+$ DC, pDC, and MoDC have been shown to process and present Ag delivered by DEC-205 to CD4$^+$ and CD8$^+$ T cells *in vitro* (20, 31–33), limited direct comparisons suggest CLEC9A$^+$XCR1$^+$ DC to be more effective at cross-presentation (20). This is likely due to the preferential trafficking of DEC-205 to late endosomes, which typically favors Ag processing via the MHC II pathway (34), whilst still allowing cross-presentation by CLEC9A$^+$XCR1$^+$ DC (20).

An attractive approach is to more specifically deliver Ag to CLEC9A$^+$XCR1$^+$ DC using Ab or ligands specific for CLEC9A (3, 4) or XCR1 (35). Studies utilizing Clec9A for Ag delivery in mice observe effective CD8$^+$ T cell responses and, surprisingly, superior CD4$^+$ T cell immunity when directly compared to DEC-205, even in the absence of adjuvant (3, 4, 36). Key reasons for the efficacy of targeting Clec9A include its intracellular trafficking, as Clec9A delivers Ag to early and recycling endosomes (27), and the persistence of anti-Clec9A Ab in serum, resulting in prolonged Ag presentation (36). Determining the molecular interactions of CLEC9A following internalization and how this influences Ag trafficking and processing, will undoubtedly shed light on the basis for Clec9A targeting efficacy.

Anti-human CLEC9A Ab can deliver Ag to human CLEC9A$^+$XCR1$^+$ DC for processing and presentation to both CD4$^+$ and CD8$^+$ T cell lines *in vitro* (37). This provides proof-of-principle and a strong rationale to further develop anti-human CLEC9A Ab for vaccines and more comprehensively compare with DEC-205 Ab and other approaches that target multiple DC subsets. Such studies have been limited due to difficulties in obtaining sufficient numbers of human CLEC9A$^+$XCR1$^+$ DC for detailed functional analysis, but are now feasible with the development of new humanized mouse models, where functional human CLEC9A$^+$XCR1$^+$ DC develop and can be targeted with CLEC9A or DEC-205 Abs *in vivo* (38).

ADJUVANTS FOR ACTIVATION OF CLEC9A$^+$XCR1$^+$DC

Early DC clinical trials and mouse studies investigating Clec9A or DEC-205 targeting Ab have clearly demonstrated a requirement for DC activation in order to induce optimal CTL responses (31, 39). TLR ligands are some of the most promising adjuvants currently being evaluated in the clinic and differential expression of TLR by DC subsets could profoundly affect the choice of adjuvant. This is a particularly important consideration for the preclinical evaluation of vaccines targeting CLEC9A$^+$XCR1$^+$ DC since TLR expression varies in mouse and human DC subsets. The TLR9 ligand, CpG, has been widely used as an adjuvant in mice, including with Clec9A Ab (36) and has been evaluated clinically, with limited adverse effects, as an adjuvant in cancer chemotherapy and *ex vivo* DC vaccines (40). Whilst TLR9 is widely expressed in mice, including by CLEC9A$^+$XCR1$^+$ DC, in humans it is restricted to pDCs (39). However, activation of human pDC by CpG induces large amounts of type I IFN that could potentially play an important bystander function for activation of CLEC9A$^+$XCR1$^+$ DC and subsequent induction of anti-tumor responses (41, 42). In contrast to their mouse counterparts, human CLEC9A$^+$XCR1$^+$ DC also lack expression of TLR4 but express TLR8, which is not functional in mice (39).

A TLR7/8 ligand, R848 or resiquimod, has been FDA approved for topical use and is currently undergoing clinical trials with DEC-205 (CDX-1401, CellDex) (43). It also activates CD1c$^+$ DC via TLR8 and pDC via TLR7. Its potential to be used in vaccines remains to be determined, with murine studies indicating that its short half-life and formulation may not be ideal for activating DC locally to initiate adaptive immune responses, and it has been implicated in severe side effects observed in clinical trials (43).

The TLR3 ligand, polyI:C, is emerging as an attractive adjuvant to combine with DC targeting Ab, as TLR3 expression is conserved across human and mouse CLEC9A$^+$XCR1$^+$ DC. PolyI:C was found to be the optimal adjuvant to use in combination with DEC-205 targeting Ab in mice (44). The poly I:C derivatives Hiltonol and Ampligen are well-tolerated in humans and induce a type I IFN response mimicking that of a viral infection (45). These are now being evaluated in clinical trials in conjunction with DEC-205 targeting Ab (CellDex Therapeutics; NCT00948961).

CONCLUSION

There remains a great need for the development of vaccines that elicit effective antiviral and anti-tumor CTL responses. The discovery of the CLEC9A$^+$XCR1$^+$DC in mice and humans, as a subset specialized for Ag cross-presentation and cross-priming CTL, has revealed promising new avenues for vaccine design. Yet, the contribution of other DC subsets to the efficacy of this process is still to be determined. Thus, the questions remain: is it more effective to deliver Ag to the CLEC9A$^+$XCR1$^+$DC that are best-equipped for cross-presentation, or will co-delivery to other DC subsets provide help? Which receptors will best deliver the Ag to the required intracellular compartments, and which adjuvants will best enhance immune responses? Studies to date suggest that targeting CLEC9A$^+$XCR1$^+$ DC *in vivo*, together with adjuvants to specifically activate these DC, offers great promise. The advancement of humanized mouse models allowing for development of CLEC9A$^+$XCR1$^+$ DC and other DC subsets, will enable these and other questions to be answered, and facilitate translation from bench to bed-side.

ACKNOWLEDGMENTS

Mireille H. Lahoud and Kristen J. Radford are supported by project grants from the National Health and Medical Research Council of Australia (NHMRC 604306 and 1025201) and the Prostate Cancer Foundation of Australia (PG2110). Kristen J. Radford holds a NHMRC CDF level 2 fellowship. Kirsteen M. Tullett is the recipient of a University of Queensland International PhD Scholarship. This work was made possible through Victorian State Government Operational Infrastructure Support and Australian Government NHMRC Independent Research Institute Infrastructure Support Scheme.

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Conflict of Interest Statement: Mireille H. Lahoud and Kirsteen M. Tullett are listed as inventors on patent applications relating to Clec9A. Kristen J. Radford has no conflicts of interest to report.

Received: 24 March 2014; paper pending published: 14 April 2014; accepted: 08 May 2014; published online: 22 May 2014.
*Citation: Tullett KM, Lahoud MH and Radford KJ (2014) Harnessing human cross-presenting CLEC9A$^+$XCR1$^+$ dendritic cells for immunotherapy. Front. Immunol. **5**:239. doi: 10.3389/fimmu.2014.00239*
This article was submitted to Antigen Presenting Cell Biology, a section of the journal Frontiers in Immunology.

frontiers in
IMMUNOLOGY

REVIEW ARTICLE
published: 11 April 2014
doi: 10.3389/fimmu.2014.00165

Paradigm shift in dendritic cell-based immunotherapy: from *in vitro* generated monocyte-derived DCs to naturally circulating DC subsets

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Dendritic cell (DC)-based immunotherapy employs the patients' immune system to fight neoplastic lesions spread over the entire body. This makes it an important therapy option for patients suffering from metastatic melanoma, which is often resistant to chemotherapy. However, conventional cellular vaccination approaches, based on monocyte-derived DCs (moDCs), only achieved modest response rates despite continued optimization of various vaccination parameters. In addition, the generation of moDCs requires extensive *ex vivo* culturing conceivably hampering the immunogenicity of the vaccine. Recent studies, thus, focused on vaccines that make use of primary DCs. Though rare in the blood, these naturally circulating DCs can be readily isolated and activated thereby circumventing lengthy *ex vivo* culture periods. The first clinical trials not only showed increased survival rates but also the induction of diversified anti-cancer immune responses. Upcoming treatment paradigms aim to include several primary DC subsets in a single vaccine as pre-clinical studies identified synergistic effects between various antigen-presenting cells.

Keywords: dendritic cell vaccination, immunotherapy, naturally circulating dendritic cells, melanoma, monocyte-derived dendritic cells, plasmacytoid dendritic cells, myeloid dendritic cells

INTRODUCTION

Melanoma is a malignant transformation of melanocytes – the pigment producing cells of the epidermis – and the most aggressive cancer of the skin (1). Over the past years, the number of melanoma incidences rose worldwide and reached 232,130 diagnosed cases in 2012 (2–4). Once melanoma patients develop metastatic disease, life expectancy drops and survival rates are low (1, 5, 6). Traditional treatment methods focus on chemotherapy and radiation therapy, which are highly invasive and often fail to induce objective clinical response (6).

Novel treatment strategies focus on melanoma patients that carry an activating mutation in protein kinases involved in MAPK or AKT signaling (7). Recently approved small molecule inhibitors, such as vemurafenib, allow specific targeting of these mutated kinases and lead to rapid tumor regression and prolonged survival in treated patients (7–9). However, due to the prompt development of resistance in many cases, and major cutaneous side effects, including the induction of neoplastic lesions, small molecule inhibitors are so far of limited clinical use (6, 8).

As pharmacological treatment paradigms fail to induce lasting responses, researchers, clinicians, and patients turn to immunotherapy, which – due to major advances – was recently declared as breakthrough of the year 2013 by scientific journal *Science* (10).

The ability of the immune system to fight tumors was first described by William B. Coley, who in the nineteenth century observed cancer regression in patients suffering from inoperable sarcoma after injecting bacterial toxins into neoplastic lesions (11). Today, cytotoxic $CD8^+$ T lymphocytes (CTLs) are considered to be the fundamental mediators of anti-cancer immunity (12–16). *In vitro* experiments and studies in mice showed that CTLs are able to specifically target cancerous cells and destroy them by inducing apoptosis (12, 13, 17). Clinical evidence confirmed the importance of CTLs in patients suffering from melanoma and other cancers, as infiltrating $CD8^+$ T cells found in tumor biopsies were strongly associated with improved life expectancy (18–20). Furthermore, melanoma patients with tumor-specific T cells in peripheral blood displayed increased clinical response rates (21). Immunotherapy hence aims to induce a potent and lasting T cell response against malignant cells.

One approach to potentiate the patient's own immune response is to prolong the activity phase of the T cell response. Immunomodulatory drugs, such as the CTLA-4-blocking antibody ipilimumab or the PD-1-blocking antibody nivolumab, aim to unleash the patients' natural anti-cancer T cell responses by interfering with inhibitory pathways (22–27). Neoplastic cells frequently exploit, e.g., the PD-1 pathways to suppress the immune system leading to immune escape and disease progression (28, 29). Notably, ipilimumab was the first treatment agent to provide survival benefit for patients suffering from melanoma and is now

Abbreviations: APC, antigen-presenting cell; CTL, cytotoxic T lymphocyte; DC, dendritic cell; FSME, Frühsommer-meningoencephalitis; HLA, human leukocyte antigen; i.d., intradermal; IFN, interferon; IL, interleukin; i.v., intravenous; KLH, keyhole limpet hemocyanin; mDC, myeloid dendritic cell; MHC, major histocompatibility complex; moDC, monocyte-derived dendritic cell; NK cell, natural killer cell; pDC, plasmacytoid dendritic cell; PRR, pathogen recognition receptor; TAA, tumor-associated antigen; TLR, toll-like receptor.

standard treatment for this type of cancer (10, 26, 28). Although only effective in a minority of patients, ipilimumab frequently induces objective responses that are remarkably long lasting (26, 30). Due to their broad mechanism of action, immunomodulatory antibodies can, however, cause severe and potentially fatal side effects by activating autoreactive T cells. Patients with, e.g., skin rash, colitis, hypophysitis, or high-grade hepatic adverse events were reported (6, 30). To overcome these side effects, targeted therapies that only activate cancer-specific T cells are desired.

Specific T cell responses are naturally induced by dendritic cells (DCs) (31, 32). DCs are professional antigen-presenting cells (APCs) that sample the body for antigens and danger signals derived from pathogens or tumors (33). After encountering such signals, DCs become activated and migrate to the lymph node, where they activate naïve T cells to become CTLs or helper T cells (32, 33). Due to their great regulatory capacities and outstanding ability to activate antigen-specific T cells, DCs have become an attractive target in several immunotherapeutic approaches in cancer.

Cellular vaccination therapies were developed in the mid 1990s, when new laboratory techniques allowed the enrichment of DCs from peripheral blood (34–37). Murine DCs were isolated from peripheral blood by density gradients, loaded *ex vivo* with tumor antigens, and injected back into the blood (17, 38). This technique was rapidly transferred to the clinical setting when in 1996 pioneer Frank Hsu treated patients suffering from B-cell lymphoma with autologous, antigen-loaded DCs (39). Strikingly, clinical response could be detected in a majority of patients, kickstarting the field of therapeutic DC vaccination (**Figure 1**).

However, only after Sallusto and colleagues discovered a method to differentiate DCs from monocytes *in vitro*, sufficient cellular material was available to start clinical trials that went further than pure proof of principal (40). Following this development, Nestle and colleagues conducted the first DC vaccination trail in melanoma patients in 1998 (41). In this study, the group isolated autologous monocytes from peripheral blood of the patients and generated DCs *ex vivo*. Monocyte-derived DCs (moDCs) were subsequently pulsed with tumor-associated antigen (TAA) peptides or tumor lysate, and injected into the lymph nodes of the patients to activate the immune system. The results of this study were promising, as complete and partial responses could be observed in a number of patients. Furthermore, tumor-specific T cells were found in vaccinated patients, indicating the induction of a melanoma-specific immune response.

In the following years, a considerable number of phase I/II clinical trials explored the impact of various vaccination parameters on the treatment outcome. In this review, we will give an overview of the major advances in the field of therapeutic DC vaccination against melanoma since the initial study by Nestle. Further, we will highlight current developments focusing on natural DC subsets and their impact on immunotherapy, and we will conclude with an outlook on future vaccination strategies including the synergistic effects of DC subsets.

MATURATION OF DCs

A major disadvantage of the DC vaccination protocol employed by Nestle et al. was the lack of activation signals. After differentiation, most moDCs possess an immature phenotype, which is dominated by high antigen uptake capabilities and poor T cell stimulatory abilities (42–45). Activation of DCs leads to the development of a mature phenotype characterized by upregulation of co-stimulatory molecules, major histocompatibility complex (MHC) molecules, and certain chemokine receptors (33, 46, 47). Especially the latter is of great importance for vaccination efficacy, as expression of the chemokine receptor CCR7 promotes the migration of injected DCs to the lymph nodes where the activation of T- and B-cells occurs (42, 47, 48). In addition to their inferior stimulatory capabilities, immature DCs were shown to induce antigen-specific tolerance, proposing that injection without activation signals is not only ineffective but also potentially detrimental (49).

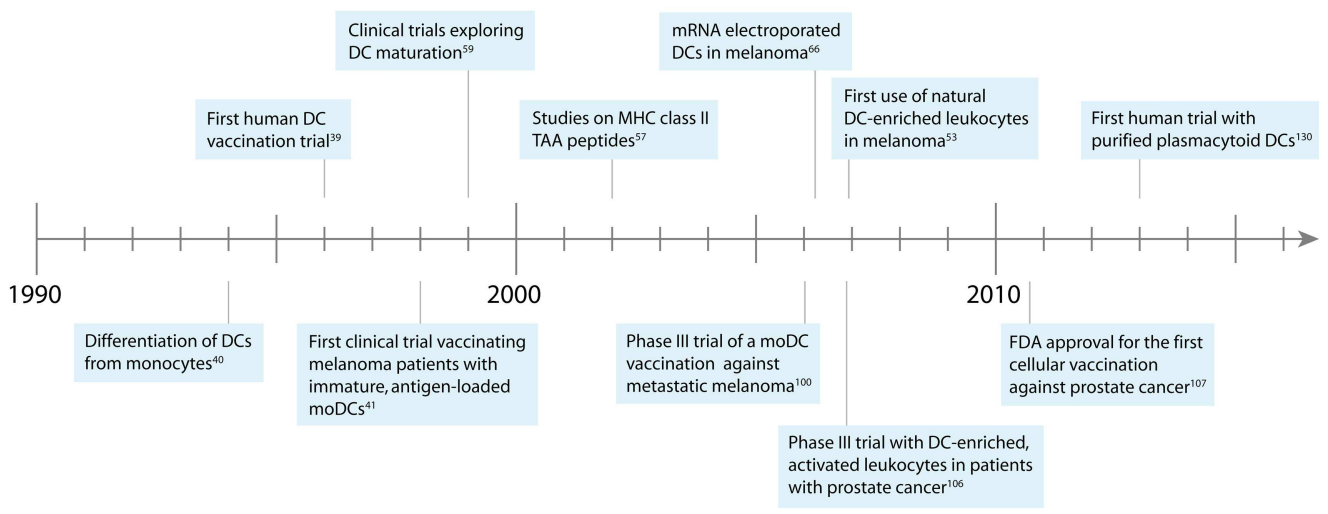

FIGURE 1 | Development of DC-based immunotherapy against melanoma.

While *in vivo* maturation signals primarily come from contact with pathogens or tissue injury, immature DCs can be matured by incubation with pathogen recognition receptor (PRR) agonists or cytokines such as TNF-α, and prostaglandin E_2 (50, 51). In a clinical setting, CD40 ligation has also been used for DC activation (52, 53).

In 2003, a phase I/II clinical trial treating stage IV metastatic melanoma patients with autologous, antigen-loaded moDCs confirmed the superiority of mature DCs to induce strong immunity, as the immunological response against both included TAAs and the control antigen keyhole limpet hemocyanin (KLH) was improved in the majority of patients treated with mature DCs, as opposed to immature DCs (54). Strikingly, tumor regression could only be observed in patients of the mature DC arm, indicating that activating DCs prior injection improves clinical response as well. Other groups that employed modified maturation cocktails made the similar observations that DC maturation is necessary for the induction of a superior immune response (55–59). These results confirmed in a clinical setting what was already known for *in vitro* models: infused DCs need to express potent stimulatory molecules to generate a strong T cell response, especially when presenting cancer antigens with low immunogenicity. Nevertheless, as proper homing to the lymph nodes is a prerequisite for DC-mediated T cell activation, upregulation of CCR7 may also partly explain the observed differences (42).

ROUTE OF ADMINISTRATION

In addition to maturation-induced upregulation of CCR7, the route of administration has a major impact on the migration of DCs to the T cell rich zones in the lymph nodes (42). Since intravenously (i.v.) injected, *ex vivo* generated DCs fail to induce potent skin-homing T cells in mice and appeared to be less efficient in inducing T_H1 responses in humans, previous clinical trials focused on subcutaneous or intradermal (i.d.) administration of the vaccines (60–62). However, using ^{111}In-labeling and scintigraphy, we could show that most of the injected DCs remain at the injection site, where they rapidly die to be phagocytosed by macrophages (42, 63, 64). Pretreatment of the skin with cytokines, toll-like receptor (TLR) ligands, or activated DCs did not lead to increased migration (64). Interestingly, Aarntzen et al. identified the number of injected DCs as an important factor for migration as a low cell density at the injection site correlated with high migration efficiency (64).

