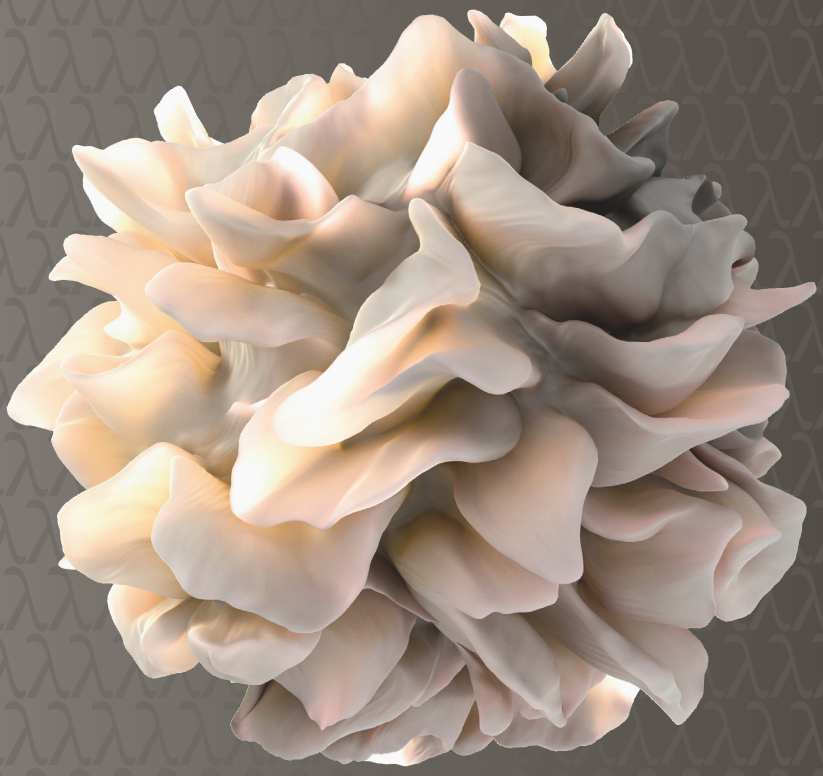
BDCA3^{+} Dendritic Cells and Their Function in Chronic Hepatitis B Virus Infection



Evelyn van der Aa

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Colophon

The studies described in this thesis were performed at the Deparment of Gastroenterology and Hepatology, Erasmus MC University Medical Center, Rotterdam, The Netherlands.

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BDCA3^{+} Dendritic Cells and Their Function in Chronic Hepatitis B Virus Infection

BDCA3^{+} dendritische cellen en hun functie in chronische hepatitis B virus infectie

Proefschrift

ter verkrijging van de graad van doctor aan de Erasmus Universiteit Rotterdam op gezag van de rector magnificus Prof.dr. H.A.P. Pols en volgens besluit van het College voor Promoties.

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Introduction

Chapter 1

Hepatitis B virus

Hepatitis B virus (HBV) belongs to the hepatotropic hepadnaviridae, which represent a family of small enveloped DNA viruses that primarily infect the liver. Worldwide, approximately 250 million people are chronically infected and thereby at increased risk to develop liver fibrosis, cirrhosis and hepatocellular carcinoma over a course of 20-50 years [1]. Hepatitis B virions, also known as Dane particles, consist of a nucleocapsid surrounded by core antigens (HBcAg) that is in turn enveloped by an outer lipid membrane containing small, middle and large surface antigens (HBsAg) (Figure 1) [2]. The nucleocapsid comprises the double-stranded relaxed-circular (RC)-DNA of approximately 3200 base pairs that contains four major open reading frames: C, encoding both HBcAg and hepatitis B e antigen (HBeAg); S, encoding the surface antigens (Ag); P, encoding polymerase, the reverse transcriptase enzyme; and X, encoding the HBV X protein (HBx), which has been described to be involved in regulation of virus replication [3, 4].

HBV specifically targets hepatocytes. Upon entry of HBV via the sodium taurocholate cotransporting polypeptide (NTCP) into these cells, the nucleocapsid is released into the cytoplasm and transported to the nucleus [5, 6]. The RC-DNA is released into the nucleus and "repaired" to form covalently closed circular (ccc)DNA [7]. cccDNA is a minichromosome that can persist in the liver for years. Since it functions as a template for all viral RNAs, incomplete eradication of cccDNA can lead to reinitiation of an infection. The small mRNAs transcribed from the cccDNA are translated into viral proteins and over-length pregenomic (pg)RNA is packaged together with the viral polymerase into a newly-formed nucleocapsid. In this core particle reverse transcriptase takes place to synthesize viral DNA. The RC-DNA-containing nucleocapsids are either transported to the nucleus to amplify cccDNA or enveloped and released as Dane particles via the endoplasmic reticulum [8]. In addition to infectious virions, also soluble HBeAg proteins and virus-like particles (VLPs) consisting of small, middle and large HBsAg, are secreted by virus-infected hepatocytes. HBeAg represents a non-structural, secreted form of the nucleoprotein, which is not required for viral replication, but is detected during the early stages of chronic hepatitis B (CHB) and exhibits immune-modulating functions [2]. HBsAg VLPs can accumulate to more than 100 µg/ml in peripheral blood of HBV patients and outnumber infectious virions at least 100-fold [9] and like HBeAg exhibit immune-modulating functions [2].