To further improve migration of DCs to lymph nodes and enhance the induced immune responses, different routes of administration have been explored in various studies (65, 66). Direct injection of DCs into the lymphatic system of the skin appeared to be a promising approach, as it ensures that most of the DCs reach the T- and B-cell rich zones of the lymph nodes. To test this hypothesis, our group conducted a phase I/II clinical trial and vaccinated melanoma patients with *ex vivo* generated, antigen-loaded, mature moDCs that were injected either intranodally or intradermally (65). Although intranodal vaccination led to increased DC migration to efferent lymph nodes, no difference in the frequency of tetramer-specific T cells could be detected. Furthermore, melanoma-specific T cells induced by i.d. vaccination turned out to be more functional, which might be caused by bystander activation of APCs at the injection site. Similar results have been found by Kyte et al. using mRNA transfected moDCs (66). Taking the complicated procedure of intranodal vaccination into account, intradermal injection of DCs appears to be the optimal route of administration in case of sufficient cellular material.

T CELL HELP

In the late 90s several groups independently discovered that, in absence of a strong inflammatory stimulus, DCs need to interact with $CD4^+$ T cells to induce potent cytotoxic $CD8^+$ T cells – a process called DC licensing (67–70). These findings, together with other important discoveries in the early 2000s, shifted the focus of therapeutic anti-cancer vaccination toward the $CD4^+$ T cells and the impact of helper responses (71–73). Besides licensing DCs, T cell help plays a crucial role in memory generation and maintenance as well as affinity maturation of tumor-specific antibodies (72, 74, 75). Additionally, $CD4^+$ T cells were shown to activate the innate immune system, to enhance the cytolytic function of macrophages, to induce senescence in malignant cells, and to destroy neoplastic cells directly (76, 77). The latter is of particular importance in the melanoma setting, where transformed melanocytes tend to constitutively express MHC class II molecules (78, 79). In particular, T_H1 cells appear to be associated with favorable clinical outcome and overall survival (80). Despite this knowledge, integration of $CD4^+$ T cell help in clinical trials was hampered due to the lack of defined TAA peptides binding to MHC class II molecules. To partly overcome this limitation, DCs were pulsed with unrelated antigens such as KLH or tetanus toxoid. The $CD4^+$ T cells generated against these antigens were supposed to secrete interleukin (IL)-2 and pro-inflammatory cytokines, and to further activate the injected DCs, leading to an improved priming of cancer-specific CTLs (81). Whether or not the antigen-independent $CD4^+$ T cell help had a strong effect on T cell priming could however not been definitely proven.

This changed when several groups characterized immunogenic melanoma-associated MHC class II epitopes of the tumor antigens gp100 and tyrosinase leading to a comparative study of melanoma patients treated with moDCs pulsed with both MHC class I and class II epitopes or MHC class I epitopes alone (79, 82–84). Analysis of patient samples showed that the simultaneous administration of TAAs restricted to both MHC classes lead to a broader anti-cancer T cell response with higher functionality compared to patients who received DCs loaded with epitopes for MHC class I only (79). Importantly, the tumor-specific $CD4^+$ T cells were Foxp3 negative and displayed a T_H1 phenotype, indicating that the vaccination did not induce regulatory T cells. This trend was reflected in the clinical response, as patients of the MHC class I/II arm showed increased progression free and overall survival, whereas no clinical benefit could be detected in patients of the MHC class I arm. The results thus indicate that antigen-specific $CD4^+$ T cell help is indeed beneficial for the induction of a strong cancer-specific immune response, which is in line with a number of other studies (57, 85).

ANTIGEN LOADING AND HETEROCLITIC PEPTIDES

Antigen loading was revolutionized when clinical grade mRNA electroporated moDCs became available. MRNAs coding for

full-length TAA proteins containing multiple immunogenic epitopes were synthesized and used to transfect DCs (86, 87). In this approach, the transfected DCs translate the injected mRNA into full-length proteins, which are subsequently degraded by the proteasome and presented on MHC class I molecules (86). Adding an MHC class II targeting tag to the mRNA leads to the transport of translated proteins to exosomes and presentation on MHC class II molecules, necessary for priming $CD4^+$ T cells (88, 89). Using electroporated DCs, several problems were solved: due to the presence of multiple immunogenic epitopes within the same protein, $CD8^+$ and $CD4^+$ T cells could be stimulated at the same time, and the induced immune responses became broader. The same effect rendered human leukocyte antigen (HLA)-restriction obsolete, as the various epitopes contained in each protein are able to bind to different HLA molecules. This made the enrollment of a much larger number of melanoma patients possible and increased the number of individuals potentially benefiting from this treatment (90, 91). These improvements however come with the price of reduced viability, which can turn into a serious problem when cellular material is scarce (92).

Studies using electroporated moDCs conducted by our group and others indeed showed the induction of specific $CD4^+$ and $CD8^+$ T cells in patients suffering from metastatic melanoma (63, 90, 91, 93). Interestingly, T cells specific for epitopes different from the TAA peptides employed in previous vaccines were readily detected in a number of patients, thus indicating an increased breadth of the immune response (93).

Soon after the first studies with electroporated moDCs were published, Bonehill et al. simplified the loading and activation process for moDCs distinctly. In their approach, they transfected DCs with mRNA, not only coding for TAA proteins, but also for the maturation-inducing molecules, CD40L and caTLR4 (constitutively active form of TLR4), as well as the T cell co-stimulatory molecule, CD70. This led to prolonged and enhanced maturation of DCs (90, 94, 95).

In parallel to the development of mRNA-based DC vaccines, various groups tried to improve the immunogenicity of the traditional peptide-pulsing approach to load DCs. Using rational design, researchers modified known TAA peptides by replacing single amino acids to improve binding to the MHC groove creating so called heteroclitic peptides (96–98). Due to tighter binding, heteroclitic peptides are presented for an extended time period, supposedly leading to stronger T cell activation. However, whereas many pre-clinical studies showed increased immunogenicity *in vitro*, clinical trials directly comparing modified and wild type peptides failed to measure any positive effect of heteroclitic peptides and even showed decreased frequencies of TAA-specific T cells in some patients (98). It appeared that the modified epitopes differed too much from the wild type peptide leading to the induction of T cells that were unable to detect endogenously presented antigens (99).

In summary, the development of mRNA electroporated moDCs simplified anti-cancer immunotherapy significantly as transfection of DCs not only induces a broad, HLA-independent $CD4^+$ and $CD8^+$ immune response but also reduces the time and costs for vaccine preparation. In contrast, heteroclitic peptides failed to prove superior immunogenicity in immunotherapy against melanoma.

EFFICACY OF DC IMMUNOTHERAPY

Although various vaccination parameters could be optimized and lasting responses were observed in selected patients, so far none of the conducted clinical trials using moDCs could demonstrate statistically supportable evidence for survival benefits in vaccinated patients. This became especially evident when in 2006 Schadendorf et al. published the first and so far only randomized phase III trial designed to demonstrate the clinical efficacy of moDC therapy in melanoma patients (100). The study was aborted early, as the Data Safety and Monitoring Board did not expect the group to reach the study goal. Analysis of the preliminary data could demonstrate the induction of an anti-cancer immune response in various patients but failed to show improved overall survival. Further, objective response was lower in the group of patients treated with DC vaccination as opposed to chemotherapy with dacarbazine (DTIC); thus no clinical benefit of moDC therapy could be detected.

One explanation for the observed lack of clinical response could be the inferior capacity of moDCs to induce effective anti-cancer immunity. However, as the study was already initiated in 1999 – thus only 1 year after the publication of the first phase I trial on moDC-based vaccines in melanoma by Nestle et al. – many of the aforementioned developments, including proper maturation of DCs, were not yet translated to the clinics (54, 100–103). Furthermore, several studies suggest that the employed maturation cocktail based on pro-inflammatory cytokines might not have been optimal for the induction of a strong anti-cancer immune response (51). DCs solely activated by these cytokines show only limited capabilities to produce polarizing cytokines that further decrease soon after activation – a phenomenon called exhaustion (51, 104, 105). At the time of injection, DCs thus might have possessed only limited capabilities to induce T_H1 cells and CTLs. Additionally, the employed clinical protocols were not suited for multicenter trials leading to highly variable maturation levels and low numbers of generated DCs (100).

Interestingly, in the same year as Schadendorf et al. published their moDC study, Small et al. presented the results of a placebo-controlled phase III trial on DC-based immunotherapy in patients with metastatic asymptomatic hormone refractory prostate cancer (106). In contrast to Schadendorf et al., the authors employed a heterogeneous mixture of readily isolated leukocytes enriched for naturally circulating DCs by gradient centrifugation, thus avoiding long term *in vitro* culture. The leukocytes were activated and antigen-loaded using a recombinant fusion protein consisting of granulocyte-macrophage colony-stimulating factor and the TAA protein prostatic acid phosphatase. The prepared leukocytes were subsequently injected i.v. – <48 h after isolation. Strikingly, significantly increased overall survival and prolonged time to disease progression could be observed among patients of the treatment arm, thereby proving clinical efficacy of DC-based immunotherapy. Together with supporting studies, these results finally led to the first FDA approval for a cell-based therapy, Provenge®, in 2010 (107).

NATURALLY CIRCULATING DCs

Inspired by the promising results of the Provenge® trial, we postulated that purified naturally circulating DCs would be superior in anti-cancer immunotherapy against melanoma (51). Not only are these DCs efficient in generating CTLs, they can also be readily isolated from the blood (108, 109). This allows immediate activation and antigen loading, thus avoiding long incubation periods and enabling robust standardization for use in multicenter trials. Therefore, natural DCs, despite their rare occurrence in peripheral blood, display various advantages over moDCs that are making them an attractive target for anti-cancer therapy.

Human naturally circulating DCs can be divided into two main subsets: plasmacytoid DCs (pDCs) and myeloid DCs (mDCs), each with distinct phenotype and function during the immune response (**Figure 2**) (110). MDCs can be further subdivided in CD1c$^+$ (BDCA1) DCs, CD141$^+$ (BDCA3) DCs, and CD16$^+$ cells, where the latter are considered to be more monocyte-like (111–115). MDCs are specialized in immunity against fungi and bacteria and have an enhanced ability to sense tissue injuries (110, 111). They are able to capture environmental- and cell-associated antigens and show high phagocytic activity (116).

CD141$^+$ DCs are specialized in the detection and uptake of necrotic cells and excel in cross-presenting these antigens to T cells (117–120). Remarkably, CD141$^+$ DCs uniquely express the C-type lectin CLEC9A (DNGR-1), which allows sensing of damaged cells by binding to exposed actin filaments (121, 122). In addition, CD141$^+$ DCs can be activated using a distinct set of TLRs including TLR1, 2, 3, 6, and 8 (117, 123). Especially, TLR3 is strongly expressed and leads to upregulation of co-stimulatory molecules, as well as the secretion of pro-inflammatory cytokines and chemokines (117, 123). Upon activation, CD141$^+$ DCs are able to secrete IFN-γ and IL-12, which allows the effective induction of T_H1 and CTL responses (117, 119). However, due to the limited availability in blood and lack of GMP-grade isolation reagents, CD141$^+$ DCs are currently not feasible for cellular immunotherapy. Several developments focusing on improved isolation and culturing, nevertheless, might allow their employment in future DC vaccination.

CD1c$^+$ DCs are responsive to a great variety of microbial and fungal stimuli (124). Triggering of TLRs 1/2/6 by bacterial ligands leads to the activation of CD1c$^+$ DCs and secretion of large amounts of the T_H1-skewing cytokine IL-12 (123, 125, 126). Due to their potent antigen processing and presentation machinery, activated CD1c$^+$ DCs are able to induce T_H1 cells and cytotoxic T cells leading to a potent cellular immune response (108, 112, 117, 123, 126, 127). Moreover, *in vitro* studies showed that CD1c$^+$ DCs isolated from healthy donors and prostate cancer patients are able to prime tumor-specific CD8$^+$ T cells (108, 128).

In contrast to mDCs, pDCs are specialized in the detection and control of viral infections (110, 129). Viral infections are rapidly detected by pDCs via the engagement of TLR7 and/or TLR9 (116, 129). TLR triggering by viral agents leads to a rapid burst of type I interferons (IFNs) and induces cytotoxic functions in pDCs as well as natural killer (NK) cells (110, 130, 131). These outstanding antiviral activities make pDCs the key effector cells in early antiviral immunity (110). In a steady state, pDCs are characterized by low expression of MHC class II and co-stimulatory molecules (111). This phenotype is associated with tolerance induction and T_H2 immunity, properties that are unfavorable for anti-cancer immunity (132). However, activation of

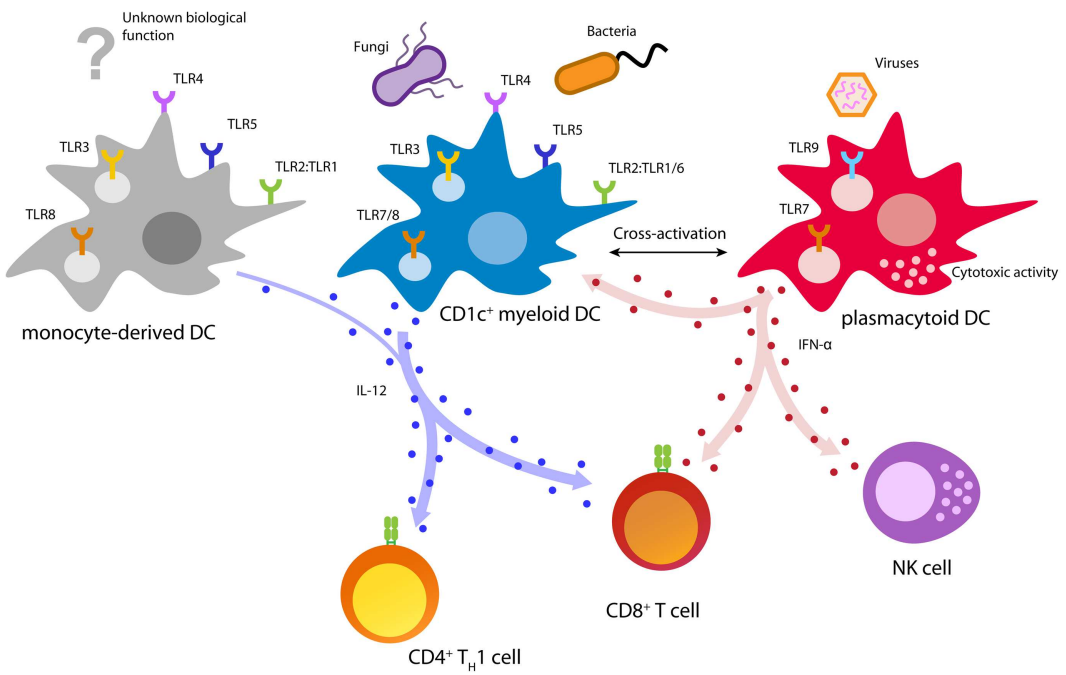

FIGURE 2 | Biology of immunotherapy-relevant human DC subsets. Depicted are major DC functions relevant for pathogen recognition and DC activation, T cell priming, and anti-cancer immunity.

pDCs leads to an upregulation of these proteins, turning pDCs into professional APCs that efficiently prime both, $CD4^+$ and $CD8^+$ T cells (108, 110, 131, 133). The strong release of type I IFN by pDCs leads to an IL-12 independent T_H1 polarization characterized by strong secretion of IFN-γ and IL-10 (110, 134–136). Despite low antigen uptake and limited phagocytosis, pDCs isolated from blood, tonsils, and spleen were shown to efficiently cross-present antigens to $CD8^+$ T cells (113, 120, 127, 137). Moreover, several studies reported that pDCs are able to prime potent melanoma-specific $CD8^+$ T cells, which produce IFN-γ and are able to locate to melanoma lesions (108, 120, 138, 139). Finally, pre-clinical mouse models showed that pDCs are able to induce a tumor-specific T cell response *in vivo*, leading to control of tumor growth (138, 140).

NATURALLY CIRCULATING DC-BASED IMMUNOTHERAPY

Due to the low occurrence of naturally circulating DCs in blood, conclusive clinical evidence on their usability for immunotherapy is lacking. In 2006, a small-scale study by Davis et al. reported on a vaccine that employed Flt3 ligand (Flt3L)-mobilized naturally circulating DCs (53). The treatment was safe and strong immune responses were detected in several patients. However, the purity of the employed DCs was generally low and, as it turned out, the administration of Flt3L induced the expansion of regulatory T cells in melanoma patients (53, 141).

Encouraged by the promising pre-clinical data, we initiated the first clinical trial on a cellular vaccine based on purified pDC in 2008 at RadboudUMC in the Netherlands (142). PDCs were isolated from leukapheresis products using MACS separation kits and cultured overnight in IL-3. On the next morning, pDCs were activated with a conventional Frühsommer-meningoencephalitis (FSME; English: tick-borne encephalitis) vaccine, which has the benefit of sustained secretion of T cell stimulatory cytokines due to natural triggering of TLRs (143). Subsequently, pDCs were loaded with TAA peptides, and injected intranodally.

Initial tests revealed only mild side effects of pDC vaccinations and the toxicity was even lower as compared to moDC vaccinations (142). Further, pDCs were able to activate the innate immune system, indicated by a systemic type I IFN signature. PDCs were also shown to efficiently migrate to efferent lymph nodes and FSME-specific adaptive immune responses were detected in 14 of 15 enrolled patients. The potent stimulatory capacities of pDCs were reflected in the cancer-specific immune response, as 7 of 15 patients showed increased frequencies of gp100-specific T cells after vaccination. Strikingly, TAA-specific T cell clones with high avidity could be identified after vaccination, indicating the induction of a strong functional response. Nevertheless, the overall magnitude of the induced melanoma-specific immune response appeared to be limited compared to previous moDC vaccination trials, as the total frequency of specific T cells in blood of pDC-vaccine patients was rather low (65, 93). Further analysis of skin-infiltrating lymphocytes obtained from delayed-type hypersensitivity reactions against tumor antigens – a sensitive assay to analyze functionality, migration, and specificity of anti-cancer T cells – showed positive responses in only 2 out of 15 tested patients (142, 144). Despite this, the overall survival of patients treated with pDCs was greatly increased in comparison to matched controls treated with standard chemotherapy. However, assumptions on clinical efficacy have to be taken with caution, as the study was primarily designed to assess the safety and toxicity of pDC-based immunotherapy.

Nevertheless, the prominent survival benefit of vaccinated patients is especially interesting in respect to the low frequency of TAA-specific T cells. Two explanations for this phenomenon are likely: (I) T cells induced by pDCs might be more potent and functional as compared to moDC primed T cells. This could be due to different cytokine secretion patterns, differential expression of co-stimulatory molecules, improved migratory capacities, or prolonged survival. (II) Alternatively, instead of – or in addition to – inducing T cell responses, the focus of pDC-mediated anti-cancer immunity might lie on the activation of NK cells and the innate immune system. Evidence for this comes from the lasting type I IFN signature induced in vaccinated patients (142). Strikingly, various studies report on pDC-dependent, IFN-α-mediated activation of natural DC subsets in arteriosclerosis, autoimmunity, and infections (145–147). Furthermore, it could be shown that type I IFNs are able to activate NK cells, induce IFN-γ secretion, and enhance cytotoxicity (148, 149). However, in comparison to subjects that underwent recombinant IFN-α therapy, patients vaccinated with pDCs showed longer overall survival indicating that the observed clinical benefits were not induced by type I IFNs alone (150–152). Interestingly, it was shown that contact-dependent interactions between pDCs and lymph node DCs greatly enhance Ag presentation and priming of anti-herpes simplex virus CTLs (153). The authors identified CD2–CD2L and CD40–CD40L as key mediators of this effect. PDCs can thus activate other DC subsets, for instance mDCs, to potentiate the immune response. However, this synergy not only acts in one direction: mDCs were shown to mature pDCs and enhance their Ag presentation capabilities during bacterial exposure (116, 154). Interestingly, in one scenario pDCs only act as APCs without instructing T cells with polarizing cytokines (116). Together, these results show that natural DCs of various subsets cooperate with each other to enhance the immune response and that the roles in this regulatory network are variable and depending on the stimulus. However, the studies also indicate a hierarchical organization within natural DC synergies, with one DC subset orchestrating and polarizing the immune response, and the other merely acting as "zombie" APC without instructive capabilities (116).

Strikingly, mouse experiments demonstrated that injection of a mixture of *ex vivo* activated and antigen-loaded mDCs and pDCs induces a superior immune response against tumors (155). Moreover, therapeutic efficiency, as assessed by overall survival and tumor burden, was greatly improved when mice received simultaneous injections of both subsets compared to injections of one subset alone (155). The observed synergistic effect was mainly based on enhanced antigen presentation by mDCs induced by contact-dependent interactions with pDCs. These observations might explain why patients in our pDC vaccination trial showed significantly increased overall survival despite low frequencies of vaccination-specific CTLs (142, 155). Injected pDCs might have activated mDCs present at the site of injection leading to the induction of a T_H1 and CTL response. As the *in situ* activated mDCs then would present naturally processed melanoma antigens expressed

at the site of the tumor, the subsequently induced anti-cancer immune response would not be fully detectable when examining the vaccine-specific T cell response only.

Subsequent to the pDC-based vaccine, we conducted a phase I trial vaccinating metastatic melanoma patients with *ex vivo* activated and antigen-loaded autologous blood $CD1c^+$ mDCs. Preliminary results confirm the safety and feasibility of mDC-based vaccines and could identify clinical responses in a number of patients (manuscript in preparation). Considering the results of these studies and the synergistic effects of pDCs and mDCs observed in mice and in *in vitro* models, the next step would be to initialize a human vaccination trial using a cocktail of activated and antigen-loaded mDCs and pDCs. Once injected in, e.g., the lymph node, these natural DC subsets might synergize and potentiate the T cell response.

Importantly, before clinical trials can exploit the synergy between mDCs and pDCs a number of questions need to be addressed: first: what ratio of mDCs and pDCs should be chosen and should one DC subset dominate the immune response? How should both DC subsets be activated *in vitro*? How does the simultaneous secretion of two different T cell polarizing cytokines (IFN-α by pDCs, IL-12 by mDCs) influence naive T cell priming? And what impact does this have on other immune cells? In addition, does the synergy between mDCs and pDCs also help to induce tumor-specific antibodies by B-cells? Does it increase the anti-cancer activity of the innate immune system?

In vitro studies and pre-clinical mouse models suggest answers to some of these questions. Mouse models, for instance, indicate that activated pDCs need to be cocultured with immature mDCs to induce maximal expression of IL-12 as well as co-stimulatory molecules CD40, CD80, and CD86 (**Table 1**) (155). This was cell–cell contact-dependent and also crucial for the induction of a superior $CD8^+$ T cell response. Secretion of IFN-α by pDCs did not influence the secretion of IL-12 by mDCs, indicating that mDCs retain their strong T_H1 polarizing capacities when administered together with pDCs. *In vitro* studies on human DCs, however, are not as conclusive and report on both, impaired and increased production of IL-12 by mDCs when cultured in IFN-α supplemented media (156–159). The induction of $CD8^+$ T cells, however, seems to be augmented by the combined effect of IFN-α and IL-12 as comprehensive and lasting immune responses including effector

Table 1 | Controversial effect of IL-12 and IFN-α on immune activation and T cell priming.

Species	Experimental setup	Observation	Reference
Mouse	Isolated pDCs were activated and cocultured with immature mDCs. This mixture or single DC subsets were then injected in tumor-bearing mice	The coculture of pDCs and mDCs induced strong expression of co-stimulatory molecules CD40, CD80, and CD86 on mDCs and led to superior secretion of IL-12 by mDCs. This process appeared to be contact-dependent. The induced T cell response was superior when both subsets were injected together and also led to improved tumor control	(155)
Human	Coculture of irradiated allogeneic moDCs and naive $CD4^+$ T cells in αCD3-coated wells	Addition of type I IFNs to the cocultures led to decreased IL-12p40 production by DCs and the induction of IL-10 producing T cells	(156)
Human	PDCs and mDCs were isolated from blood and cocultured with cytokines. Subsequently, DCs were cultured with allogeneic, naive $CD4^+$ T cells	IFN-α induced mDC maturation leading to IL-10 but not IL-12 production. IFN-α matured mDCs further induced IL-10 producing T cells	(157)
Human/ mouse	MoDCs were activated in cytokine-supplemented media	The presence of type I IFNs at low levels augmented the production of IL-12p70	(158)
Human	MoDCs were activated using TLR ligands. IFN-α was added at different stages and secretion of IL-12 was measured	The presence of IFN-α during maturation increased the secretion of IL-12p70 by moDCs. When added after maturation IFN-α inhibited the secretion of IL-12p70	(159)
Human/ mouse	Naive $CD4^+$ T cells were activated in cytokine-supplemented media	In contrast to IL-12, IFN-α was not sufficient to induce stable T-bet expression and thus T_H1 differentiation. However, no significant reduction in T_H1 induction could be observed when both cytokines were administered together	(162)
Human	Naive $CD8^+$ T cells were cultured and activated in αCD3/αCD28-coated plates. The media was supplemented with polarizing cytokines	Whereas IL-12 induced fast-dividing, IFN-γ secreting effector memory T cells, IFN-α primed slowly dividing central memory T cells. For a comprehensive T cell response, both cytokines were necessary	(161)
Human/ mouse	Naive $CD8^+$ T cells were cultured and activated via αCD3/αCD28-coated beats. The media was supplemented with polarizing cytokines	Priming of naive $CD8^+$ T cells in IFN-α-supplemented media induced stem cell-like memory T cells with increased ability to respond to homeostatic cytokines, increased persistence upon adoptive transfer, and reduced effector functions. These T cells were able to mount robust recall responses and showed superior ability to contain tumor progression after adoptive transfer	(160)

and memory T cells could only be detected when T cells were cocultured with both cytokines (160, 161).

Although many studies report synergistic effects of IFN-α and IL-12 on T cell priming and immune activation, it is hard to predict how these and other factors integrate in the complex microenvironment found in neoplastic lesions of melanoma patients. Following initial clinical trials focusing on safety and feasibility, future studies thus need to explore the interactions between DC subsets in patients and improve various vaccination parameters.

CONCLUDING REMARKS

Although randomized clinical trials are needed to further prove the clinical efficacy of vaccination with natural blood DCs, DC therapy has major advantages over treatment with FDA-approved checkpoint inhibitors like ipilimumab, as DC therapy with natural DC is less costly and associated with only very mild side effects. Before anti-cancer therapy with natural DCs can be implemented as standard therapy for melanoma, some issues still need to be overcome. First, DC vaccination, in particular DC vaccination with natural DCs, is currently performed only in a limited number of medical centers. However, the isolation technique with magnetic beads is FDA-approved for stem cell isolation and common practice, thus enabling robust standardization for use in multiple centers in the future. In addition, as it is not feasible yet to perform mRNA electroporation on these rare cells, antigen loading still depends on HLA-binding tumor-peptides, thus excluding patients that do not have the matching HLA-phenotype. Efforts are made to enable peptide-loading for a broader HLA-repertoire, including MHC class II epitopes, to induce broader immune responses and enable inclusion of more patients.

As the field of moDC vaccinations appears to have reached some level of maturity, naturally circulating DC-based vaccinations are just at the beginning of their clinical development. However, the lessons learned from moDC-based vaccination trials will surely contribute to accelerate the development of mDC/pDC-based vaccines, hopefully leading to highly efficient DC-based immunotherapies and benefits for an increasing number of cancer patients.

ACKNOWLEDGMENTS

This work was supported by grants from the Dutch Cancer Society (KUN2010-4722, KUN2009-4402), The Netherlands Organization for Scientific Research (NWO-95103002 and NWO-95100106), the Swedish Research Council, and a Radboud University Medical Center PhD grant. Carl G. Figdor received the NWO Spinoza award and an ERC Adv grant.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 03 February 2014; paper pending published: 01 March 2014; accepted: 28 March 2014; published online: 11 April 2014.
*Citation: Wimmers F, Schreibelt G, Sköld AE, Figdor CG and De Vries IJM (2014) Paradigm shift in dendritic cell-based immunotherapy: from in vitro generated monocyte-derived DCs to naturally circulating DC subsets. Front. Immunol. **5**:165. doi: 10.3389/fimmu.2014.00165*
This article was submitted to Antigen Presenting Cell Biology, a section of the journal Frontiers in Immunology.

frontiers in
IMMUNOLOGY

REVIEW ARTICLE
published: 13 February 2014
doi: 10.3389/fimmu.2014.00055

Self-antigen presentation by dendritic cells in autoimmunity

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The operation of both central and peripheral tolerance ensures the prevention of autoimmune diseases. The maintenance of peripheral tolerance requires self-antigen presentation by professional antigen presenting cells (APCs). Dendritic cells (DCs) are considered as major APCs involved in this process. The current review discusses the role of DCs in autoimmune diseases, the various factors involved in the induction and maintenance of tolerogenic DC phenotype, and pinpoints their therapeutic capacity as well as potential novel targets for future clinical studies.

Keywords: self-antigen presentation, peripheral tolerance, tolerogenic dendritic cells, dendritic cell subtypes and autoimmunity

INTRODUCTION

Immune reaction against self-antigens is primarily prevented within the thymus in a process called central tolerance (1). Despite the rigorous screening of the evolving T-cell repertoire, some autoreactive T cells escape from the thymus (1). To avoid autoimmunity, multiple operations ensure the control of the "escaped" T-cell repertoire at the periphery such as induction of anergy, deletion of autoreactive T cells, and activation or induction of regulatory T cells (Tregs) (2, 3). The presentation of self-antigens at the periphery, similarly to the thymus, is carried out by multiple antigen presenting cells (APCs) such as stromal cells and dendritic cells (DCs) (4). This review focuses on DCs as principal APCs involved in this process.

Dendritic cells are present in all tissues and involved in the initiation of immune responses (5). They are capable of recognizing pathogens and various danger signals, which leads to the upregulation of their co-stimulatory molecules, production of cytokines, and activation and effector differentiation of pathogen-specific T cells. Additionally, via communicating with various immune cells [e.g., natural killer cells (NKs), natural killer T (NKT) cells] they bridge the innate and adaptive arm of the immune response (5).

Dendritic cells are a heterogeneous cell population consisting of multiple subtypes (6). Major populations of DCs present in murine secondary lymphoid organs (SLOs) are $CD8^+$, $CD8^-$ DCs, and plasmacytoid DCs (pDCs). The $CD8^-$ DCs can be further subdivided into three groups: $CD4^+$, double negative (DN) ($CD11c^+$ $CD11b^+$ $CD4^-$ $CD8^-$), and triple negative (TN) subset ($CD11c^+$ $CD11b^-$ $CD4^-$ $CD8^-$) (**Table 1**) (6, 7). Differences in gene signature and consequently in functional characteristics exist among DC subsets regarding antigen processing, T-cell stimulatory capacity, and how they respond to pathogens (7, 8). $CD8^+$ DCs are efficient in cross-presentation, induction of $CD8^+$ cytotoxic T-lymphocyte (CTL) response while $CD4^+$ DCs are mainly involved in the activation of $CD4^+$ T cells and in the induction and homeostasis of Tregs (5). Moreover, pDCs are the major source of type-I interferon (IFN) and play important role in the induction of antiviral immunity and in regulating the activity of NKs (9, 10). Parallel to the lymphoid organs, three types of DCs are present in most non-lymphoid organs [except the lamina propria and dermis (7, 11)] (**Table 1**): the $CD103^+$DCs ($CD45^+$ PDCA-1^- $CD11c^+$ MHC-II^+ $CD103^+$ $CD11b^-$), the $CD103^-$DCs ($CD45^+$ PDCA-1^- $CD11c^+$ MHC-II^+ $CD103^-$ $CD11b^+$), and pDCs ($CD45^+$ $CD11c^+$ PDCA-1^+) (7, 11). The $CD103^+$ DCs resemble lymphoid tissue $CD8^+$ DCs and can efficiently cross-present cell-associated antigens (7, 11). The $CD103^-$ DCs display a heterogeneous population containing cells from both the DC and monocyte lineage (7, 11). Their specific role is less characterized. Of note, non-lymphoid tissues as well as SLOs contain not only fully differentiated DCs but also pre-DC population ($CD45^+$ Lin^- MHC-II^- $CD11c^+$) that provide source for DC development and homeostasis *in situ* (12, 13).

Due to their functional heterogeneity and central spot in antigen presentation, DCs seem to carefully balance between

Abbreviations: Batf3, basic leucine zipper transcription factor, ATF-like 3; Bim, Bcl-2 interacting protein; CCR7, chemokine (C–C motif) receptor 7; CCR9, chemokine (C–C motif) receptor 9; CCL18, chemokine (C–C motif) ligand 18; CTL, cytotoxic T-lymphocyte; CTLA-4, cytotoxic T-lymphocyte antigen 4; CXCL12, chemokine (C–X–C motif) ligand 12; Flt3L, FMS-like tyrosine kinase-3 ligand; FoxP3, forkhead box P3; GM-CSF, granulocyte macrophage-colony-stimulating factor; IFN, interferon; IL, interleukin; MHC-II, major histocompatibility complex II; LPM2 and 7, proteasome beta subunits; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; Notch1, notch homolog 1, translocation-associated (*Drosophila*); PD1, programmed cell death protein-1; PDL-1, programmed cell death 1 ligand; SIRP1α, signal regulatory protein-1 alpha; STAT-3, signal transducer and activator of transcription 3; TGFβ, transforming growth factor β; Th1, T helper type 1; TLR, toll like receptor; TNFα, tumor necrosis factor-alpha; Tr1 cells, type 1 T regulatory cells; zbtb46, zinc finger and BTB domain containing 46; XCR1, chemokine (C motif) receptor-1.

Table 1 | Murine DC subsets and their role in tolerance.

	Subgroups	Surface markers	Function in tolerance	Reference
LOs				
$CD8^+$ DCs		$CD11c^+$ $CD8\alpha^+$ $CD4^-$ $CD11b^-$	Induce $CD8^+$ T-cell-tolerance Induce *de novo* generation of Foxp3 Tregs	(14–19)
$CD8^-$ DCs	$CD4^-$ DCs	$CD11c^+$ $CD8\alpha^-$ $CD4^+$ $33D1^+$ $DC11b^+$	Efficient in activating existent Foxp3 Tregs	(17, 18)
	DN DCs	$CD11c^+$ $CD8\alpha^-$CD4 $CD11b^+$	Unknown	
	→mcDCs	($CD11b^{lo/-}$)	Presentation of apoptotic cell derived antigens	(20)
	TN DCs	$CD11c^+$ $CD8\alpha^-$ $CD4^-$ $CD11b^-$	Breaking of T-cell tolerance in diabetes	(21)
	Thymic migratory DC	$CD11c^+$ $CD8\alpha^{low}$ $CD11b^+$ $SIRP1\alpha^+$ $XCR1^+$	Unknown	
	regDCs	$CD11c^{lo}$ $MHCII^{lo}$ $CD11b^{hi}$ ($CD45RB^{hi}$)	Acquire antigens at the periphery, migrate to the thymus	(22–25)
	L-DCs	$CD11c^{low}$ $MHCII^-$ $DC8\alpha^-$ $CD11b^{hi}$	Involved in deletion and Treg induction Production of IL-10 and inducing Tr1 cells and Tregs Diminish experimental autoimmune hepatitis Unknown	(26–31)
pDCs		$CD11c^-$ $MHCII^{int}$ $B220^+$ $PDCA\text{-}1^+$ $CCR9^+$ $PDCA\text{-}1^+$ $B220^-$	Regulate breach of self-tolerance in arthritis Induce anergy or deletion of T cells during oral tolerance Aberrant activation promote diabetes and lupus	(32–36)
			Acquire antigens at the periphery, migrate to the thymus Involved in deletion and Treg induction	(22, 24, 37)
eTACs		$CD45^{low}$, $CD11c^{low}$, $MHC\text{-}II^{hi}$, $CD357^+$, $DC80^{int}/86^{int}$	Induction of tolerance through AIRE-mediated expression of self-antigens Induction of T-cell unresponsiveness of $CD4^+$ T cells independent of Tregs Prevention of autoimmune diabetes	(38)
NON-LOs				
$CD103^+$		$CD11c^+$ $MHCII^+$ $CD11b^-$ $CD103^+$ LP: $CD103^+CD11b^+$	Cross-presentation of self-antigens to maintain $CD8^+$ T-cell-tolerance Induce and enhance the *de novo* generation of Foxp3 Tregs	(39–44)
$CD11b^+$		$CD11c^+$ $MHCII^+$ $CD11b^+$ $CD103^-$	Need further clarification	
pDC		$CD11c^-$ $B220^+$ $PDCA\text{-}1^+$	Aberrant activation of pDCs promote diabetes and lupus	(34, 36)

Arrow indicates that mcDCs belong to the DN DC subset. ($CD45RB^{hi}$) indicates that this marker was investigated and associated with some regDCs only. regDCs, regulatory DCs; DCs, dendritic cells; pDC, plasmacytoid DC; DN/TN DCs, double/triple negative DCs; mcDCs, merocytic DC; eTACs, extrathymic Aire-expressing cells; Los, lymphoid organs; non-Los, non-lymphoid organs; LP $CD103^+$ $CD11b^+$, refer to the additional DC subset present in lamina propria besides the $CD103^+$ and $CD11b^+$ subsets.

immunity and tolerance. Considering the substantial amount of data available, there are at least five contrasting points to contemplate in order to understand what features describe a tolerogenic DC (tDC) and therefore their influence in autoimmune diseases: (a) Maturation status of DCs, (b) intrinsic characteristics of DCs (involving intracellular signaling, antigen presentation capacity of DCs, and expression of effector molecules), (c) division of labor among DC subsets in tolerance induction, (d) interaction between DCs and other immune or stromal cells, and (e) the effect of the microenvironment to generate DCs with tolerance-inducing potential (e.g., soluble factors).

SELF-ANTIGEN PRESENTATION BY DCs: DOES DC MATURATION MATTER?