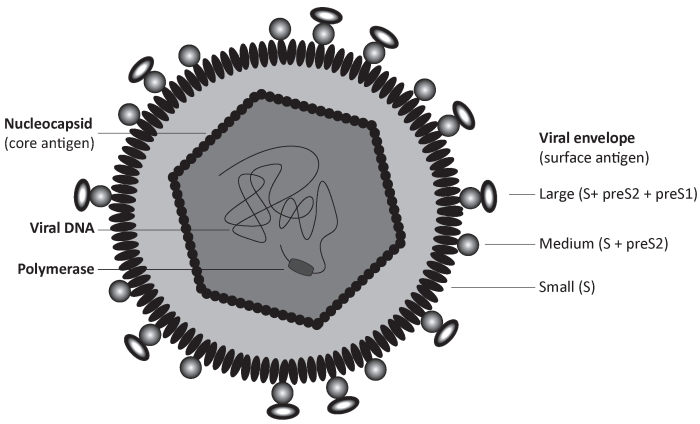

Figure 1. Schematic representation of an HBV virion.
An HBV virion consists of a nucleocapsid of core antigens, which contains viral DNA and polymerase, and is enveloped by an outer lipid membrane made up of small, middle and large surface antigens.

Natural course of HBV infection

HBV can be transmitted from mother to child (vertical transmission) or via exposure to blood or infected body fluids (horizontal transmission). During the first 4-7 weeks post-infection, HBV DNA and HBV Ags are not detectable in serum or the liver. After this lag phase HBV starts expanding exponential, and can reach levels up to 10^9-10^{10} copies per ml [10]. Infection with HBV leads to acute hepatitis, which can be either symptomatic or asymptomatic. During the acute phase an adequate innate and adaptive immune response, strong and broad antiviral $CD4^+$ and $CD8^+$ T cell responses as well as HBsAg-specific antibodies, are induced, which control and eradicate the virus [11-14]. However, lack of an effective antiviral immune response leads to the development of CHB [14-16]. Approximately 90% of the people that encounter infection during adulthood are able to clear the virus and develop lifelong protective immunity, while 90% of perinatal infections in contrast result in CHB. Factors determining the risk of development of CHB in adulthood are route of infection, age, dose of inoculum and HLA class II genetic profile of the individual [17-20]. Thus far, it is unknown what factors cause that especially neonates cannot clear the infection.

HBV in itself is a non-cytopathic virus, but the ineffective and persistent immune response in CHB patients induces liver damage [21]. The natural history of CHB follows 4 clinical phases that differ in levels of HBeAg , viral replication, and liver damage as indicated by alanine aminotransferase (ALT). The 4 phases include the immune tolerant (IT) phase, the immune active (IA) phase, inactive carrier (IC) phase and HBeAg-negative (ENEG) CHB phase, also called reactivation phase [8, 22]. The IT phase is characterized by HBeAg positivity, high HBV DNA titers (>20,000 IU/ml) as well as ALT levels within the normal range (<40 IU/L), and the IA phase is also characterized by HBeAg positivity but with low HBV DNA titers and elevated ALT levels as a result of the recognition of HBV by the immune system. After the IA phase seroconversion from HBeAg to anti-HBe antibodies can take place which leads to the control of viral replication and again a reduction of immune damage. The IC phase is therefore characterized by lack of HBeAg, low or undetectable HBV DNA titers, and ALT levels within the normal range. The IC phase can be followed by the ENEG phase, in which viral replication is reactivated. This phase is thus characterized by elevated levels of HBV DNA and ALT, as well as a lack of HBeAg.

Although an effective prophylactic vaccine is available, there is no effective treatment for established infections and the few drugs that are beneficial, often are not accessible in poor countries where the prevalence of HBV infection is highest. Treatment with pegylated interferon α (PEG-IFNα), which has both antiviral and immunomodulatory activity, induces persistent immune control in 20-30% of patients but is associated with severe side effects. Treatment with nucleos(t)ide analogues, which interfere with viral reverse transcriptase, can reduce HBV DNA replication, but has to be given life-long as it does not eliminate HBV cccDNA and is prone to selection of escape mutants [23]. Therefore, there is still a high demand for more effective therapies that induce immune control of viral replication in a more lasting and patients-friendly manner or fully clear the virus. In order to develop such immunomodulatory treatment strategies, it is crucial to better understand why adequate HBV-specific immune responses are lacking in CHB patients.

Antiviral immunity in general

Upon viral infection, the innate immune system forms the first line of defense. The innate immune system involves monocytes, macrophages, natural killer (NK) cells, the complement system, and cytokines. Innate immune cells sample the environment for invading pathogens and recognize these via pattern recognition receptors (PRR). Upon pathogen recognition, the cells become activated and start producing chemokines, to attract other immune cells, as well as antiviral and/or immunoregulatory cytokines, such as interferons (IFNs) [24]. IFNs play a crucial role in the host defense against viral infections as they possess a direct antiviral function by inhibiting cell infection and virus replication, as well as an immunoregulatory function by affecting the activation and cytokine production of immune cells [25]. Different types of IFN exist, which can be divided into three main classes; type I IFNs, including 13 IFN-α homologs, IFN-β and several poorly defined subtypes, type II IFN, i.e. IFN-γ, and type III IFNs, including IFN-λ1, IFN-λ2, IFN-λ3 and IFN-λ4.
In addition, innate recognition can trigger further adaptive immune responses, involving B cells that produce antibodies, $CD8^+$ cytotoxic T lymphocytes (CTL) that can eradicate virus-infected cells, and $CD4^+$ T helper cells that can regulate B cell and CTL activation [26]. In contrast to the innate immune system, which acts in a generic way, the adaptive immune system acts specific and is required for induction of long-lasting and protective immunity.

The role of DCs in antiviral immunity

As stated above, effective antiviral immunity involves the production of antiviral cytokines such as IFNs, and the induction of a virus-specific adaptive immune response to clear virus-infected cells. Dendritic cells (DCs) play an essential role in induction of antiviral immunity because they can both produce high levels of type I and type III IFNs and are the most potent Ag-presenting cells that can activate virus-specific naïve T cells [27-29]. In contrast to other Ag-presenting cells such as macrophages, DCs harbor the unique capacity to migrate from peripheral tissues to lymphoid tissues, allowing efficient communication with LN-resident adaptive immune cells. In the periphery, DCs continuously sample their environment for invading pathogens, including viruses and virus-infected cells. Upon recognition of such foreign antigens by PRR, such as Toll-like receptors (TLRs) and C-type lectins, DCs internalize this material and become activated as a result of signaling cascades emanating from ligand-bound PRRs [30]. DC activation is associated with upregulation of maturation markers and/or co-stimulatory molecules such as CD40, CD80, CD83 and CD86, the production of cytokines and with the migration of DCs from the periphery to the draining lymph nodes. In addition to direct activation by pathogens via PRR ligation, DCs can also be activated indirectly by signals from other immune cells or virus-infected non-immune cells (e.g. by cytokines or surface receptors on these cells) [31].
Concomitant to PRR-triggered DC activation, ingested pathogens or infected cells are processed intracellularly by DCs into peptides and DCs present these peptides on major histocompatibility complexes (MHC) to activate T cells. Peptides presented on MHC class II can activate $CD4^+$ helper T cells (direct presentation) and those on MHC class I can activate cytolytic /IFN-γ-producing $CD8^+$ T cells (cross-presentation) [32].
Optimal activation of T cells by DCs requires recognition of peptides in MHC molecules by the T cell receptor (TCR) (signal 1) combined with co-stimulatory molecules such as CD40

and CD86 (signal 2) and cytokines such as IL-12, IL-4, and IFN-α (signal 3) that polarize T cells towards a specific type of immune response (e.g. antibody mediated/humoral, cytolytic, tolerogenic) [33-36]. Cross-presentation of exogenous antigens in MHC class I is especially important for the defense against viruses that do not infect DCs themselves, such as HBV. It is required for the induction of an effective $CD8^+$ T cells response against such antigens. An extensive overview of the mechanisms underlying MHC class I presentation of viral antigens by DCs is provided in chapter 2.

Activation and skewing of $CD4^+$ T cells in combination with the right cytokines is also important because IL-12-induced T helper 1 (Th1) cells provide adequate help for $CD8^+$ T cell activation by producing cytokines such as IFN-γ and licensing signals such as CD40 [37]. IL-4-induced T helper 2 (Th2) cells in contrast will skew the immune response more towards the production of antibodies. Similarly, other T helper cells including Th17, follicular helper T cells (Tfh) or regulatory T cells (Treg) may be induced by IL-17, IL-21 and IL-10/TGF-β, respectively, that each have a different effect on the resulting immune response [38]. A wrong balance between T cell skewing cytokines could divert an immune response into the wrong direction. Moreover, when DCs are not fully mature and lack sufficient T cell activation/skewing signals, T cell stimulation can also result in induction of tolerogenic/regulatory T cells instead of immunogenic T cells [39].

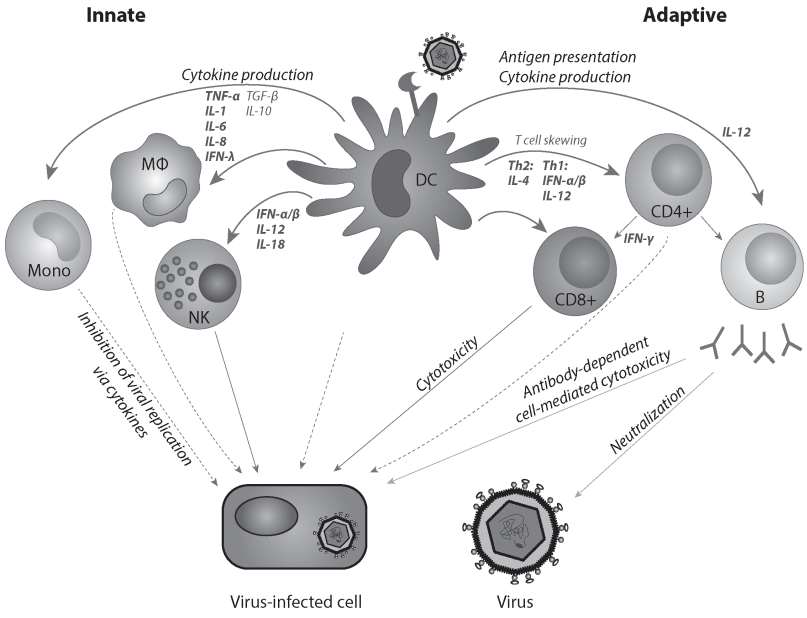

Figure 2. The role of DCs in induction of antiviral immunity.

Upon recognition of a virus, DCs become activated and start producing cytokines that can inhibit viral replication, and attract as well as activate (***bold***) or inhibit (*regular*) cells of the innate and adaptive immune system, including monocytes, macrophages, NK cells and B cells. In addition, DCs can process and present antigens in MHC molecules, which together with cytokine production can induce $CD4^+$ and $CD8^+$ T cell responses. These activated immune cells can in turn also secrete cytokines that inhibit viral replication. Furthermore, activated B cells produce antibodies that neutralize the virus and activated T cells and NK cells can kill virus-infected cells via induction of apoptosis.
Mono, monocyte; Mϕ, macrophage; NK, NK cell; DC, dendritic cell; CD8+, CD8+ T cell; CD4+, CD4+ T cell; B, B cell.

Besides induction of T cell responses, DCs can interact with and activate other immune cells, including B cells, NK cells, monocytes and macrophages (Figure 2). Furthermore, DCs regulate the magnitude and quality of immune responses via production of pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6 and IL-8 or anti-inflammatory cytokines such as IL-10 and TGF-β [40]. Because of this capacity to bridge and control innate and adaptive immune responses, DCs play an essential role in induction of virus-specific immunity.