The early groundbreaking studies have demonstrated in a series of transgenic animal models that cell-associated antigen expressed in peripheral tissues resulted in $CD8^+$ T-cell deletion (14, 15). These studies identified DCs as major APCs involved in peripheral tolerance. In these models, DCs acquired cell-associated antigens under non-inflammatory condition from apoptotic cells at the periphery and migratory DCs carried these antigens to the draining lymph node (LN) where $CD8^+$ T-cell deletion was initiated (14, 15). This so-called cross-tolerance toward autoantigens involved CD95-signaling (45, 46), Bcl-2 interacting protein (Bim)-dependent apoptosis of T cells (47), and was controlled by cognate $CD4^+$ T-cell help (48). The importance of cross-tolerance was additionally demonstrated in an animal model where phagocytosis of apoptotic cells was inhibited in $CD11c^+$ cells (16). Transfer of polyclonal $CD8^+$ T cells from these animals to Rag1 deficient recipients resulted in an autoimmune phenotype (16). Moreover, viral epitope genetically targeted to CD11c expressing cells caused $CD8^+$ T-cell unresponsiveness that was dependent

on the engagement of programmed cell death protein-1 (PD1) and cytotoxic T-lymphocyte antigen 4 (CTLA-4) (49). Subsequent studies have similarly demonstrated that model antigen targeted to DCs using C-type lectin receptors (CLRs) such as Dec205 and dendritic cell immunoreceptor (DCIR) induced peripheral $CD8^+$ T-cell tolerance and resulted in $CD4^+$ Treg induction in the steady state (17, 18). Overall, above data led to the widely accepted notion that immature DCs present self-antigens under non-inflammatory condition and this result in peripheral tolerance. These immature DCs were defined as cells expressing low level of co-stimulatory molecules (CD80, CD86, MHC-II) and failed to produce pro-inflammatory effector molecules such as interleukin (IL)-12 (50) (**Figure 1**). This notion was underlined by the fact that the same self-antigen presentation by resident DCs using targeting strategy toward, e.g., Dec205, DCIR, or DC NK lectin group receptor-1 (DNGR1) in the presence of anti-CD40 resulted in DC maturation and efficient T helper type 1 (Th1) immunity (17, 18, 51, 52). These mature DCs capable of inducing immunogenic response exhibited high expression of co-stimulatory molecules (CD80, CD86, CD40), upregulated MHC-I and II, and produced pro-inflammatory cytokines such as IL-6, IL-12, and TNF (5) (**Figure 1A**). Thus, DCs seemed to remain in an immature state during tolerance while they fully mature during induction of immunity. This view was challenged by multiple consecutive studies. $CCR7^{hi}$ $MHC\text{-}II^{hi}$ DCs could develop without pathogen within peripheral tissues, after disruption of cell adhesion via E-cadherin and despite their phenotypic maturation; they failed to secrete inflammatory cytokines and elicited a tolerogenic T-cell response *in vivo* (53). Moreover, increasing number of $MHC\text{-}II^{hi}$ matured DCs could be observed in draining LN prior to the detection of the autoreactive T and B-cell responses in arthritis (54). Transfer of these matured DCs caused autoimmunity in recipient animals indicating that these cells were responsible for the breaching of self-tolerance (54). Thus, tDCs are not necessarily remaining in an immature state for tolerance induction. Accordingly, it has been suggested by Reis and Sousa that immature DCs could give rise to several different types of "effector" DCs (55). In this model, each type of "effector" DC is functionally distinct and can drive various T-cell responses, such as T helper cell differentiation, induction of CTL, and T-cell tolerance (55). This suggests that tolerance-inducing capacity of DCs is associated with another entity of DCs that is distinct from their immature state (**Figure 1B**). According to this model, two important questions remain: (i) what features define "effector" DCs with tolerance-inducing capacity and (ii) what signals influence the generation of this "effector" tDC phenotype?

WHAT FEATURES ARE ASSOCIATED WITH THE TOLEROGENIC CAPACITY OF DCs? – INTRINSIC CHARACTERISTICS OF tDCs

INTRACELLULAR SIGNALING EVENTS AFFECTING THE ACTIVATION AND SURVIVAL OF DCs

There is increasing evidence that tDC phenotype (or development of "effector" tDCs) is an active process and the result of the operation of multiple signaling pathways. In agreement with this, several recent studies have identified key signaling molecules necessary for the tolerogenic function of DCs. One of the prominent pathways involved in this process is the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway. The central role of the NF-κB pathway was demonstrated in DCs specifically lacking A20, a ubiquitin-editing enzyme, which induces the degradation of various signaling molecules that activate NF-κB signaling such as receptor interacting protein-1 (RIP1) (56–59). In these

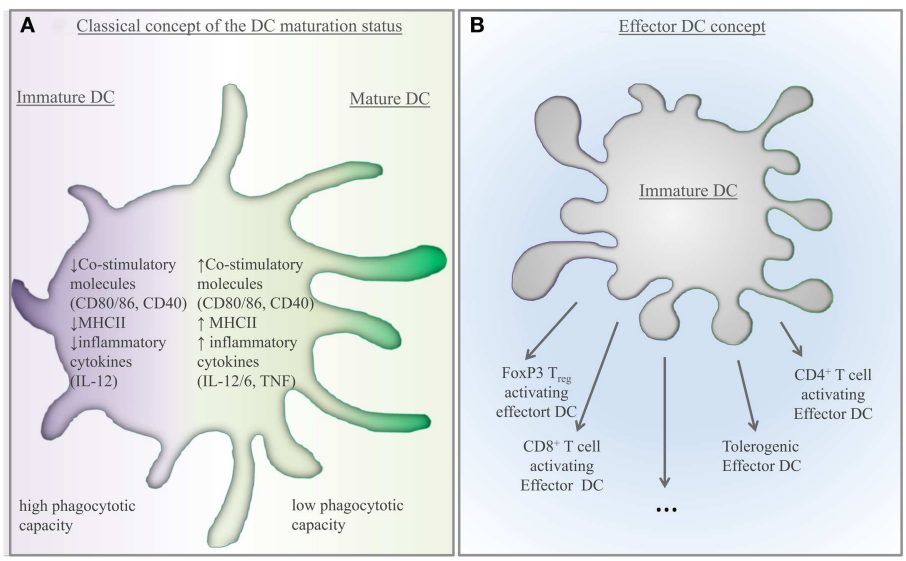

FIGURE 1 | Scientific concepts: how to characterize tolerogenic DCs? (A) Previous concept described DCs as a cell type existing in two different states: immature and mature DCs. These categories were based primarily on their co-stimulatory molecule expression, effector cytokine production, and T-cell stimulatory capacity. According to this model, immature DCs were able to induce tolerance. **(B)** Based on novel observations, the existence of multiple effector DCs has been suggested by Reis and Sousa (55). According to this model, immature DCs develop into various types of effector cells. Consequently, effector DCs, capable of inducing tolerance, are the effector tolerogenic DCs.

animals, colitis and arthritis developed spontaneously (56). Additionally, milk-fat-globule-EGF VIII (MFG-E8), a secreted molecule that determines the recognition of apoptotic cells, supported the tolerogenic activity of DCs. Mechanistically, MFG-E8 activated signal transducer and activator of transcription 3 (STAT-3) and A20 and decreased pro-inflammatory cytokine production (60) further suggesting the supportive role of the decreased activity of NF-κB pathway in promoting tolerance. Consequently, inhibition of NF-κB and notch homolog 1, translocation-associated (*Drosophila*) (Notch1) by miR-23b promoted tDC differentiation of murine bone marrow dendritic cells (BMDCs) (61). Therefore, over-expression of miR-23b in BMDCs produced less IL-12, increased level of IL-10, and demonstrated enhanced Treg inducing capability *in vitro* (61). NF-κB plays a significant role in DC activation (62) and consequently inhibition of this pathway likely shifts the balance toward tolerance. Surprisingly, unstimulated NF-κB1 deficient DCs pulsed with self-antigen were able to mount $CD8^+$ T-cell response and induced autoimmunity (63). This indicates that some degree of activation of this pathway is required for maintaining tolerance as well. It is possible that a combination of pathways will determine whether finally tolerance or immunity occurs and which effector DC phenotype will be the end result of the various stimuli.

Furthermore, p50, active form of NF-κB1, regulated the immunogenicity and life span of DCs (64). According to this, p50 deficient DCs produced higher level of pro-inflammatory cytokines, exhibited increased T-cell stimulatory capacity, and showed longer survival (64). The lifetime of DCs provides an interesting aspect of how tolerance and immunity is regulated and it is thought to be at least partially determined by intrinsic properties of DCs (65). Under physiological condition, DCs die within 48 h after the activating stimuli (66). Significant accumulation of DCs has been observed in MRL-lpr/lpr mice suggesting a connection between apoptosis and autoimmunity (67). Moreover, over-expression of the caspase inhibitor p35 in $CD11c^+$ cells resulted in accumulation of DCs and anti-nuclear antibody production in aged mice (65). FAS (68) or Bim deficiency (69) in DCs also caused autoimmunity including autoantibody production. Thus, besides NF-κB, apoptotic pathways regulate the lifetime of DCs and they provide additional checkpoint to maintain tolerance.

Generally, intracellular signaling events, that negatively regulate DC activation, have been implicated to balance tolerance vs. immunity. These pathways primarily act through affecting the size of DC compartment or the extent of the DC activation. Accordingly, DCs deficient in protein tyrosine phosphatase-1 (SHP1) promoted strong Th1 activation that resulted in glomerulonephritis and autoantibody production in aged mice (70). Furthermore, DC-specific deletion of Lyn tyrosine kinase, a negative regulator of the myd88 pathway, resulted in spontaneous T- and B-cell activation, which caused lupus-like autoimmune disease (71). Additionally, STAT-3 deficiency in DCs was also associated with their increased T-cell stimulatory activity and caused ileocolitis resembling human inflammatory bowel disease, suggesting its role in mucosal tolerance (72). Transgenic mice, where suppressor of cytokine signaling-1 (SOCS-1) expressed only in the T- and B-cell compartment exhibited B-cell hyper activation and autoantibody production. SOCS-1 deficient DCs in these animals produced more B-cell activating factor (BAFF), which contributed to the observed autoimmune phenotype (73). Negative regulatory motifs such as immunoreceptor tyrosine-based inhibitory motif (ITIM) containing molecules could affect the numbers and activity of the DCs and thereby tolerance as well. DCIR, a C-type lectin, has been identified as a negative regulator of DC expansion in spleen (74). Consequently, DCIR deficient mice spontaneously developed autoimmune sialadenitis and enthesitis (74).

ANTIGEN CAPTURE, PROCESSING, AND PRESENTATION

Dendritic cells acquire antigens via phagocytosis, receptor mediated endocytosis, and macropinocytosis that lead to the presentation of these antigens to T cells (5). Autoimmune diseases are associated with multiple autoantigens against which the tolerance is broken (75–78). Therefore, the ability of DCs to obtain, process, and present self-antigens is key in understanding tolerance and to closer define the tDC phenotype. Along this line, the antigen-uptake, the nature of antigen, and the specialized machinery associated with tolerance or autoimmunity need to be considered.

Firstly, the mechanism of antigen capture can influence the outcome of the response induced by DCs. Indeed, apoptotic cells (unlike necrotic cells) or soluble proteins, as major sources of self-antigen presentation at the periphery, resulted in tDC activation (50, 79). In case of apoptotic cells, TAM receptor tyrosine kinases (Tyro3, Axl, and Mer) expressed in apoptotic cell membranes triggered SOCS-1 and SOCS-3 expression in DCs, which inhibited the toll like receptor (TLR) and cytokine-induced signaling cascades and therefore the immunogenic DC maturation (80). Underlining this, TAM triple gene (Tyro3, Axl, Mer) deficient mice possessed hyperactive DCs and developed systemic autoimmunity (81). Moreover, the uptake of apoptotic cells triggered transforming growth factor β (TGFβ) release, which led to DC-mediated Treg induction (82, 83). Accordingly, the DC-specific loss of TGFβ activating integrin (αvβ8) resulted in the failure of Treg development initiated by DCs *in vitro* and caused autoimmune colitis *in vivo* (84).

For the uptake of soluble proteins as source of self-antigens, an important antigen-uptake receptor group is the CLRs. They play a role in the uptake of glycosylated antigens. The recognition of most CLRs was not pathogen-restricted, as they often interacted with self-glycoproteins (85–87). Thus, CLRs were involved in the clearance of multiple soluble self-antigens such as thyroglobulin by the mannose receptor (87). In particular, CLRs directed antigen to both MHC-I and MHC class II to prime $CD4^+$ and $CD8^+$ T-cell responses (88, 89). Targeting antigen to Dec205 or DCIR on DCs is a classical example of inducing antigen specific tolerance toward the antibody coupled soluble protein (17, 18). Additionally, engaging the mannose receptor by mannosylated myelin peptide inhibited EAE (90). Similarly, oral administration of mannose-enriched antigens can induce oral tolerance and favor the generation of IL-10-producing type 1 T regulatory cells (Tr1 cells) via SIGNR1 expressed on DCs of the lamina propria (91). It is less understood which is the exact self-antigen recognition repertoire for each CLRs, and to what extent CLRs on DCs influence autoimmunity. Nevertheless, their intracellular signaling motifs (either ITIM or ITAM motifs) could greatly influence

DC activation and effector cytokine production (86) and thereby could influence the tDC phenotype upon antigen capture.

Secondly, in several autoimmune disorders multiple post-translational protein modifications have been observed resulting in alteration of self-antigens and neoantigen formation against which the immune system has not been exposed and tolerized. Multiple autoimmune disorders were dependent on the presence of such post-translational modifications of autoantigens (77). Acetylation of myelin basic protein was required for the development of EAE as non-acetylated peptides failed to stimulate T cells or induce the disease (92, 93). Similar post-translational modifications were involved in the autoimmune process in lupus, celiac disease, and psoriasis (75, 77). Importantly, these modified proteins could be produced and/or taken up by DCs for presentation to T and B cells. So far there is limited understanding of how these modified proteins are captured or produced by DCs, what are the exact consequences of this presentation in disease development, and whether specific DC subsets could be skewed toward presenting these modified proteins during autoimmunity. In line with this, certain modifications such as citrullination could alter the peptide generation of DCs for MHC-II by altering the susceptibility of antigen to cathepsin D (94). On the opposite end of the spectra are the enzymes involved in creating these post-translational modifications. They could affect tolerance such as the *N*-acetyl glucosaminyl transferase (Mgat5) involved in glycosylation process. Mtga5 deficient animals exhibited profound autoimmune disease due to the decreased threshold for T-cell activation (95, 96). Accordingly, increasing N-glycan branching inhibited TCR activation in autoimmune models of EAE and diabetes (97). It will be important for future studies to dissect the involvement of this and similar enzymes in autoimmunity in a cell specific manner especially focusing on DCs.

Thirdly, differences in antigen processing machinery might affect tolerance toward self-antigens. Accordingly, murine $CD8^+$ ($Dec205^+$) and $CD4^+$ ($DCIR^+$) DCs differed in their antigen processing machinery, as $CD8^+$ DCs were specialized in cross-presentation while $CD4^+$ DCs were more potent inducers of $CD4^+$ T-cell activation (17). These differences were based on a distinct expression of antigen processing components such as TAPs, cathepsins, and HLA-DM (17).

The proteasome is involved in the production of most MHC-I ligands and therefore considered as main component of the antigen processing machinery (98). Interestingly, autoimmune disorder such as scleroderma was associated with allele variants of immunoproteasome subunits, LMP2 and LMP7 (99). Also, local immunopathology could be explained by tissue specific differences in the proteasomal processing of MHC-I epitopes in a colitis model (100). Additionally, during inflammation the upregulation of LMP7 immunoproteasomal subunit at the periphery was associated with the prevention of diabetes (101). As opposite to this, over-expression of the LMP7 in splenocytes was required for $CD8^+$ T-cell auto-reactivity (102). While above studies demonstrate the clear participation of the proteasome in autoimmune processes, it is less understood how cell specific (DC-specific) changes in these components influence disease development. Such cell or subset specific alterations could be especially interesting, as in the thymus, different sets of the proteasome subunits are expressed in mTECs and cTECs suggesting specialization for presentation of self-antigen repertoire for tolerance induction (103). In scleroderma, DC-specific alteration in proteasomal processing was associated with the disease (76, 104). In this case, the unusual processing of topoisomerase-I by the nucleoproteasome in DCs was connected with autoantibody production and clinical manifestation of this autoimmune disorder (76, 104).

The proteasome generates peptides some of which are further trimmed by aminopeptidases. Some of the trimming takes place in the cytoplasm but a large proportion is located within the endoplasmic reticulum (ER). One of the primary enzymes in the ER is the ER associated aminopeptidase (ERAP) (105). These trimming enzymes in humans were associated with susceptibility to various autoimmune diseases (106). For example, based on genetic studies, ERAP1 was highly associated with ankylosis spondylitis (107) and ERAP2 was linked to Crohn's disease (108). Whether specific alterations in such peptide processing are associated explicitly with DCs needs further evaluation.

In terms of the presence of specialized intracellular compartments associated with tolerance or autoimmunity, merocytic DCs (mcDCs) that were able to breach self-tolerance (20)(**Table 1**) possessed specialized vesicles where they could store apoptotic cellular material for autoantigen presentation for an extended period of time. Nevertheless, the understanding of these intracellular organelles is limited so far.

Hence, it remains to be further explored whether altered antigen presentation machinery exists and would be associated with DCs inducing tolerance and/or with DCs breaching tolerance.

Taken together, various signaling pathways and processes influencing antigen handling and processing determine the capacity of DCs for tolerance induction and dysregulation in these pathways could result in alteration of tDC "effector" phenotype toward promoting autoimmunity.

WHAT FEATURES DEFINE tDCs? – EFFECTOR CHARACTERISTICS OF tDCs

The tolerogenic effector capacity of DCs predominantly has been analyzed in functional co-culture assays (induction of Tregs or Tr1 cells), determining how DC-transfer affected disease outcome or via using transgenic animal models (3, 50). Moreover, increased expression of IL-10 or TGFβ and reduced expression of pro-inflammatory cytokines (e.g., IL-12, IL-1, IL-6, TNF) and co-stimulatory molecules (e.g., CD80, CD86) are typically considered as hallmark of tDCs (3, 50) (**Figure 1B**). In NOD mice, DC-derived IL-2 was required for $CD8^+$ T-cell deletion and for protection from diabetes (109). Additionally, DC-derived IL-2 together with CD40–CD40L interaction were involved in Treg homeostasis (110–112) establishing IL-2 as novel effector molecule for tDCs. Besides, variety of enzymes such as retinaldehyde dehydrogenase-2 (RALDH2) involved in retinoid acid (RA) metabolism and indolamine 2,3 dioxygenase (IDO) altering tryptophan metabolism were associated with tDCs (3, 113). RA was involved in Treg induction primarily in the gut and skin while IDO could inhibit the proliferation of activated T cells and enhanced the induction of Tregs (3, 113). It has been recently demonstrated that the non-enzymatic activity of IDO upon TGFβ challenge in pDCs was involved in maintaining their regulatory phenotype (114).

IDO mediated intracellular signaling in pDCs, evoked the capacity of these cells to suppress Th1 immunity, and resulted in increased Treg differentiation *in vivo* (114).

Apart from this, there is increasing evidence suggesting a high level of complexity associated with the tDC "effector" phenotype. The effect of dexamethasone and vitamin D on human DCs has been recently characterized at a molecular level (115). Both compounds alone and in combination induced tDCs and have been widely used to generate these cells *in vitro* (113). Interestingly, the tDC phenotype was associated with unique protein profiles with severe impact on metabolic pathways (115). These pathways affected lipid, glucose, and oxidative phosphorylation in tDCs. Moreover, they altered the production of ROS, the survival of DCs, and the dependence of DCs on available nutrients (115). This is in line with the observation that after TLR stimuli, the metabolic status of DCs transitioned from oxidative phosphorylation to glycolysis (116). This transition was partially inhibited by IL-10, a cytokine associated with tolerance (116). Hence, it is likely that the tolerogenic potential of DCs is associated with a specific metabolic fingerprint that supports DC function in maintaining immune homeostasis. Also, blocking mammalian target of rapamycin (mTOR) signaling via rapamycin during DC maturation resulted in tDCs, which promoted alloantigen specific tolerance (117). Although, mTOR affects multiple cellular processes and only one aspect of them is associated with metabolism, further studies are needed to clarify whether the above effect of rapamycin was due to specific metabolic changes associated with tDCs.

Overall, it seems that the specific features of "effector" tDCs are more complex than previously thought. The broader determination of the switch in metabolic status, the checkpoints regulating this change together with the intracellular pathways, and secretome profile of tDCs might provide more precise specifications of what the tDC phenotype means. It is plausible that these features are slightly different dependent on the microenvironmental factors affecting DCs and might show organ or even DC subset specific amendments.

DIVISION OF LABOR FOR TOLERANCE INDUCTION

Various animal models have demonstrated the importance of $CD11c^+$ cells in the maintenance of tolerance (3, 14–18, 49, 50). Supporting this notion, transient depletion of $CD11c^{+/hi}$ cells aggravated immune pathology and inflammation (118). Rather surprisingly, the constitutive ablation of $CD11c^+$ cells showed myeloproliferative disorder associated with elevated serum Fms-like tyrosine kinase-3 ligand (FLT3L) level (119, 120). Between the two pioneering studies on constitutive DC depletion, only Ohnmacht et al. found impaired negative selection of $CD4^+$ T cells and the development of inflammatory bowel disease (120). Although the ultimate role of DCs in autoimmunity could not be demonstrated in these studies, they pinpointed an important regulatory circuit within the myeloid cell compartment. Importantly, unlike CD11c, novel molecules such as the transcription factor zinc finger and BTB domain containing 46 (zbtb46) and DNGR1 were exclusively expressed by DCs and were absent in NK cells, pDCs, or monocytes (121, 122). It will be interesting to investigate in future studies how short- or long-term depletion of DCs using the above-mentioned markers would affect autoimmunity and peripheral tolerance.

Given the heterogeneity of DCs, genetic models where certain DC subtype was missing provided interesting insight into the process associated with immune homeostasis. *Batf3* deficient mice lack $CD8^+$ DCs in SLOs and $CD103^+$ DCs at the periphery (123). Despite this loss in these subtypes, the mice under steady state have no obvious autoimmune phenotype (123). Nonetheless, renal LN $CD8^+$ $XCR1^+$ DCs were absent in *Batf3* deficient animals and therefore failed to induce tolerance against soluble antigen concentrated in the kidney (19). Additionally, pulmonary tolerance toward inhaled antigen correlated with the ability of $CD103^+$ DCs to upregulate RALDH2, which promoted forkhead box P3 (FoxP3) expression in Tregs (39). Correspondingly, *Batf3* deficient mice failed to induce tolerance toward inhaled antigen (39). Besides the lung, the $CD103^+$ DC subpopulation in the gut prevented colitis and was efficient in inducing Tregs via production of TGFβ, RA, and induction of IDO (40–42). Similarly, $CD103^+$ skin migratory DCs were responsible for tolerance induction by transporting skin-associated antigens into draining LNs (43). Since *Batf3* deficient animals showed no obvious autoimmune phenotype, it is likely that other subsets took over the tolerance-inducing function of the missing DC subtype.

Also, pDCs have been identified as guardians of immune homeostasis in arthritis (32) and oral tolerance (33). Despite these data, transient depletion of pDCs did not result in spontaneous autoimmune disorder (10, 124). Additionally, pDCs have been associated with multiple autoimmune disorders (34–36). Thus, the question is whether do individual subsets of DCs specialized in tolerance exist and is there functional redundancy among the DC subsets?

A novel subset of tDCs has been recently identified within SLOs localized at the T–B cell border: the extrathymic Aire-expressing cells (eTACs) (38). These cells were $CD45^{lo}CD11c^{lo}$ and positive for zbtb46, therefore could be identified as DCs (38). Besides, eTACs expressed high level of Epcam and MHC-II but low level of co-stimulatory molecules (**Table 1**). Importantly, eTACs functionally inactivated autoreactive $CD4^+$ T cells independent from Tregs and were unresponsive to a variety of inflammatory stimuli (38).

Moreover, Wakkach et al. have distinguished in the spleen, the IL-10 secreting tDCs harnessing $CD11c^{lo}CD45RB^{hi}$ surface markers (**Table 1**) (26). These cells showed immature phenotype and induced the differentiation of Tr1 cells (26). Additionally, they were resistant to various inflammatory maturation stimuli and upon adoptive transfer they induced antigen-specific unresponsiveness in recipient mice (26). Further studies demonstrated that the differentiation of $CD11c^{lo}CD45RB^{hi}$ tDCs from hematopoietic precursors could be instructed by splenic stromal cells (27) and via utilizing neuropeptides such as vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating polypeptide (PACAP) (125).

Specific peripheral DC populations could migrate via blood to the thymus and contributed to central tolerance. Approximately 50% of the thymic DCs arrived from the peripheral blood and represented the migratory DC population in this organ (126). This migratory DC population in the murine system consisted of $CD11c^+CD8\alpha^{low}CD11b^+SIRP1\alpha^+$ conventional DCs, $CCR9^+$

pDCs, and distinguished from the resident thymic DC population ($CD11c^+CD8\alpha^{hi}CD11b^-SIRP1\alpha^-$) (22–24, 37, 126, 127) (**Table 1**). Importantly, similar DC subsets were described in humans as well (128). The three murine DC subsets differed in their thymic localization, chemokine receptor requirement for their intrathymic positioning, and their origin (25, 37, 129). The migratory thymic DC populations are especially interesting for tolerance induction. $SIRP1\alpha^+$ DCs and pDCs sampled blood borne antigens and transported them to the thymic cortex area where they contributed to clonal deletion and Treg induction (23, 24, 127). Additionally, $SIRP1\alpha^+$ DCs has been implicated in negative selection toward circulating tumor antigens thereby promoting tumor tolerance (130). Moreover, in an experimental system where model antigen was expressed in cardiac myocytes in a membrane-bound form, autoantigen presentation depended on VLA4-mediated recruitment of migratory peripheral DCs to the thymus (22) suggesting that cell-associated antigen was transported by migratory DCs to the thymus. Also, pDCs could acquire particulate antigens injected subcutaneously from the skin and transported to the thymus for tolerance induction (37). Regardless, it remains to be elucidated how these DCs sample antigens from peripheral organs before migrating to the thymus, what is their exclusive physiological contribution in tolerance induction, and what regulatory circuits play a role in their migration. Interestingly, TLR ligands downregulated the capacity of these DCs to reach the thymus (22, 37), thus separating the immunogenic response toward pathogens from the thymic tolerance. Another intriguing possibility about these cells is that they may transport antigens from the digestive tract that could potentially result in tolerance toward food-related antigens (103). This possibility needs further investigation in the future.

Importantly, in balancing tolerance and immunity, tDCs represent one side of the spectrum and on the other side are the DC subtypes, which are specifically promoting autoimmunity. Such DC subtype has been also found in NOD mice (20). This subtype of DCs is called mcDCs (**Table 1**). The frequency of mcDCs was elevated in spleen and pancreatic LNs of NOD mice possessing insulitis (20). Importantly, these cells could acquire apoptotic cellular materials and induce T-cell activation that reversed the deletion of self-reactive T cells (20). Moreover, upon transfer to young NOD recipients, antigen loaded mcDCs could break peripheral tolerance toward β-cell antigens (20). The number of mcDCs within the spleen was negatively regulated by the Idd13 locus which was previously associated with diabetes prevention (21). It remains to be elucidated whether mcDCs could break tolerance toward other antigens than β-cell related ones *in vivo* thus indicating a general tolerance breaking DC subtype.

Long-term culture of splenic stromal and hematopoietic cells could also result in the generation of a novel DC subtype, the L-DCs (**Table 1**). L-DCs were superior in cross-presentation of soluble antigens *in vitro* compared to $CD8^+$ DCs (131). Interestingly, adoptive transfer of these DCs induced immunogenic $CD8^+$ T-cell activation *in vivo* (131). It will be interesting to see whether according to their immunogenic properties they could manifest DC subtypes breaching $CD8^+$ T-cell tolerance.

Future studies should illuminate whether there might be a functional cross-talk among tDCs, the tolerance breaching DC population, and the well-established lymphoid organ resident DCs during prevention and development of autoimmune disease.

INTERACTIONS BETWEEN tDCs AND IMMUNE OR STROMAL CELLS FOR TOLERANCE INDUCTION

Dendritic cells were capable of inducing or activating Tregs in multiple ways (3, 113). The rather surprising discovery was the participation of DCs in maintaining homeostasis of Tregs. According to this, transient depletion of $CD11c^+$ cells reduced the frequency of Tregs (118) while the expansion of DCs using FLT3L resulted in increased Treg numbers *in vivo* (118, 132, 133). Moreover, MHC-II expression by DCs was required to maintain the Treg population at the periphery (118). These results suggested that the DC-Treg feedback would set the tone for tolerance. Accordingly, human diabetes patients displayed lower DC numbers than healthy ones (134). Also the expansion of Tregs, due to increased DC numbers, reduced severity of colitis and arthritis (118, 133, 135). Despite these data, in some cases autoimmunity was associated with increased number of DCs, but was not accompanied with altered Treg numbers (65, 74). This could indicate additional factors, which might influence Treg homeostasis or could pinpoint functional alterations of the expanding DC population. Indeed, recent study demonstrated that DCs generated using FLT3L lacked the ability to induce Tregs *in vitro* (136). Moreover, the expansion of donor liver DCs, using FLT3L before transplantation, abrogated liver allograft acceptance and resulted in graft rejection (137). The discrepancies in the effect of FLT3L as well as the exact relation in DC and Treg numbers *in vivo* remain to be determined.

Another type of cellular cross-talk between Tregs and DCs has been recently demonstrated in the murine model of contact hypersensitivity (138). Here Tregs conditioned DCs to induce regulatory $CD8^+$ T cells that could protect against the disease (138). how and what is the molecular mechanism of this imprinting needs further clarification.

Autoimmune arthritis induced in B-cell deficient mice resulted in exacerbation of the inflammatory response. In this model, DCs produced higher amount of inflammatory cytokines due to the missing control by the $IL\text{-}10^{hi}$ B-cell subpopulation (139). Similar phenomenon exists between human B cells and DCs, where B cells in soluble and cell contact dependent manner regulated DC activation and IL-12 production (140). This suggests a close interplay between these two cell types while maintaining homeostasis.

Not only B cells but also innate cells such as NKT could control tDC function. Treatment of NOD mice with NKT activating ligand such as α-galactosyl ceramide resulted in the accumulation of tDCs in draining LN (141). These tDCs anergized autoreactive T cells and therefore prevented diabetes (141). The interaction between NKT cells and DCs was rather complex, bidirectional, and not restricted to only tolerogenic outcome (142, 143). The exact circumstances when NKT cells act toward the development of tDCs remain to be elucidated.

In the last few years, multiple studies have demonstrated that LN stromal cells are capable of inducing T-cell tolerance (144–147). Anatomically, stromal cells within SLOs are positioned in close proximity with lymphoid resident DCs (148) and guide migratory DCs within SLOs (149). In addition to this, stromal cells inhibited the capacity of DCs to activate T cells (150, 151).

Furthermore, they prompted hematopoietic progenitors to differentiate toward regulatory IL-10 producing tDCs (27). During *Leishmania* infection, splenic stromal cells upregulated chemokine (C–X–C motif) ligand 12 (CXCL12) and CCL8 to specifically attract hematopoietic precursors and induced tDC differentiation *in situ* (152). Importantly, tDCs have been identified under steady state in lung (28), spleen (26, 27, 29), and liver (30). Moreover, stromal cells directed the differentiation of not only hematopoietic precursors but also matured DCs toward regulatory ones. These tDCs produced nitric oxide and IL-10 and consequently dampened T-cell responses (31). Importantly, in adoptive transfer experiment, the tDCs promoted by liver stromal cells diminished experimental autoimmune hepatitis (30). Thus, it is likely that these DCs not only provide important negative regulatory circuit during T-cell activation but also contribute to maintain tolerance. It still needs to be clarified what is the exact role of these DCs under steady state and whether they could play a role in balancing autoimmunity and tolerance.

WHICH SOLUBLE MOLECULES ENDORSE THE tDC PHENOTYPE?

The soluble molecules involved in inducing tDC phenotype can be generally divided into two groups: the ones which promote differentiation of tDCs from hematopoietic precursors or peripheral blood monocytes and the ones that directly act on immature DCs (113, 153). Not only natural biomolecules but also multiple pharmacological compounds have been used to generate tDCs *in vitro* (113, 153). These experiments generally combined basic differentiation factors, such as granulocyte macrophage-colony-stimulating factor (GM-CSF) for murine BMDCs, with the variety of soluble molecules and characterized the tolerogenic phenotype of the developed DCs *in vitro* (113, 153). IL-10, TGFβ, TNF, IL-6, hepatocyte growth factor, prostaglandins, and vitamin D were identified as effective molecules in inducing tDC phenotype *in vitro* (113). Hormones could also affect DC maturation and the tolerance-inducing competence of DCs. In particular, glucocorticoids suppressed DC maturation and generated tDCs *in vitro*. Glucocorticoids acted via nuclear receptors followed by the induction of glucocorticoid induced leucine zipper (GILZ) (154). GILZ is a transcription factor, which was absolutely required for glucocorticoid-mediated tDC differentiation (154). DC-specific transcript (DC-SCRIPT), a corepressor of GILZ has been recently identified in DCs (155), indicating a network of transcription factors that counterbalances the effect of glucocorticoids in immunity vs. tolerance. It remains to be identified whether changing the balance of these transcription factors can be used as therapeutic target for generating tDCs for therapy as well.

Although most of the above-mentioned compounds were used in a combination with GM-CSF *in vitro*, the effect of GM-CSF itself in tolerance is not straightforward. GM-CSF deficient animals developed lupus-like systemic autoimmune disorder and GM-CSF together with IL-3 promoted diabetes (156, 157). On the other hand, in the absence of GM-CSF, mice were protected against collagen-induced arthritis (158).

Pro-inflammatory mediators such as IFNγ and TNF could transform DCs into inhibitory IDO expressing tDCs. Such IDO$^+$ DCs induced oral tolerance and prevented arthritis and colitis (41, 159). IDO expression and induction of tDCs could be initiated by chemokine (C–C motif) ligand 18 (CCL18) as well (160). The role of cytokines affecting DC function under steady state could be of relevance, as these molecules could actively maintain the tolerogenic environment. This could be underlined by the fact that asthmatic patient exhibited reduced CCL18 binding to its receptor suggesting a protective role of CCL18 under steady state (160). As opposite to this, cytokine signaling could also contribute to the breaking of tolerance. Indeed, IL-1R1 signaling in DCs promoted autoreactive CD4$^+$ T-cell expansion and caused autoimmune myocarditis (161).

There are increasing examples of novel soluble molecules, with known primary function unrelated to DC biology that can incite immature DCs with a tolerogenic capability. Adiponectin, which is an adipocytokine with anti-inflammatory properties, increased programmed cell death 1 ligand (PDL-1) expression of DCs and thereby intensified their Treg inducing capacity (162). Likewise, adiponectin deficient mice exhibited severe cardiac transplant rejection (163). Further studies are required to delineate its effect in tolerance induction.

Thrombomodulin (TM), a cofactor of thrombin, turned BMDCs to secret IL-10 independent of its thrombin and coagulation related function (164). Importantly, transfer of TM$^+$ DCs protected recipient animals against airway hypersensitivity (164). Another novel molecule involved in DC biology is adrenomedullin, a calcitonin related neuropeptide. This molecule induced IDO in immature BMDCs and thereby promoted the conversion of CD4$^+$ T cells to CD4$^+$ CD25$^+$ Foxp3^{hi} Tregs *in vitro* (165).

Additionally, it has been also recently identified that Wnt3a and Wnt5a directly induced immunoregulatory cytokine expression by DCs and promoted Treg development. Interestingly, Wnt3a acted via β-catenin signaling while Wnt5a triggered other signaling pathways (44, 166). β-Catenin signaling in intestinal DCs induced RALDH2, inhibited the expression of pro-inflammatory cytokines, and promoted their Treg inducing capacity (44).

In mucosal sites, secretory IgA encompasses protective role against invasion of various pathogens but it seems that it exhibits further functions within the circulation. SIGNR1 binding to secretory IgA on BMDCs rendered these cells resistant to TLR dependent maturation (167). IgA primed BMDCs showed higher capacity to induce Tregs via their IL-10 production and were able to inhibit autoimmunity in animal models of diabetes and EAE (167).

Taken together, a long line of biomolecules is available with the capacity to either alter DC function or promote tDC differentiation. It is not clear yet whether all *in vitro* defined tolerogenic signals truly induce similar DC activation *in vivo* or other factors might intervene with their effect *in vivo*. Most of these soluble molecules are in the focus of tDC research to utilize them for generating human tDCs from autologous bone marrow or from peripheral blood monocytes. Autologous transfer of tDCs has been tested in clinical trials and was well tolerated in diabetic patients (168, 169). Although this approach provides attractive therapeutic possibilities, more research is needed to evaluate and understand the complexity of tolerance, such as the stability of the tDC phenotype *in vivo* and the dose and route of tDC vaccine used for the treatments of autoimmune patients.

SUMMARY AND CONCLUSION

In peripheral tolerance, similarly to the thymus, various APCs are involved to guard immune homeostasis. DCs are the major APCs involved in this process. Multiple components are implicated in maintenance and/or induction of tolerogenic effector DC phenotype (**Figure 2**). Intrinsic signaling and antigen processing properties of DCs together with the impact of the microenvironment influence the tolerogenic adeptness of DCs. It is itself intriguing that a variety of active processes seem to be necessary for mediating immune homeostasis and it is clearly not a passive effect of the missing maturation signal as previously thought. The picture is further complicated with the fact that various subtypes of DCs seem to possess different capacity for tolerance. Similarly in immunity, sequential antigen presentation by different DC types resulted in different aspects of T-cell activation and effector differentiation (170). Thus, maintenance of immune homeostasis is a result of a complex interaction of soluble and cell-associated components. Understanding this network and thereby influencing DCs provide important targets for treatment of autoimmune disease.

AUTHOR CONTRIBUTIONS

Ann-Katrin Hopp contributed with literature search, prepared the table and the figure and figure legend, and critically read the manuscript. Anne Rupp contributed with literature search. Veronika Lukacs-Kornek developed the concept of the manuscript, supervised, and wrote the manuscript.