Dendritic cell subsets

Human DCs consists of several subsets that differ in ontogeny, localization, phenotype and specialized immune functions [41, 42]. The blood DC subsets can be subdivided into two main categories; $CD11c^+$ myeloid DCs (mDCs) and $BDCA2^+BDCA4^+CD123^+$ plasmacytoid DCs (pDCs). pDCs are mainly found in peripheral blood and lymphoid organs and are characterized by expression of CD123, CD303 (BDCA2) and CD304 (BDCA4). pDCs are known to produce high amounts of antiviral type I interferon (IFN) upon endosomal TLR7 or TLR9 ligation by nucleic acid derivatives [43]. mDCs are located in peripheral blood and peripheral and lymphoid tissues and comprise two main populations: $CD1c^+$ ($BDCA1^+$) DCs and the more recently discovered $CD141^{hi}$($BDCA3^{hi}$)$CLEC9A^+XCR1^+$ DCs (further referred to as $BDCA3^+$ DCs). Compared to pDCs, mDCs are better inducers of T cell activation. $BDCA1^+$ DCs are known for their high production of IL-12 in response to TLR ligation [44]. $BDCA3^+$ DCs are the most potent producers of IFN-λ in response to viruses or the synthetic RNA polyinosinic:polycyticylic acid (polyI:C) [45-48]. In addition, $BDCA3^+$ DCs are superior at cross-presentation of cell-derived Ag to $CD8^+$ T cells [49-53]. Because of these capacities $BDCA3^+$ DCs are thought to be of importance for anti-viral responses. The presumed murine equivalents of human $BDCA3^+$ DCs and the main cross-presenting cells in mice are the $CD8\alpha^+$ DCs and $CD103^+$ DCs [54, 55] which have been shown to be crucial for generating optimal virus-specific $CD8^+$ T cell responses to for example herpes simplex virus, influenza virus, vaccinia virus and West Nile virus [56-59]. In addition, lymph node-resident $CD8\alpha^+$ DC have been shown to uniquely function as a platform for activation of antiviral CTL and the associated delivery of $CD4^+$ T cell help [60, 61]. Compared to other DC subsets, $BDCA3^+$ DCs are very rare, and were more recently discovered and are thus least well studied. In chapter 3 it is extensively described what is currently known of the developmental, phenotypic and functional characteristics of $BDCA3^+$ DCs.

Dendritic cells in HBV infection

As outlined above, DCs are key players in induction of adaptive immunity. Consequently, these cells are expected to also play an important role in induction of HBV-specific immunity. In the liver, $BDCA1^+$ DCs and pDCs are mainly located at the portal tract and central veins where they sample the environment for invasive pathogens [62]. Upon capture of antigens, DCs can translocate to the space of Disse and migrate to the secondary lymphoid organs [63, 64]. As the liver is continuously exposed to foreign antigens that are present in blood coming from the gastrointestinal tract, it has a tolerogenic microenvironment to prevent chronic inflammation. In line with this, intrahepatic DCs have been shown to be less immunogenic than their counterparts in blood [65, 66].

Several studies have previously assessed the frequency and function of BDCA1$^+$ DCs and pDCs in HBV-infected patients [67]. This has revealed that the frequencies of both BDCA1$^+$ DCs and pDCs in the blood of CHB are either unaffected or decreased, depending on the phase of disease. Both DC subtypes however, were found at increased numbers in the liver of CHB patients. [68-73]. This is not completely surprising as it can be expected that the frequency of intrahepatic DC populations will be affected by liver inflammation leading to the increased recruitment of immune cells to the liver and/or the migration of DCs from the liver to secondary lymphoid organs.
DCs themselves are not permissive to HBV infection [74, 75]. However, DCs containing HBsAg have been detected in liver and blood of HBV patients, indicating that DCs can interact with, and ingest viral material [74, 76]. It remains to be determined whether HBV virions or viral proteins activate DCs and if so, via which receptors this occurs. Interaction between HBV and DCs could also impair the function of DCs. Functional analysis of circulating BDCA1$^+$ DCs showed that in CHB patients these cells are impaired in their capacity to mature, produce TNF-α and activate T cells [70, 77, 78]. For pDCs it was demonstrated that IFN-α-production was impaired in CHB patients [68, 70, 79, 80]. This reduced pDC function may be (partially) due to an effect of the virus or viral proteins, because HBV virions, HBsAg and HBeAg reduced the IFN-α production by pDCs in vitro [79, 81, 82]. HBV and HBsAg were shown to inhibit IFN-α production by interfering with TLR9-induced mTOR-mediated S6 phosphorylation that in turn drives phosphorylation of IRF7 and IFN-α production [79, 83]. Because it is difficult to obtain liver tissue for research of intrahepatic DCs, most studies on DC function in HBV infection so far were performed with peripheral blood DCs and were restricted to BDCA1$^+$ DCs and pDCs. DCs have been characterized in the liver of healthy controls, but except for HBsAg uptake, no information is available on intrahepatic DC function in HBV infection [76]. Furthermore, as outlined above, BDCA3$^+$ DCs may be of particular importance in anti-viral responses, yet BDCA3$^+$ DCs have been least well studied in general and their function in HBV infection is still unknown. It remains to be determined whether HBV also interferes with BDCA3$^+$ DC function. More insight into the frequency, distribution and function of BDCA3$^+$ DCs in healthy donors as well as CHB patients may help to better understand their role in intrahepatic immune regulation both in steady state and HBV infection. Moreover, knowledge on the local presence and functional state of these cells may aid the development of immunotherapeutic strategies exploiting these cells to improve immune responses to HBV (see below).