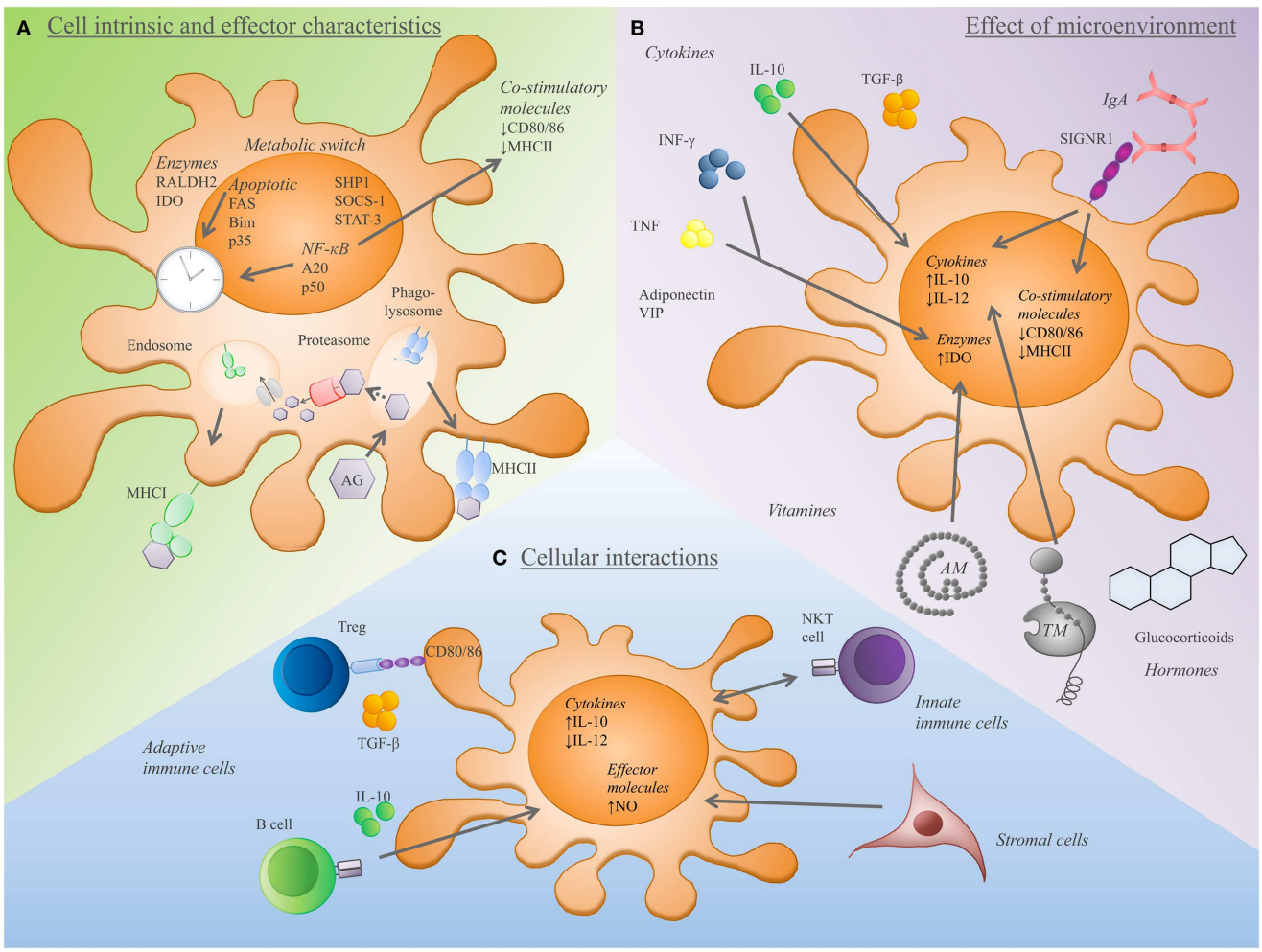

FIGURE 2 | Components that determine the establishment and tolerance-inducing capacity of tolerogenic effector DCs. **(A)** DCs expressing high level of anti-inflammatory cytokines (IL-10, TGFβ) and low level of co-stimulatory molecules (DC80/86) show a tolerogenic rather than an immunogenic phenotype. Additionally, the capacity of DCs to express Raldh2 or IDO is associated with tolerance. Furthermore, the activity of several pathways is linked to tDCs, such as metabolic, apoptosis, and NF-κB pathway, or activity of SHP1 and STAT-3. Additionally, the antigen capture and processing machinery (uptake of apoptotic cells, antigen-uptake receptors such as CLRs together with the MHCI and II processing machinery) greatly influence the T-cell inducing and tolerogenic capacity of DCs. **(B)** A variety of biological substances have an impact on tDC differentiation and function. Cytokines, vitamins, hormones as well as antibodies, thrombomodulin (TM), adrenomedullin (AM), and VIP induce tDCs. **(C)** The dialog of DCs with other immune cells and stromal cells provides additional checkpoints for the maintenance of tolerance. DC-Treg crosstalk involves the regulation of Treg homeostasis, the activation and induction of Tregs. Tregs, IL-10 expressing B cells, and natural killer T (NKT) cells favor a tDC phenotype. In addition, stromal cells promote tDC differentiation toward IL-10 or nitric oxide (NO) producing regulatory tDCs.

ACKNOWLEDGMENTS

This work was supported by the Alexander von Humboldt Foundation, Sofja Kovalevskaja Award to Veronika Lukacs-Kornek.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 01 December 2013; accepted: 30 January 2014; published online: 13 February 2014.
*Citation: Hopp A-K, Rupp A and Lukacs-Kornek V (2014) Self-antigen presentation by dendritic cells in autoimmunity. Front. Immunol. **5**:55. doi: 10.3389/fimmu.2014.00055*
This article was submitted to Antigen Presenting Cell Biology, a section of the journal Frontiers in Immunology.

frontiers in
IMMUNOLOGY

MINI REVIEW ARTICLE
published: 08 May 2014
doi: 10.3389/fimmu.2014.00203

Metabolic control of dendritic cell activation and function: recent advances and clinical implications

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Dendritic cells (DCs) are key regulators of both immunity and tolerance by controlling activation and polarization of effector T helper cell and regulatory T cell responses. Therefore, there is a major focus on developing approaches to manipulate DC function for immunotherapy. It is well known that changes in cellular activation are coupled to profound changes in cellular metabolism. Over the past decade there is a growing appreciation that these metabolic changes also underlie the capacity of immune cells to perform particular functions. This has led to the concept that the manipulation of cellular metabolism can be used to shape innate and adaptive immune responses. While most of our understanding in this area has been gained from studies with T cells and macrophages, evidence is emerging that the activation and function of DCs are also dictated by the type of metabolism these cells commit to. We here discuss these new insights and explore whether targeting of metabolic pathways in DCs could hold promise as a novel approach to manipulate the functional properties of DCs for clinical purposes.

Keywords: metabolism, oxidative phosphorylation, mitochondria, glycolysis, TLR signaling, immunogenic dendritic cells, tolerogenic dendritic cells, immunotherapy

INTRODUCTION

Dendritic cells (DCs) play a crucial role in the development of adaptive immune responses during infections and inflammatory diseases, as well as in the regulation of immune homeostasis during steady state, by governing the activation and maintenance of T cell responses. In response to many viral and bacterial infections, DCs promote the generation of effector $CD4^+$ T helper 1 (Th1) and $CD8^+$ T cell-dominated immune responses, while fungal and parasitic worm infections are predominantly associated with Th17 and Th2 responses, respectively. In addition to these effector responses, DCs can be instructed to become tolerogenic and promote regulatory T cells (Tregs), which regulate effector T cell responses, a process that is crucial for maintenance of immune homeostasis and control of autoimmune disorders and allergies. Because of the powerful immunoregulatory functions of DCs, there has been great interest in delineating the cellular processes that control the different properties of these cells, to ultimately identify ways to manipulate the function of DCs for the rational design of DC-based immune-interventions.

It has long been appreciated, especially in the cancer field, that changes in cellular activation coincide with, and are underpinned by, alterations in cellular metabolic state (1, 2). Importantly, over the last couple of years it is becoming increasingly clear that immune cell activation is also coupled to profound changes in cellular metabolism and that their fate and function are metabolically regulated (3). This has led to the idea that manipulation of cellular metabolism of immune cells can be used to shape innate and adaptive immune responses to our advantage. While most of our understanding in this area has been gained from studies with T cells (4–6) and macrophages (7, 8), evidence is emerging that metabolic processes also control the activation and immune-priming functions of DCs. In the current review, we will discuss these recent findings and explore whether targeting of metabolic pathways in DCs could hold promise as a novel approach to manipulate their functional properties for DC-based immunotherapy.

ROLE OF CELLULAR METABOLISM IN DC FUNCTION

Under non-inflammatory conditions, most DCs reside in peripheral tissues where they exist in a resting immature state. In this quiescent state, DCs are poorly immunogenic. However, upon triggering of a set of germline-encoded pattern recognition receptors, including Toll-like receptors (TLRs) by pathogen-derived products or inflammatory stimuli, DCs undergo a well-characterized process of cellular activation, termed DC maturation, which renders them highly immunogenic. This process involves an increase in capturing and processing of antigens for antigen presentation in context of major histocompatibility complex I (MHC-I) and MHC-II and the induction of expression of chemokine receptors, pro-inflammatory cytokines, and costimulatory molecules. This activation program endows DCs with the capacity to traffic, via tissue-draining lymphatics, to T cell zones of secondary lymphoid organs to efficiently prime and control effector T cell responses (9).

In T cells, catabolic metabolism centered around mitochondrial oxidative phosphorylation (OXPHOS) is associated with cellular longevity and quiescence, whereas cellular activation and proliferation are accompanied by a switch to glycolytic metabolism to support anabolic pathways needed for biosynthesis (4–6). Consistent with these observations, DCs when activated by TLR agonists, undergo a robust metabolic switch characterized by an increase

in glycolysis and a concomitant progressive loss of OXPHOS (10–13). We have shown that in inflammatory DC subsets, such as murine GM-CSF-derived bone marrow DCs (14), this switch from OXPHOS to glycolysis is a direct consequence of TLR-induced inducible nitric oxide synthase (iNOS) expression that through the production of nitric oxide (NO) poisons the mitochondrial respiratory chain in an autocrine fashion (15). In this setting, in the absence of functional OXPHOS, TLR-agonist activated inflammatory DCs depend heavily on glycolysis as their sole source of ATP for survival both *in vitro* and *in vivo* (12). Consistent with this, *in vitro* and *ex vivo* TLR-activated *Nos2*$^{-/-}$ inflammatory DCs still have functional OXPHOS and as result do not display a long-term increase in glycolytic metabolism (12). Likewise, we did not observe a switch to glycolytic metabolism following TLR stimulation of conventional DCs (cDCs) *ex vivo* (12), which do not express iNOS in response to TLR stimulation. However, a more recent *in vivo* study showed that TLR-activated cDCs do display long-term diminished mitochondrial activity and enhanced glycolysis (13). They found that this metabolic shift is iNOS-independent and instead driven by TLR-induced autocrine type I interferon production. Despite the differences in mechanism underlying the metabolic switch, similar to inflammatory DCs, cDCs seem to rely on the glycolytic shift for ATP production for their survival (13) (**Figure 1**).

These studies suggest that the metabolic reprograming toward glycolytic metabolism is a consequence of TLR-driven DC activation, rather than a prerequisite for it. However, given the fact that TLR stimulation results in a rapid activation program in both cDCs and inflammatory DCs, we recently tested the hypothesis that rapid metabolic reprograming needs to occur in both types of DCs to meet the bioenergetic and anabolic needs of TLR-driven DC activation itself. Indeed, we observed that TLR stimulation in both cDCs and inflammatory DCs results within minutes in an increase in glycolytic rate that is maintained for several hours after which it returns to prestimulation levels in the absence of iNOS (16). Inhibition of this early metabolic reprograming blunts DC activation, migration, and T cell priming both *in vitro* and *in vivo*, illustrating its importance for DC biology. Functionally, as opposed to the long-term glycolytic commitment, the rapid increase in glycolysis appears not to be important as a rapid source of ATP, but rather to serve a central anabolic role by acting as a carbon source for both the pentose phosphate pathway (PPP) and the tricarboxylic (TCA) cycle to support the generation of NADPH and citrate, respectively, that are used for *de novo* fatty acid (FA) synthesis. Moreover, glycolysis-supported *de novo* FA synthesis plays a crucial role in DC activation and function at the posttranscriptional level, by allowing for the synthesis and expansion of membranes including Golgi and ER that are required for synthesis, transport, and secretion of proteins associated with TLR-driven DC activation (16). These findings share strong parallels with activated T cells that heavily rely on glycolysis as a carbon source for *de novo* FA synthesis to support the need for membrane synthesis required for cellular proliferation (17). However, in contrast to T cells, DCs do not proliferate and seem to use this pathway to expand the cellular machinery necessary for increased production and secretion of the mediators that are integral to DC activation (**Figure 1**). This is consistent with a recent study positively correlating lipid content with immunogenicity of DCs in the liver and showing that the immunogenicity of DCs with high lipid content is dependent on FA synthesis (18). Taken together, these

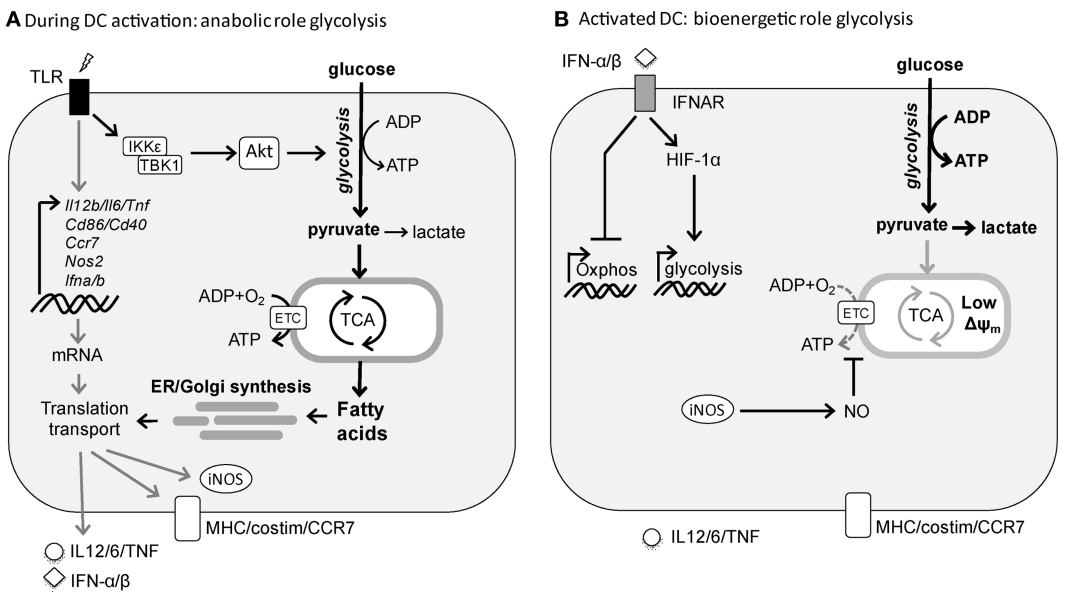

FIGURE 1 | TLR-induced metabolic changes in dendritic cells. **(A)** Rapid induction of glycolysis in DCs by TLR stimulation serves an anabolic role in DC activation, by generating lipids for synthesis of additional membranes including ER and Golgi to support the increased demands of synthesis and transport of proteins required for DC maturation. **(B)** Following activation, DCs sustain high glycolytic rates to generate ATP to compensate for the loss of mitochondrial function. In cDCs this process appears to be driven by autocrine type I interferon, while in inflammatory DCs this is a direct consequence of iNOS-derived NO that blocks OXPHOS.

studies illustrate that the induction of glycolysis plays a central role for DCs to acquire immunogenic properties as well as their survival following activation.

While the metabolic features of immunogenic DCs are becoming more well characterized, there is still little known about the metabolism of tolerogenic DCs. Tolerogenic DCs, as opposed to immunogenic DCs, are generally characterized by the absence of traditional signs of activation, are maturation-resistant, and express increased levels of immunoregulatory factors, important for controlling Treg responses (19–21). Consistent with this immature, maturation-resistant phenotype, proteomic analysis of human DCs treated with dexamethasone and vitamin-D3, two well known immunosuppressive drugs that induce tolerogenic DCs, revealed increased expression of genes associated with mitochondrial metabolism and OXPHOS (22, 23). Furthermore, DCs conditioned by IL-4 acquire a phenotype highly reminiscent of alternatively activated (M2) macrophages and expression of M2-associated activation markers on DCs is required for optimal induction of IL-10-secreting T cells (24). The fact that M2 activation by IL-4 is dependent on increased fatty acid oxidation (FAO) and OXPHOS (25–27) makes it conceivable that there is a causal link between mitochondrial metabolism fueled by FAO and the acquisition of a tolerogenic phenotype by DCs. The observations that direct inhibition of glycolysis in TLR-activated DCs favors the induction of Foxp3-expressing Th cells at the expense of IFN-γ-producing Th1 cells (16), and that resveratrol and rosiglitazone, drugs known to promote FAO (28) and mitochondrial biogenesis (29), respectively, interfere with TLR-induced DC activation and can render them tolerogenic (30–33), would support this idea. However, these studies are mostly correlative and more work will be needed to elucidate whether there is a direct functional link between mitochondrial catabolic metabolism and the acquisition of tolerogenic properties of DCs.

REGULATORS OF DC METABOLISM

In recent years, major advances have been made in unraveling the signaling pathways in immune cells that regulate their metabolic state. The conserved kinase mammalian/mechanistic target of rapamycin (mTOR) and its upstream activators PI3K-Akt have been identified as central regulators of cellular activation and proliferation due to their ability to control glycolysis and anabolic metabolism (34–36). Consistent with a role for mTOR in regulating DC metabolism as well, cDCs isolated from mice with a DC-specific deletion of tuberous sclerosis 1 (Tsc1), a negative regulator of mTOR, display enhanced mTOR activity, an increase of expression of glycolytic and lipogenic genes, and of maturation markers at steady state (37). Also in response to TLR ligands inflammatory DCs depend on signaling through PI3K, Akt, and mTOR for their long-term commitment to glycolysis (10, 38). mTOR promotes anabolic pathways and glycolysis by driving expression and stabilization of transcription factors such as sterol-regulatory element binding protein (SREBP) (39, 40) and hypoxia-inducible factor (HIF)-1α (41), that control expression of genes involved in lipogenesis and glycolysis, respectively. While it remains unknown whether SREBP plays a role in DC metabolism, several studies have documented an important role for HIF-1α in supporting the long-term commitment in glycolytic metabolism of both inflammatory DCs and cDCs in response to TLR stimulation (11, 13). Moreover, consistent with its well-recognized role in regulating innate immune cell function under inflammatory conditions (42), TLR-induced DC activation and T cell priming appear to rely on HIF-1α (11, 13, 43). However, whether this is a consequence of the role of HIF-1α in promoting glycolytic metabolism and thereby cell survival, or a reflection of the direct control of expression of inflammatory cytokines independently from glycolytic regulation (44, 45), remains to be addressed. In addition to direct transcriptional regulation of glycolysis through HIF-1α, mTOR may regulate the TLR-induced commitment to glycolysis indirectly in inflammatory DCs, through induction of iNOS expression and NO production (46, 47) that forces these cells to switch to glycolysis in the absence of mitochondrial respiration (12).

In contrast to the clear role for the mTOR-HIF-1α axis in regulating TLR-induced long-term metabolic changes, recent evidence suggests that the early TLR-driven induction of glycolysis to support the anabolic demands of DC activation itself does not depend on mTOR or HIF-1α signaling (16, 48). Instead, there is a critical role for Akt in this response that directly enhances the enzymatic activity of rate-limiting glycolytic enzyme hexokinase-II (HK-II) by promoting its association with the mitochondria. Interestingly, Tank-Binding Kinase 1 (TBK1) and IκB kinase-(IKK) but not the canonical Akt activators PI3K or mTORC2 appear to be the crucial upstream regulators of Akt activation in this TLR-driven rapid induction of glycolysis (16). Taken together based on these recent findings a picture is emerging that TLR-signaling drives two functionally and temporally distinct waves in glycolytic metabolism in DCs that are controlled by largely separate signaling pathways (**Figure 1**).

While the signaling pathways that promote the shift to glycolysis and anabolic metabolism required for TLR-induced activation and immunogenicity of DCs are starting to be characterized, much less is known about the signals in DCs that may antagonize these responses and that are potentially important for induction and function of tolerogenic DCs. In this respect, in T cells and in macrophages the metabolic sensor AMP Kinase (AMPK) is known to play a central role in antagonizing biosynthetic pathways, including lipogenesis, and has instead been shown to promote catabolic metabolism by, amongst other pathways, the activation of peroxisome proliferator-activated receptor gamma coactivator (PGC)-1α that promotes mitochondrial biogenesis to increase mitochondrial OXPHOS (7, 35). Consistent with these observations, pharmacological activation of AMPK suppresses TLR-induced glucose consumption and activation of DCs, while knockdown of AMPK has the opposite effect (10, 49), suggesting an important role for AMPK signaling in the metabolic control of DC activation. Furthermore, systemic administration of drugs activating AMPK signaling to promote catabolic metabolism drives induction of tolerogenic immune responses in several inflammatory disease models (50–52). However, it remains to be determined whether these treatments exert their effects through direct induction of tolerogenic DCs. Moreover, resveratrol, a drug that has been linked to induction of tolerogenic DCs, is thought to favor catabolic metabolism through activation of the histone deacetylase Sirtuin 1, which is known to suppress HIF-1α function as well as enhance PGC-1α activity (29, 32, 53). In addition,

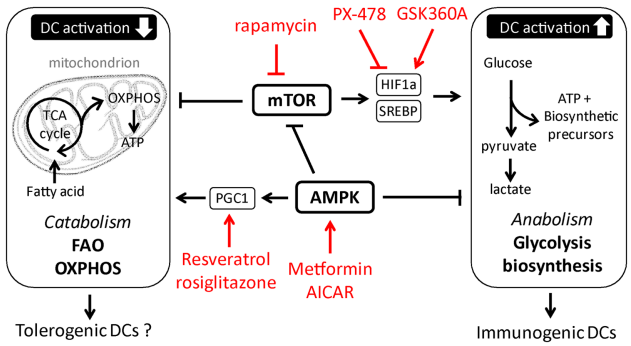

FIGURE 2 | Putative metabolic pathways and upstream regulators in tolerogenic versus immunogenic dendritic cells. In red examples are depicted of pharmacological approaches currently tested or used in other therapeutic settings, that could be used to manipulate DC metabolism.

DCs deficient for Nuclear factor-erythroid 2 p45-related factor-2 (NRF2) or PPAR-γ, downstream targets of PGC-1α, display increased maturation and T cell priming capacity (31, 54, 55). Hence, these studies may point toward an important role for the AMPK-PGC-1α axis in promoting mitochondria-centered catabolic metabolism in DCs, which may be crucial for the acquisition of a tolerogenic phenotype (**Figure 2**). However, how these signaling pathways are regulated under physiological conditions and to what extent the effects of these factors on DC biology can be attributed to direct regulation of DC metabolism are still unresolved questions.

MANIPULATING DC METABOLISM FOR THERAPEUTIC PURPOSES?

There is a great interest in the use of DCs as targets for immune-intervention and for vaccine strategies, because of their powerful immune stimulatory as well as regulatory functions (56). The use of highly immunogenic DCs can be used to promote robust cellular and humoral immunity that is central for improving vaccination efficacy against a variety of infectious diseases and tumors, while the use of tolerogenic DCs will allow for induction of regulatory immune responses in settings where unwanted effector T cell responses need to be controlled, such as to prevent rejection following transplantation. It is of pivotal importance to identify and characterize the regulatory processes underpinning these different functions of DCs. It is becoming clear from the aforementioned studies that the activation and T cell-priming function of DCs is tightly regulated by their metabolic fate. What can we learn from these new metabolic insights in DC biology and would there be ways to use this knowledge in developing approaches to enhance DC-based immunotherapies? The idea of manipulating cellular metabolism for therapeutic purposes is not a new concept. In fact, in the cancer field there is great interest in the use of pharmacologicals that inhibit anabolic metabolism or glycolysis to reduce tumor growth (57–60). Likewise, studies in T cells have provided a clear proof of principle that targeting of cellular metabolism can provide a viable means for improving the efficacy of vaccinations (61, 62).

Based on the importance of anabolic metabolism and glycolysis in supporting DC activation and immunogenicity, and the possible role of catabolic metabolism in supporting tolerogenic DC function, it will be of great interest to assess whether promoting these types of metabolism in DCs can be used as a strategy to enhance the immunogenicity or tolerogenicity of DCs in therapeutic settings. It should be noted that some of the pharmacological approaches currently used to manipulate the immunogenicity of DCs, such as dexamethasone, Vitamin-D3, and rapamycin (63–66) that are known for their capacity to induce tolerogenic DCs, have been described to influence DC metabolism (22, 23, 38). Thus it is possible that direct targeting of metabolism of DCs as a single treatment may not be superior to some other already existing manipulations that also affect metabolism. It is therefore more conceivable that manipulation of metabolism of DCs for immunotherapy will be most effective when used in conjunction with existing approaches to complement and enhance their therapeutic efficacy. A second important advantage of direct enforcement of certain types of metabolism in DCs is that is it may render them more resistant to environmental metabolic manipulation. This is highly relevant since a key parameter that determines the efficacy of immunotherapies is how long targeted DCs retain their phenotype following their functional manipulation. The microenvironment, which DCs become exposed to *in situ*, may lead to the loss of immunogenicity or tolerogenicity and would significantly affect the outcome of the therapy. For instance, the immunostimulatory capacity of DCs is often suppressed in a tumor microenvironment (67). Given the important role for cellular metabolism in regulating DC function, many of the suppressive effects of tumors appear to be attributable to effects on DC metabolism. It has been shown that tumor-derived IL-10 can suppress glycolysis in DCs through down regulation of glycolytic enzyme pyruvate kinase (68). Additionally, yet unidentified tumor-derived factors can promote aberrant lipid accumulation in DCs, resulting in impaired T cell priming (69, 70). Moreover, immunogenic DCs are likely to be impaired in their function in a microenvironment where glucose will be scarce due to the high glycolytic rates of tumors themselves (71). Finally, caloric intake and mitochondrial activity are important determinants of organismal as well as cellular lifespan (72, 73). Therefore targeting DCs metabolism can also be used to manipulate DC longevity to affect their immunostimulatory potential. For example, mTOR inhibition has shown to increase the lifespan of TLR-activated DCs and enhance their capacity to induce protective tumor immunity (38).

Several agonist and antagonists of metabolic enzymes and upstream signaling pathways that could be used to manipulate DC metabolism have already been developed and tested for safety and efficacy in other systems (58–60, 74) (**Figure 2**). In addition to pharmacological approaches, genetic manipulation through introduction of small hairpin RNAs has shown to be a successful strategy to alter DC immunogenicity (75, 76) and could provide a feasible alternative to target DC metabolism. In recent years, there has been a major focus on manipulating the immunostimulatory properties *ex vivo* generated DCs for autologous DC vaccination. Some of these vaccines have made it to the clinic (77) or are currently in clinical trials (78–80). In addition, widespread enthusiasm has been generated by results from the *in vivo* use of

nanoparticles, consisting of antibody covered micelles carrying antigens and potentially drugs or shRNA, that can be specifically targeted to DCs *in situ* (81, 82). Given the amenability of cellular metabolic intervention, it seems feasible that metabolism-targeted manipulations to DCs could be implemented in protocols for DC-based vaccinations.

CONCLUDING REMARKS

It is becoming increasingly clear that the metabolic phenotype of DCs dictates their activation and immunogenicity. However, many of the details and underlying mechanisms of how cellular metabolism controls the functional properties of DCs remain to be determined. For instance, the precise metabolic processes that underpin the function of tolerogenic DCs are still poorly defined. Moreover, do different *in vivo* DC subsets have different metabolic characteristics and are unique metabolic processes required for DCs to perform particular functions, such as cross presentation or the induction of Th1/2/17 cell responses? Addressing these and other questions will not only contribute to a better fundamental understanding of the biology of DCs, but will also aid in the rational design of metabolism-based approaches to enhance the efficacy of DC-based immunotherapies.

ACKNOWLEDGMENTS

The work was supported by the National Institutes of Health grants to Edward J. Pearce (AI53825, CA164062), and by a Veni grant from Netherlands Organisation for Scientific Research to Bart Everts.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 01 February 2014; paper pending published: 02 April 2014; accepted: 24 April 2014; published online: 08 May 2014.
*Citation: Everts B and Pearce EJ (2014) Metabolic control of dendritic cell activation and function: recent advances and clinical implications. Front. Immunol. **5**:203. doi: 10.3389/fimmu.2014.00203*
This article was submitted to Antigen Presenting Cell Biology, a section of the journal Frontiers in Immunology.

frontiers in
IMMUNOLOGY

REVIEW ARTICLE
published: 19 May 2014
doi: 10.3389/fimmu.2014.00218

Dendritic cell therapy in an allogeneic-hematopoietic cell transplantation setting: an effective strategy toward better disease control?

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Hematopoietic cell transplantation (HCT) is a last treatment resort and only potentially curative treatment option for several hematological malignancies resistant to chemotherapy. The induction of profound immune regulation after allogeneic HCT is imperative to prevent graft-versus-host reactions and, at the same time, allow protective immune responses against pathogens and against tumor cells. Dendritic cells (DCs) are highly specialized antigen-presenting cells that are essential in regulating this balance and are of major interest as a tool to modulate immune responses in the complex and challenging phase of immune reconstitution early after allo-HCT. This review focuses on the use of DC vaccination to prevent cancer relapses early after allo-HCT. It describes the role of host and donor-DCs, various vaccination strategies, different DC subsets, antigen loading, DC maturation/activation, and injection sites and dose. At last, clinical trials using DC vaccination post-allo-HCT and the future perspectives of DC vaccination in combination with other cancer immunotherapies are discussed.

Keywords: DC-vaccination, hematopoietic cell transplantation, disease control, relapse, T-cell responses

INTRODUCTION

Allogeneic-hematopoietic (stem) cell transplantation (HCT) is the last treatment resort and only potentially curative treatment option for several hematological malignancies resistant to chemotherapy. Although the survival rates improve after HCT for selected indications, relapses remain a major cause of death after allogeneic HCT. In these high-risk hematological malignancy patients, the estimated 5-year survival rates vary between 10 and 80% (1–3). As such, novel immune therapeutic strategies are being developed aimed at getting better disease control to prevent relapse after HCT.

Currently, the most widely used type of additional immunotherapy combined with allo-HCT is the donor lymphocyte infusion (DLI), where allo-reactive T cells can help to eradicate residual tumor cells. Unfortunately, this "non-specific" strategy suffers from severe toxic side effects, such as Graft-versus-Host Disease (GvHD) (4). Novel immunotherapeutic approaches aim to increase innate or adaptive anti-tumor responses by transferring *ex vivo*-generated effector cells, such as natural killer (NK) cells, chimeric antigen receptor (CAR)-modified cytotoxic T lymphocytes (CTLs), or transgenic T-cell receptor expressing tumor-specific CTLs (5). Although initial results seem promising, the production procedures of these cell therapies are often time-consuming (up to months) and have limitations, severe acute toxicities ("cytokine-release syndrome": e.g., in CARs), long-term B-cell deficiency (in CD19-CAR), uncertain functionality, and limited or no induction of lasting immunity. Since dendritic cells (DCs) are potent and professional antigen-presenting cells (APC), which induce activation of the adaptive immune system, vaccination strategies could be used post-allo-HCT for the induction of lasting immunity against the tumor. Several vaccination strategies have been used post-allo-HCT like the vaccination with autologous tumor cells either directly transduced to express GM-CSF (6) or coinjected with fibroblast expressing transgenic CD40L and IL-2 (7).

The use of DCs as vaccines showed beneficial effects in an autologous setting (8), which led to the first FDA approved immunotherapy (9). In this review, we will explore the use of DCs as vaccination strategy for the induction of anti-malignancy responses when combined with an allo-HCT (**Figure 1**). More specifically, we will focus on the use of donor-derived DCs as part of this immunotherapy.

ALLO-HCT IN CANCER IMMUNOTHERAPY

Allo-HCT is the sole curative option for many patients with high-risk hematologic malignancies and even some solid tumors (4, 10, 11). A variety of different allo-HCT grafts, including bone marrow (BM) or mobilized peripheral blood stem cells (PBSC), as well as unrelated umbilical cord blood (CB) are currently used as a cell source in the treatment of malignancies (12). The therapeutic success of the allo-HCT is not only due to the replacement of the diseased BM but also due to Graft-versus-Leukemia (GvL) or Graft-versus-Tumor (GvT) effects. However, as a trade-off, the potentially life-threatening complication GvHD can occur. In this regard, it is interesting that the use of CB as cell source is associated with lower relapse-rates suggesting stronger GvL-effects, despite

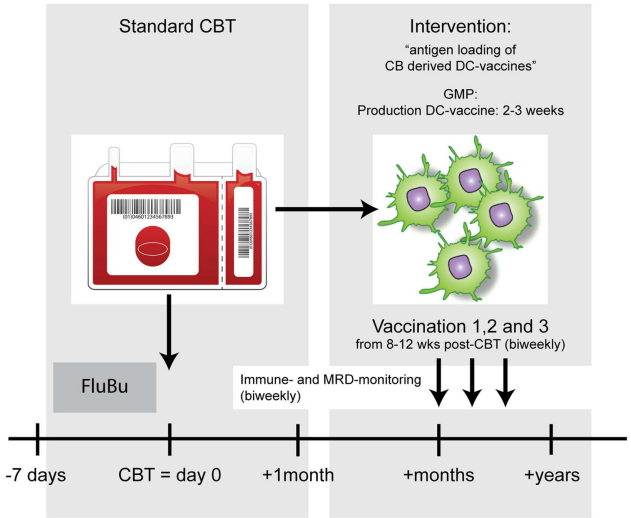

FIGURE 1 | Example of a DC vaccination strategy to enhance anti-tumor immunity after allo-HCT. After standard conditioning (FluBu: Fludarabine + Busulfan) and cord blood transplantation (CBT) patients will receive biweekly antigen-loaded-DC vaccines. The timing of vaccinations will be dictated by the chances that most CBT-associated complications are solved or are very unlikely to occur and the T-cell compartment has time to recover.

lower GvHD-rates compared with BM or peripheral blood as cell source in HCT (2, 13). As such, therapeutic interventions aimed at enhancing the GvT will not necessarily lead to higher rates of GvHD, whereas the active inhibition of GvHD will not necessarily affect the GvT effects (14).

The importance of CTLs in the GvT effects is supported by the observation that an increase in leukemic antigen (WT1) specific CTLs correlated negatively with the WT1 mRNA expression, as a measure of minimal residual disease (MRD) (15). Moreover, the absence of T cells specific for different tumor associated antigen (WT1, MUC1, and proteinase-3) was related to relapses post-allo-HCT in patients with hematological malignancies (16). These data show that tumor-antigen-specific CTLs can be induced after HCT and failure to induce these cells may hamper GvT responses. This strengthens the idea that the active enhancement of tumor-antigen-specific immunity is a viable treatment option to prevent relapses after HCT (17). The development of tumor-antigen-specific CTLs strongly relates to the general immune recovery (especially T cells and DCs) after HCT, a process that is both complex and dynamic, and is affected by a variety of patient and graft-related factors. These include graft source, graft manipulation, age of recipient and donor, conditioning regimen, recovery of thymic output, the occurrence of infections, and GvHD, and their treatment (11, 18–23). Some of these factors will be difficult to control, whereas there are some factors, like the conditioning regimen [especially the serotherapy component: anti-thymocyte globulin (ATG) or Alemtuzumab], which can be more carefully controlled to enhance or get a more predictable immune reconstitution after HCT. In this regard, detailed immune recovery studies showed that the T-cell recovery can be very fast after HCT depending on that timing, dosing, and/or omission of ATG (24). This occurred without causing mayor effects on the development of GvHD [in particular chronic-GvHD (cGvHD)] but with significantly reduced occurrence of viral reactivation, which is strongly dependent on post-HCT T-cell recovery.

A predictable immune reconstitution is of importance to establish an optimal effect of the applied vaccine. Thus vaccination strategies early after allo-HCT, in a setting of a better-predicted immune reconstitution, aiming to prime and/or stimulate tumor-specific CTLs may be an attractive and effective treatment modality.

DCs AND THEIR ROLES IN GvHD AND GvT POST-ALLO-HCT

As professional APCs, DCs have been well recognized for their role in the induction of GvHD on the one hand and GvT responses on the other. Whereas host-derived DCs have shown to be essential for the induction of acute GvHD (aGvHD) in mice, donor-derived DCs intensify aGvHD and may be involved in the development of cGvHD (25, 26). The role of the different DCs in the GvT response after HCT is still poorly understood. From mouse studies, it is known that host DCs may play an important role in GvT responses (27), especially those that are able to cross present tumor-specific antigen (TSA) from tumor cells to the donor T cells (28). The role of host DCs in GvT in humans has been supported in a study where the combination of donor T cells and mixed chimerism in DC subsets induced a potent GvL effect in association with GvHD, whereas DLI in patients with donor chimerism in both T cells and DC subsets resulted in GvL reactivity without GvHD (29).

Largely independent of conditioning regimen and stem cell donor source, a rapid DC chimerism was detected in peripheral blood after allo-HCT (30). Fourteen days after HCT approximately 80% of the DCs were of donor origin increasing up to 95% at 56 days after HCT. With regard to DC chimerism in peripheral tissues, it was found that depending on the regimen, an average 97% of the Langerhans cells (LC) were donor-derived with full intensity conditioning, while 36.5% was donor-derived with reduced intensity conditioning 40 days after allo-HCT. At day 100, at least 90% of the LC was donor-derived (100% in half of the patients) (31). In another study, donor chimerism with median of 95% was detected for LC in skin biopsies taken between day 18 and 56 after HCT (32). However, this same study also indicated that the majority of the patients with an incomplete donor chimerism suffered from aGvHD. Moreover, these data were challenged in a recent paper studying the chimerism in the skin itself, rather than in DCs that migrated from explants (33). This study showed that 3 months after HCT, at least half of the dermal DCs were still of host origin in the absence of aGvHD, suggesting that the mere presence of host DCs is not the cause of aGvHD.

As both host and donor DCs are present after HCT "regular" vaccination strategies (with epitopes from tumor antigens) or targeting DCs *in vivo* as an immunotherapy early after HCT may also be feasible. In patients with a high risk of relapse, the period early after HCT may be crucial for DC-based therapies as the tumor burden is still low and the suppressive immune environment of the tumor can still be overcome. When studies identify a specialized subtype of human DC that may increase GvT without enhancing GvHD, as was shown for $CD8\alpha^+$ DCs in mice (28), specific *in vivo*

targeting and stimulation of these cells may be a treatment option in the future. Since the *in vivo* targeting of endogenous DC as immunotherapy has recently been extensively reviewed elsewhere this will not be further discussed here (34).