Type I and type III IFNs in HBV infection

IFNs play a key role in the induction and regulation of antiviral responses. These antiviral cytokines restrict viral replication and infection of neighbouring cells by induction of interferon-stimulated genes (ISGs), and possess immunoregulatory capacities as they can stimulate responses of several immune cells, such as DCs, NK cells and Th1 cells. Type I IFNs have been thoroughly studied and their antiviral and immunoregulatory characteristics are well known. HBV infection by itself does not induce production of type I IFNs and the capacity of IFN-α to combat HBV is the rationale for the current use of PEG-IFN-α as therapy for CHB infection.
Like type I IFNs, type III IFNs (IFN-λs) are important antiviral cytokines with antiviral activity against multiple viruses, including influenza virus, hepatitis C virus (HCV), human

immunodeficiency virus (HIV), encephalomyocarditis virus, vesicular stomatitis virus (VSV), cytomegalovirus (CMV) and herplexsimplex virus (HSV) [84-90]. Moreover, IFN-λ has a crucial role in the defense against rotavirus and West Nile virus [91-93] and is the major IFN type induced upon infection with influenza virus [94]. Importantly, IFN-λ can also inhibit HBV replication, indicating IFNλ may also have therapeutic potential for HBV [88, 95, 96]. Compared to type I and type II IFNs, knowledge on the regulation of IFN-λ production is still limited. Recent studies have shown that the promotor regions of IFN-λ1-3 contain binding elements for IRF3, IRF7, and NFκB, and that these transcription factors are indeed involved in the regulation of IFN-λ1-3 production [97-103]. These studies on IFN-λ-regulating pathways have been performed with several cell types, such as hepatocytes, HEK293 cells, monocyte-derived DCs (moDCs) and colon epithelial cells. As said however, BDCA3$^+$ DCs are the major producers of IFN-λ upon recognition of synthetic dsRNA or viruses [45, 46]. Yet, the molecular pathways underlying IFN-λ regulation in primary DCs have not been studied. Insight into these regulatory mechanisms will help to better understand by what properties BDCA3$^+$ DCs excel in IFN-λ production.

The IFN-λ family consists of four subtypes, including IFN-λ1 (IL-29), IFN-λ2 (IL-28A), IFN-λ3 (IL-28B) and IFN-λ4 [104, 105]. IFN-λ4 is only expressed in individuals who have a frameshift variant in their genome, resulting in a novel gene that encodes IFN-λ4 [106].

All IFN-λ subtypes signal via the IFN-λ receptor, which forms a complex of two subunits; the IL-28 receptor α (IL-28Rα) chain and IL-10Rβ chain. Signaling via this receptor induces, like IFN-α signaling, activation of the JAK-STAT and MAPK pathways and subsequent induction of ISGs [107]. The ISGs induced by type III IFNs are comparable to those induced by type I IFNs, yet, type I and III IFN differ in expression kinetics and bioactivity, which is mostly due to the restricted expression of the IFN-λ receptor. [108-110]. In contrast to the IFN-α receptor, which is expressed by most cells, expression of the IFN-λ receptor is present only on a limited group of cell types, including epithelial cells, B cells, pDCs, macrophages, neutrophils and hepatocytes, and the latter points towards an important role for IFN-λ in the liver [105, 110-113].

In addition to direct antiviral activity by the induction of ISGs, IFN-λ also has important immunoregulatory properties, including effects on macrophages, NK cells, B cells, neutrophils, pDCs and T cells (Figure 3) [107, 114, 115]. IFN-λ has been shown to stimulate IFN-γ production by NK cells via induction of IL-12 production by macrophages, to augment TLR-mediated function of B cells, and to enhance the anti-viral response of pDCs by increasing IFN-α and IFN-λ production [116-118]. In addition, IFN-λ promotes production of the Th1 cytokine IFN-γ and reduces production of Th2 cytokines IL-4, IL-5 and IL-13, indicating that IFN-λ supports skewing towards a Th1 response, and in this way may also promote effective antiviral immunity [86, 119].

Given these antiviral and immunoregulatory functions, IFN-λ may aid the induction of HBV-specific immunity.

Recently it was shown HBV induces IFN-λ production rather than IFN-α by primary human hepatocytes via activation of the intracellular RNA receptor RIG-I [120]. However, despite the potential of HBV to induce IFN-λ in vitro, IFN-λ transcripts were hardly detectable in HBV-infected livers, and serum of CHB patients contains IFN-λ levels that are comparable to those of control individuals [121-125]. HBV is considered to be a stealth virus as it does not seem to induce type I IFN responses upon infection [126]. These data on IFN-λ now suggest that HBV intervenes not only with induction of type I IFN responses, but also with the induction of IFN-λ. Indeed, it was recently shown that HBV is able to actively inhibit IFN-λ

production in hepatocytes [120, 127]. When present however, IFN-λ may help to combat HBV infection, given that SNPs near the IL28B gene are associated with better response to PEG-IFN-α therapy in HBeAg-positive CHB patients [128]. Nevertheless, the link between these SNPs and response to PEG-IFN-α therapy has not been unraveled yet.
In a recent clinical trial patients have now also been treated directly with PEG-IFN-λ. This study showed that, in HBeAg-positive patients, during the first 24 weeks of treatment, PEG-IFN-λ induced greater reduction in HBV DNA and HBsAg levels than was seen with PEG-IFN-α. However, at end of treatment (48 weeks), serologic and virologic responses were comparable for PEG-IFN-λ and PEG-IFN-α [129]. As expected from expression of the IFN-λ receptor by only a limited group of cell types however, the rates of interferon-associated side effects of PEG-IFN-λ were lower than those of PEG-IFN-α, indicating IFN-λ may have a therapeutic advantage.

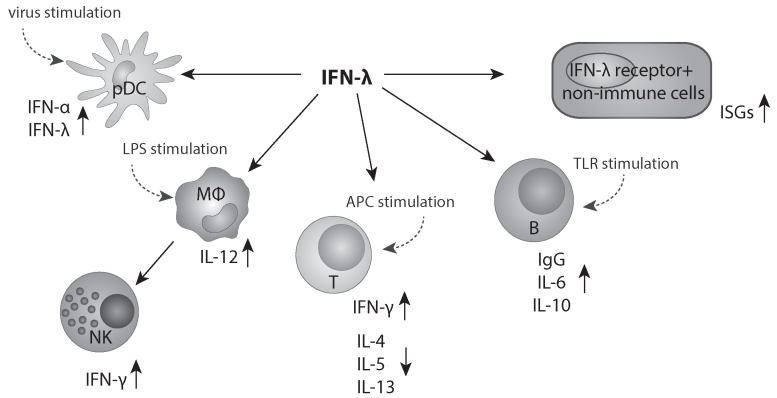

Figure 3. Effects of IFN-λ on immune cells and IFN-λ receptor-expressing non-immune cells.
IFN-λ exhibits direct antiviral effects, via induction of ISGs, and immunoregulatory effects. Depicted are the stimulatory (↑) or inhibitory (↓) effects of IFN-λ. NK, NK cell; pDC, plasmacytoid DC; Mϕ, macrophage; T, T cell; B, B cell.

Treatment opportunities for chronic hepatitis B currently under investigation

Currently, there are several new therapies proposed based on intervention with different specific stages of the HBV life-cycle or the expression of viral proteins, such as intervention with HBV entry into hepatocytes by Myrcludex B [130, 131], cleavage of cccDNA upon activation of the CRISPR-Cas9 system, cleavage of cccDNA upon activation of lymphotoxin beta receptor (LTβR) and APOBEC3 proteins [132, 133], and inhibition of HBsAg as well as HBcAg production by silencing RNA (siRNA; Figure 4) [134]. However, it remains challenging to reach the ultimate goal, complete elimination of HBV cccDNA, by these approaches.
The loss of cccDNA can also be achieved by immune-mediated killing of cccDNA-containing hepatocytes. As HBV-specific T cells together with HBV-specific antibodies can recognize and eliminate infected hepatocytes that express or present viral antigens on their surface, immunotherapy aiming to restore anti-viral B and T cell responses therefore also represents a promising treatment strategy [135-137]. The potency of the immune system to fight HBV is illustrated by the observation that upon bone marrow transplantation, CHB patients mounted effective HBV-specific T cell responses as well as HBsAg-specific antibodies, and

were able to clear the infection [138-140]. Unfortunately, this strategy is not being used widely as it is a complex and expensive procedure that is difficult to implement in developing countries and is not without risk. Therefore, alternative immunotherapeutic approaches for restoring HBV-specific immunity are currently under investigation (Figure 4).

One approach is based on the observation that in CHB patients HBV-specific T cells are functionally exhausted and express inhibitory molecules such as PD-1, CTLA-4 and Tim-3 which may impair T cell function upon ligation by their agonists on antigen presenting cells or infected hepatocytes [141-144]. Blocking these inhibitory receptors by monoclonal antibodies has been shown to enhance the function of HBV-specific T cells *in vitro* and therefore is considered as a viable approach to restore T cell function in CHB patients [145].

Another approach under investigation is restoration of virus-specific T cell immunity by adoptive T cell therapy. T cells from patients are genetically modified so that they express an HBV-specific T cell receptor (TCR) that can recognize and kill virus-infected cells [146-148]. *In vivo* studies showed that adoptive transfer of such T cells can reduce HBV replication and HBsAg levels in a patient with HBV-related HCC [148, 149]. However, the disadvantage of this technique is that it depends on autologous T cells and can thus not be generically applied to all patients, which again prohibits its use in developmental countries.

Another opportunity currently exploited is to boost antiviral immune responses by the activation of Toll-like receptor 7 (TLR7). For this treatment TLR7 agonists are administrated that activate TLR7, which is predominantly expressed by pDCs and B cells, and thereby induce an endogenous type I IFN response that has direct antiviral activity and augments innate and adaptive immune responses. Recent studies have shown that administration of the TLR7 agonist GS-9620 in chronically HBV-infected chimpanzees and woodchuck hepatitis virus (WHV)-infected woodchucks (an animal model to study infection of hepadnaviruses) resulted in reduced HBV DNA levels [150, 151]. However, subsequent administration of a low dose of the TLR7 agonist in CHB patients did not induce clinically significant changes in HBsAg or HBV DNA levels [152]. Further research is required to determine the feasibility of this treatment in CHB patients.

Many studies have tried to boost HBV-specific immunity by therapeutic vaccination with nucleic acids encoding viral proteins [135]. These studies showed that HBsAg-encoding DNA vaccines can stimulate immune responses and reduce HBV replication in some patients, but overall have limited virological and clinical efficacy [153, 154].

As CHB is associated with T cell hyporesponsiveness, and reduction of viral load by antiviral treatment has been shown to enhance HBV-specific T cell responses [155], administration of antiviral drugs prior to therapeutic vaccination was thus proposed to optimize the efficacy of immunotherapeutic strategies. Indeed, vigorous T cell response and sustained immunological control of chronic hepadnaviral infection was observed in woodchucks chronically infected with WHV that had received DNA prime-adenovirus boost vaccination and the nucleoside analogue entecavir [156]. Likewise, injection of DNA vaccines encoding multiple HBV proteins and IL-12 in combination with lamivudine treatment induced a virological response and vigorous HBV-specific memory T cell responses that persisted for 40 weeks after vaccination in half of the treated patients [157].