DC SOURCES AND SUBSETS FOR VACCINATION IN ALLO-HCT SETTING

Dendritic cells for vaccination purposes can be directly isolated from peripheral blood or can be generated from stem cells residing in the blood or BM. In the post-allo-HCT setting, DCs could be directly isolated from the peripheral blood of the donor. From the blood different subsets can be isolated, namely plasmacytoid DCs (pDCs) and conventional (c)DCs, this latter population can be further subdivided into BDCA1$^+$ and BDCA3$^+$ DCs. However, the low numbers of in particular circulating into BDCA1$^+$ and BDCA3$^+$ DCs complicates their clinical application. In an autologous non-HCT setting, promising results were obtained with isolated pDCs. Freshly isolated pDCs that were loaded and activated *ex vivo*, induced antigen-specific CD4$^+$ and CD8$^+$ T-cell responses in patients suffering from melanoma (35). Despite the low numbers, using DC subsets in current and future trials is relevant and therefore intrinsic properties of DC subsets to stimulate productive T cells should be taken into account in the DC vaccine design.

Dendritic cells may also be generated from precursor cells like CD14$^+$ monocytes (from peripheral blood) or CD34$^+$ HSC (from BM or peripheral blood), which can be differentiated *ex vivo* into monocyte-derived DCs (moDCs) or conventional DCs, respectively.

After the finding that monocytes develop DC-like features when cultured in the presence of GM-CSF and IL-4 (36), moDCs have been used in many clinical trials as a cancer immunotherapy. The use of moDCs as a vaccine is generally considered as safe, but clinical responses have only sporadically been observed (37), possibly due to maturation status or migratory capacity, discussed in more detail below. Since more research focuses on differential functionalities within DC subsets, the vaccine research shifts toward targeting of specialized DC subsets (34, 38) and *in vitro* generation of conventional DCs from CD34$^+$ precursor stem cells. Several protocols have been developed trying to mimic the different naturally occurring DC-populations (39–41), so far no clinical data are available on the efficacy of these DC cultures. The most important advantage of using CD34-derived DC, especially in the CB HCT setting, is the possibility to use an expansion step prior to DC differentiation allowing the generation a large number of DCs from a limited number of precursor cells.

Although studies directly comparing the anti-leukemic effects of CD14- versus CD34-derived DC vaccines are lacking (42), it has been suggested that CD34-derived DCs may induce better CD8 responses, compared to moDCs. This might be caused by the presence of LC in these cultures (43). The presence of LC is however strongly dependent on the presence of specific growth factors during differentiation.

DC VACCINATION STRATEGIES

Besides the type of DC, the specific antigen loading and maturations strategies have major impact on the priming capacity of the DC. In addition, the functionality of the DC vaccine is dependent on the infection site, dosing regimen, and timing of vaccination, all of which may be even more prominent when combined with allo-HCT (**Figure 2**).

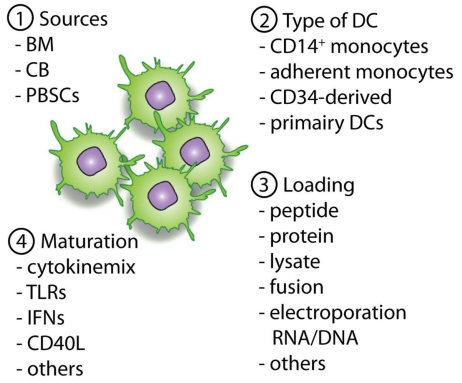

FIGURE 2 | Overview of important parameters to consider and optimize pre-clinically with regard to DC vaccines. The first important parameter is the source of the allo-HCT graft (1), which will determine the available cell sources for the generation of the DCs (2). When DCs are generated the antigen loading strategy (3) will define the presentation of (tumor)-antigens in MHC-class II and I molecules, providing the first signal for T-cell activation. Next, optimal maturation signals should be used to induce the expression of co-stimulatory molecules and the necessary cytokines (signal 2 and 3). This will enable homing of the DCs to lymph nodes followed by an optimal stimulation of antigen-specific T cells for the induction of lasting immunity.

TUMOR-ANTIGEN LOADING

Different loading strategies have been developed over the years, reviewed by Nierkens et al. (44). Exogenous MHC-class I loading with a 9-mer peptide of a pre-defined tumor antigen is frequently used. Although the analyses of T-cell specificity against that one peptide may simplify immune monitoring, this system has however some major disadvantages, such as, HLA-restriction, epitope spreading by the tumor, and lack of induction of antigen-specific CD4 T cells. Alternatively, DCs can be loaded with long-peptides, containing several MHC-class I and II restricted tumor-antigen peptides, 15-mer peptide pools covering the whole tumor antigen, or the whole tumor antigen (protein or mRNA). These approaches require the prior identification of the TSA. For several tumors specific antigens may however not be known. In these cases, whole tumor cell lysates, DC-tumor cell fusions, or apoptotic/necrotic tumor cells can be used as a source of tumor antigens (45, 46). Although vaccination with tumor cells or their lysates may induce/aggravate acute or cGvHD due to the presentation of allo-antigens shared by tumor and normal host cells, to date, none of the studies using tumor cells as part of their vaccine showed any induction of exacerbation of GvHD (6, 7, 47, 48). As such, loading DC vaccine with killed tumor cells or lysates may be an attractive alternative when specific tumor antigens are not known, when they differ between the patients with the same cancer or when the proteins are sensitive to mutations.

MATURATION

For the stimulation of antigen-specific T cells DCs require maturation, which can be induced by clinical grade maturation mixes. Classically, moDCs are matured with a mix of pro-inflammatory cytokines, e.g., IL-1b, IL-6, TNF, and PGE2, which induce strong upregulation of CD40, CD80, CD83, CD86, and CCR7 (49, 50) and are clinical grade available. PGE2 has been shown to be necessary for the migration of DCs (51), but it also induces IDO expression (52), which is involved in inducing tolerogenic responses. However, Krause et al. showed IDO expression independently of PGE2, and strong CD4 and CD8 proliferation after co-stimulation with DCs matured with PGE2, despite IDO expression (53).

Dendritic cells also express different pathogen recognition receptors, like Toll-like receptors (TLRs). Although TLR antagonists have been shown to be good candidates for DC activation, their use as maturation agent in DC vaccination trials is still limited. Currently, PAM3cys for TLR2, Poly-IC or Poly-ICLC (Hiltonol) for TLR3, LPS for TLR4, or Imiquimod TLR7 and CpG-ODN for TLR9 are clinical grade available and used in several combinations (with each other or with cytokines) in clinical trials (54–56). The effect of the maturation mixes strongly depends on the DC subset isolated or cultured, since different DC subsets express different TLRs (57). In several clinical trials, the combination of TLR agonists or cytomix is used with IFN type 1 or 2, TNF, or CD40L (58–61). This combination not only enhances their maturation efficacy, but also induces stronger cytokine production *in vitro* (59). CD40L is used to activate DCs *in vitro* before injection, and although DC maturation and IL-12 production was reported, no clinical benefit was observed (62). One could even speculate that stimulation with CD40L before the vaccination infusion may somehow activate the DC before they were able to connect with the antigen-specific T cells in the lymph nodes.

In addition to co-stimulatory molecules, DCs are also known to express co-inhibitory molecules, like PD-L1 and PD-L2, which may hamper T-cell stimulation via interaction with PD1. Targeting the expression PD-L1 and PD-L2 siRNA electroporation or transfection into DCs has been shown to enhance CTL responses *in vitro* and *in vivo* (63, 64). Since this approach can be incorporated into DC vaccines relatively easy, this has the potential to become a standard procedure in addition to the maturation for future DC vaccinations.

INJECTION SITES AND DOSING AND TIMING

When a DC vaccine is optimally loaded and matured, the next border to cross is to consider the optimal injection site. In clinical vaccination studies, DCs have been injected intravenously (i.v.), intradermal (i.d.), subcutaneously (s.c.), directly in the lymph node (i.n.) under sonographic guidance, or intratumoral (i.t.) or at different sites within the same trial. Side to side comparisons of injection sites are generally lacking making it hard to make a strong statement on which site would be preferable. Intratumoral DC vaccination has been shown to be safe (65). The question remains whether the DCs are needed at the tumor site to restimulate the tumor infiltrating lymphocytes (TILs) or that they are required to present their cargo in the lymph node for the priming of novel CTLs, in which case other sites of injection could be a better option. Furthermore, the strong immune suppressive environment in the tumor may be detrimental for CTL activation. Bedrosian et al. (66) showed in a phase I trial in metastatic melanoma patients that i.n. is superior over i.d. with regard to CTL induction. Whereas the study of Kyte et al. showed no advantage of injecting i.d. compared to i.n. in a phase I/II trial also in melanoma patients (67). The type of DC used for vaccination or disease stage could both contribute to these contradictory findings. The limited overall efficacy of DC vaccination may further hamper the proper comparison between the different injection sites.

Another variation within clinical trials is the frequency and dosing regimen, varying from 2 to 6 times. No clear comparison has been made, and therefore no strong conclusions can be drawn. According to mouse studies and some clinical trials, vaccination seems to be critical, but boosting strategies of subjects with residual disease or with tumor recurrence, should be carefully revisited (68).

MONITORING THE EFFECT OF DC VACCINATION

Over 1000 trials have been performed using DC vaccination, but read-outs are very diverse, and mainly phase I/II trials test for cytotoxicity and overall survival are studied. The immunological CTL response generated by the DC vaccination can be monitored using HLA-peptide tetramers or by assessing cytokine production after *ex vivo* antigen-specific restimulation (ELISA, ELISPOT, or intracellular flow cytometry).

Since most DC vaccinations have been performed in an autologous (HCT) setting there may be tumor-antigen-specific T cells present. To be able to differentiate between priming and reactivation of T cells, KLH is sometimes used as a reporter for the presence of priming and Influenza Matrix Protein (Flu-MP) could be added as positive control for reactivation. When combined with peptide-loaded DCs, these proteins may also be helpful in providing bystander CD4 help (69). Almost all patients receiving DC vaccination in the skin are tested at several time points after vaccination for a delayed type hypersensitivity (DTH) response however most of these responses are KLH or Flu-MP specific and might not necessarily be predictive of the induced anti-tumor responses (70).

With regard to tetramer staining to study antigen-specific CTLs, the recent development of conditional HLA-ligand peptide exchange technology combined with combinatorial coding may provide an excellent opportunity to check for a wide range of different peptide–HLA combination in limited amount of material (71, 72).

With increasing sensitivity of PCR techniques, MRD markers are increasingly used to monitor clinical efficacy of immune therapy (73), including DC vaccination (74). A more general approach is immune-phenotyping analysis for the frequency of different immune cells at several time points before and after vaccination. These kind of analysis have reported changes in NK cells and their activation status after DC vaccination (74). Since current DC vaccines are still limited in their potential to induce an effective anti-tumor immune response, the possibility to compare results from different studies could benefit from "international standardized" immuno-monitoring protocols (75).

Table 1 | Overview of DC vaccination trials after allo-HCT.

Source stem cells	Source DC	(Tumor) target	Antigen	Antigen form	Vaccination	Read-out	Immune response	Clinical response	(S)AE	Reference
BM	PBSC	AML aLL	Whole tumor	Apoptotic tumor cells	IV	Vitro CTL/MLR DTH	DTH 3/4	3/4	NR	(47)
PBSC	CD14+	Renal cell carcinoma	Autologous tumor	Lysate	ID	DTH	0/1	0/1	NR	(48)
BM/PBSC	CD14+	CMV	Pp65 pp150	Peptide	SC near LN	Tetramer peptide recall	7/17 (41%)	YES link IR?	NR	(76)
BM/PBSC?	CD14+	AML	WT1 KLH reporter	Peptide	ID (6 month after HCT)	Tetramer peptide recall	KLH yes WT1 no	0/1	NR	(78)
BM/PBSC?	CD14+	CMV	PP65	Protein	SC near ILN (6 month after second HCT)	Protein recall	1/1	1/1	NR	(77)
BM/PBSC?	CD14+ host-derived	MM	Allo-antigens MiHA KLH reporter	Protein	ID near ILN (6 month after second HCT)	Protein recall DTH	KLH 6/6	No but patients also did not respond to DLI	NR	(4)

(S)AE, (Severe) adverse events.

DC VACCINATION TRIAL IN ALLO-HCT

Although the use of DC vaccination after allo-HCT had been suggested for many years, Grigoleit and colleagues were the first to publish a phase 1/2 clinical trial using donor CD14-derived DC after HCT in patients at high risk for developing CMV disease (76) (**Table 1**). In this setting, peptide-loaded DCs were injected s.c. near the inguinal lymph node. Immune monitoring showed the induction of CMV-specific T-cell responses, which had clinical effects on CMV disease in a prophylactic as well as therapeutic setting. With regard to the potential adverse events, it was important to notice that vaccination with donor-derived DCs pulsed with HCMV peptides did not stimulate or expand allo-reactive T cells. Nor were there any long-term adverse effects of DC vaccination after HCT. Taken together, this phase 1/2 study provided the first evidence indicating that DC vaccination can be performed safely in allogeneic HCT setting. DC vaccination was also performed in a therapeutic setting in a patient suffering from recurrent CMV reactivation after a second HCT (77). As there was emerging viral resistance to the antiviral chemotherapy, DC cells were prepared from $CD14^+$ monocytes isolated from the patients PB and loaded with CMV PP65 protein. The induction of PP65 specific CD4 and CD8 cells was detected and coincided with lasting prevention of CMV recurrence. This study is strongly supportive of the use of protein instead of peptide to enable the induction of both CD4 and CD8 responses. In this study again no adverse events were reported.

The first publication using DCs to boost the GvT responses after HCT was by Fujii and colleagues (47). Four patients with hematological malignancies relapsed after allo-HCT and were treated with DCs cultured from PBSC isolated from the same donor as the HCT. These donor-derived DCs were then loaded with tumor cells from the patient that were induced to go into apoptosis by irradiation. DCs were then injected i.v. and clinical response was reported in three out of four patients characterized by the reduction in tumor load. No side effects were detected in any of the patients. In a following case report, DC vaccination was used in a patient who received an allo-PB-HCT as a treatment for renal cell carcinoma (48). However in this patient no antigen-specific recall response (DTH) or any clinical response was reported. Like the previous report, this patient also did not show any severe adverse events.

Another case report describes vaccination with CD14-derived DCs pulsed with WT1 peptide and KLH antigen for the treatment of AML relapse after allo-HCT (78). Although no WT1 peptide-specific T cells could be detected, the KLH specific DTH and ELISPOT further support the ability of DC vaccination to induce an antigen-specific immune response in a patient after allo-HCT. Host CD14-derived DCs, isolated prior to allo-HCT, were used to present minor histocompatibility antigens (MiHA) antigens in six multiple myeloma patients that had received auto-HCT followed by allo-HCT and two rounds of DLI (4). This study showed that DC vaccination using host-MoDCs was safe (no GvHD) when applied at least 6 months after HCT induced immunity (KLH). Unfortunately, no MiHA specific T cells were detected after vaccination and also clinical responses were poor, probably caused by the setup of the treatment protocol.

So, although only very limited studies have been reported using DC vaccination after allo-HCT, the data so far are promising with some clinical responses, detectable immune responses, and no increase in the adverse events normally occurring after allo-HCT, all ruling in favor of further exploration of DC vaccination in allo-HCT. In additional, ongoing or recently finished, trials patients are treated utilizing idiotype-pulsed allogeneic DCs post-allo-HCT (NCT00186316 clinicaltrials.gov) or with donor-derived

DCs pulsed with WT1 peptides in combination with DLI (NCT00923910 clinicaltrials.gov).

COMBINATION THERAPIES

The limited clinical efficacy of DC vaccination may not only be due to the vaccine or vaccination strategy since the final eradication of the tumor depends on a variety of factors within the cancer-immunity cycle (79). When antigen-specific CTLs are induced and go to the tumor site there are mechanisms in place that prevent the tumor cells from getting killed by CTLs, i.e., downregulation of activation receptors, co-stimulatory molecules, or HLA class I antigens recognized by CTLs; upregulation of co-inhibitory molecules like PD-L1 release of soluble factors that inhibit Th cells, CTLs, and APCs; and altered FAS-L expression on the tumor cells causing apoptosis resistance (80–83). Clinical trials with therapies aimed at these immune blockades, such as cytotoxic t-lymphocyte-associated antigen 4 (CTLA4) and programed cell death protein 1 (PD1), have shown some very promising results as reviewed recently (84), making some of the therapies interesting candidates to use in combination with DC vaccination. This is supported by the observation in combination with a DC vaccine, a PD1 blocking antibody enhances *ex vivo* activated T-cell responses after DC/tumor fusion stimulation (85).

Another post-HCT immune therapy that can be combined with DC vaccination is the infusion of tumor antigen or MiHA specific CTLs that can provide additional effector cells to reduce the tumor burden if disease has relapsed. In this way, it may also affect the tumor microenvironment enabling better migration and CTL function of the DC generated CTLs. The use of PBSC or BM as HCT graft has the obvious advantage that DLI can be performed as a prophylaxis or therapy combined with DC vaccination (4, 86, 87). Another possibility is the use TCR gene transfer for the formation of a large population of tumor-antigen-specific T cells that would reduce the risk of GvHD or other bystander immune responses (88, 89). All these latter techniques remain to be tested in combination with DC vaccination.

Very recently, epigenetic drugs were used in combination with DC vaccination to enhance MHC upregulation, and therefore tumor-antigen expression on the tumor cells. A very promising clinical trial in a stage IV Neuroblastoma (NB) patient showed complete remission with this combined therapy (90).

To take DC vaccination to the next level one should consider making use of these additional therapies to hopefully enhance clinical efficacy of DC vaccination in all immune therapeutic settings.

SUMMARY

Although allogeneic-hematopoietic (stem) cell transplantation (HCT) is the only potentially curative treatment option for several hematological malignancies resistant to chemotherapy, relapses remain a major problem. DC vaccination may be an attractive additional immune therapeutic option for the induction of specific anti-malignancy immune responses in the context of an allo-HCT setting. Factors like optimizing and predicting immune recovery suggest that a more personalized conditioning regimen especially considering the use of ATG is essential for optimal effect of the vaccine. Depending on the HCT graft source different DC sources can be considered, with currently no conclusive data on which source to prefer. Preclinical development of the DC vaccine should further contain the optimization of antigen loading, DC maturation as well as limitation of the expression of co-inhibitory molecules. Finally, one should carefully consider the injection site and dose and frequency of the DC vaccine. The few DC vaccinations studies after allo-HCT have shown to be safe as well as promising with regard to both clinical and immunological responses. As such the field is open for further exploration especially with the current advances in possible combination therapies to further reduce the relapse rates and improve the survival rates.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 31 January 2014; accepted: 30 April 2014; published online: 19 May 2014.
*Citation: Plantinga M, de Haar C, Nierkens S and Boelens JJ (2014) Dendritic cell therapy in an allogeneic-hematopoietic cell transplantation setting: an effective strategy toward better disease control? Front. Immunol. **5**:218. doi: 10.3389/fimmu.2014.00218*
This article was submitted to Antigen Presenting Cell Biology, a section of the journal Frontiers in Immunology.

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