Finally, efforts are made to exploit DCs to fight HBV. Mouse studies showed that DC-stimulatory signals alone, i.e. stimulation of intrahepatic DCs by TLR ligands or CD40L, already enhanced virus-specific CTL responses [158, 159]. Vaccination with HBsAg resulted in enhanced HBV-specific immune responses and lower HBV DNA titers in some patients [160-163]. In addition, clinical trials have been performed, in which HBV peptide-pulsed

DCs alone or combined with antiviral drugs were administered to patients [164-166]. This treatment resulted in suppression of virus replication and improved HBeAg seroconversion rates.

Given the central role of DCs in immunity, these cells represent an attractive target for therapeutic vaccines. Therefore, protein/peptide vaccines are being developed that are based on *in vivo* (cross-)presentation by DCs [167]. These vaccines target DCs with HBV proteins or peptides in combination with proper stimulatory signals to boost DC activation and antigen presentation, resulting in induction of IFN responses as well as HBV-specific innate and adaptive immune responses.

As BDCA3$^+$ DCs possess excellent cross-presenting and IFN-λ-producing properties, delivery of TLR3 ligands combined with HBV peptide or antigen to these cells may lead to their activation and subsequent initiation of HBV-specific CTL responses and IFN-λ production. Targeting the BDCA3$^+$ DCs in particular may thus be an interesting novel therapeutic strategy for HBV. For development of efficient DC-targeted vaccines against CHB however it is essential to know whether (BDCA3$^+$) DCs are present in livers and peripheral blood of CHB patients and what the functional state of these cells is.

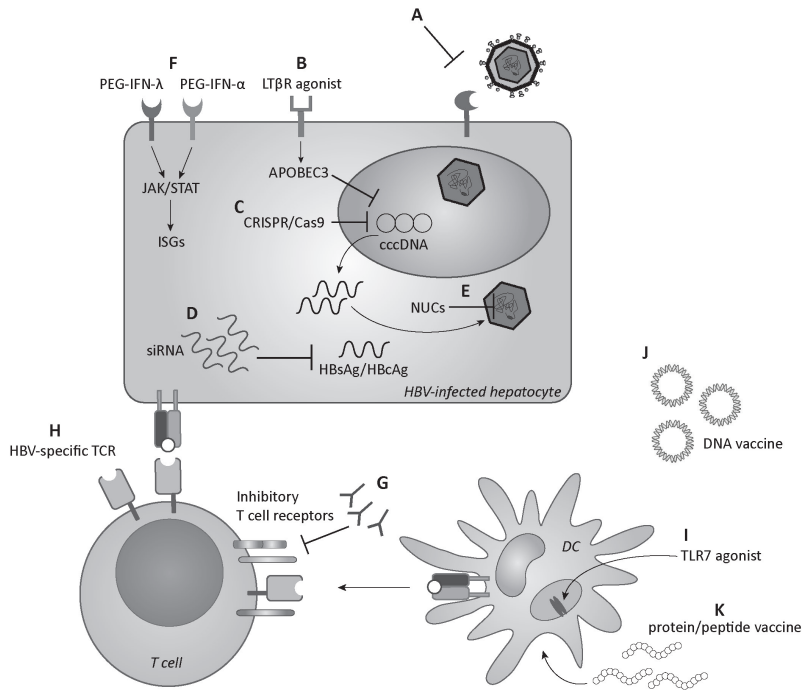

Figure 4. Therapeutic strategies for chronic hepatitis B.

Depicted are the various strategies for the treatment of chronic hepatitis B that are available or under investigation. These strategies include: A, inhibition of viral entry; B, cleavage of cccDNA upon activation of the lymphotoxin beta receptor (LTβR) and APOBEC3 proteins; C, cleavage of cccDNA upon activation of the CRISPR/Cas9 system; D, inhibition of HBsAg and HBcAg production by siRNAs; E, inhibition of HBV DNA replication by nucleot(s)ide analogs (NUCs); F, induction of ISGs and stimulation of immune response by PEG-IFN-λ or PEG-IFN-α; G, blocking of inhibitory T cell receptors by monoclonal antibodies; H, genetic modification of T cells by T cell receptor gene transfer; I, administration of TLR7 agonists; J, administration of DNA-based vaccines; K, administration of protein/peptide-based vaccines.

Aims and outline of the thesis

DCs are key players in induction of anti-viral immunity and therefore this thesis aims to better understand how DCs, and specifically BDCA3$^+$ DCs, are affected by or may help to clear chronic HBV infection.
In **chapter 2**, the different mechanisms employed by human DCs to facilitate MHC class I presentation of viral antigens and subsequent anti-viral CD8$^+$ T cell induction are discussed. This overview shows that cross-presentation is a highly efficient mechanism to initiate virus-specific immunity.
Compared to other DC subsets, BDCA3$^+$ DCs were discovered more recently and are less well studied. To get more insight into this cell type, **chapter 3** provides a summary of the developmental, phenotypic and functional characteristics of BDCA3$^+$ DCs. As the frequencies of BDCA3$^+$ DCs in the body are very low, assessment of these cells is challenging. Therefore, we attempted to generate BDCA3$^+$ DCs from CD34$^+$ progenitor cells *in vitro* in **chapter 4**. Although we were able to generate CD34$^+$-derived BDCA3$^+$ DCs that produce high levels of IFN-λ, subsequent gene expression analysis revealed that they do not completely resemble BDCA3$^+$CLEC9A$^+$ DCs *in vivo*. Nevertheless, CD34$^+$-derived BDCA3$^+$ DCs represent a valuable model to study regulation of IFN-λ production and BDCA3 expression by DCs.
BDCA3 is also (partly) expressed by other DC subsets than the typical BDCA3^{hi}CLEC9A$^+$ DCs, including BDCA1$^+$ DCs. However, whether differences exist between BDCA3$^+$ and BDCA3$^-$ cells within the BDCA1$^+$ DC population remains unknown. In **chapter 5**, we therefore studied the difference between blood BDCA3$^+$BDCA1$^+$ DCs and BDCA3$^-$BDCA1$^+$ DCs, which indicated that BDCA3$^+$BDCA1$^+$ DCs have a more activated status and produce higher cytokine levels compared to BDCA1$^+$ DCs.
In contrast to regulation of type I IFN production, regulation of type III IFN production is poorly understood. **Chapter 6** describes the study of regulation of IFN-λ production by the major IFN-λ-producing cells, BDCA3$^+$ DCs, and reveals that this process involves the canonical NFκB pathway and the PI3K-PKB-mTOR pathway.
BDCA3$^+$ DCs are considered to be important in regulating antiviral immune responses because of high IFN-λ production and efficient antiviral cytotoxic T cell induction. However, the presence and function of these cells in HBV-infected patients remains unknown. **Chapter 7** describes the frequency and function of BDCA3$^+$ DCs in liver and blood of CHB patients and control individuals. The data show that BDCA3$^+$ DCs are abundantly present in HBV-infected livers. However, blood BDCA3$^+$ DCs from CHB patients are impaired in their capacity to mature and produce IFN-λ compared to controls. This impaired function may be caused by the virus or viral proteins since we show that HBsAg inhibits IFN-λ production by BDCA3$^+$ DCs *in vitro* via its effect on other immune cells. One of the cell types of which the function is directly influenced by HBsAg are BDCA1$^+$ DCs. The HBsAg receptors on BDCA1$^+$ DCs that affect the function of these cells, however, are unknown. In **chapter 8** the interaction between HBsAg and BDCA1$^+$ DCs and its effect on BDCA1$^+$ DC function was investigated, which demonstrated that BDCA1$^+$ DCs are activated by HBsAg and that this activation depends on the presence of (soluble) CD14.
A detailed discussion and interpretation of results is given in **chapter 9**.

References

1. Schweitzer, A., Horn, J., Mikolajczyk, R. T., Krause, G., Ott, J. J. (2015) Estimations of worldwide prevalence of chronic hepatitis B virus infection: a systematic review of data published between 1965 and 2013. Lancet 386, 1546-55.
2. Dandri, M. and Locarnini, S. (2012) New insight in the pathobiology of hepatitis B virus infection. Gut 61 Suppl 1, i6-17.
3. Ganem, D. and Prince, A. M. (2004) Hepatitis B virus infection--natural history and clinical consequences. N Engl J Med 350, 1118-29.
4. Lucifora, J., Arzberger, S., Durantel, D., Belloni, L., Strubin, M., Levrero, M., Zoulim, F., Hantz, O., Protzer, U. (2011) Hepatitis B virus X protein is essential to initiate and maintain virus replication after infection. J Hepatol 55, 996-1003.
5. Yan, H., Zhong, G., Xu, G., He, W., Jing, Z., Gao, Z., Huang, Y., Qi, Y., Peng, B., Wang, H., Fu, L., Song, M., Chen, P., Gao, W., Ren, B., Sun, Y., Cai, T., Feng, X., Sui, J., Li, W. (2012) Sodium taurocholate cotransporting polypeptide is a functional receptor for human hepatitis B and D virus. Elife 1, e00049.
6. Ni, Y., Lempp, F. A., Mehrle, S., Nkongolo, S., Kaufman, C., Falth, M., Stindt, J., Koniger, C., Nassal, M., Kubitz, R., Sultmann, H., Urban, S. (2014) Hepatitis B and D viruses exploit sodium taurocholate co-transporting polypeptide for species-specific entry into hepatocytes. Gastroenterology 146, 1070-83.
7. Nassal, M. (2015) HBV cccDNA: viral persistence reservoir and key obstacle for a cure of chronic hepatitis B. Gut 64, 1972-84.
8. Rehermann, B. and Nascimbeni, M. (2005) Immunology of hepatitis B virus and hepatitis C virus infection. Nat Rev Immunol 5, 215-29.
9. Seeger, C. and Mason, W. S. (2000) Hepatitis B virus biology. Microbiol Mol Biol Rev 64, 51-68.
10. Whalley, S. A., Murray, J. M., Brown, D., Webster, G. J., Emery, V. C., Dusheiko, G. M., Perelson, A. S. (2001) Kinetics of acute hepatitis B virus infection in humans. J Exp Med 193, 847-54.
11. Thimme, R., Wieland, S., Steiger, C., Ghrayeb, J., Reimann, K. A., Purcell, R. H., Chisari, F. V. (2003) CD8(+) T cells mediate viral clearance and disease pathogenesis during acute hepatitis B virus infection. J Virol 77, 68-76.
12. Maini, M. K., Boni, C., Ogg, G. S., King, A. S., Reignat, S., Lee, C. K., Larrubia, J. R., Webster, G. J., McMichael, A. J., Ferrari, C., Williams, R., Vergani, D., Bertoletti, A. (1999) Direct ex vivo analysis of hepatitis B virus-specific CD8(+) T cells associated with the control of infection. Gastroenterology 117, 1386-96.
13. Penna, A., Chisari, F. V., Bertoletti, A., Missale, G., Fowler, P., Giuberti, T., Fiaccadori, F., Ferrari, C. (1991) Cytotoxic T lymphocytes recognize an HLA-A2-restricted epitope within the hepatitis B virus nucleocapsid antigen. J Exp Med 174, 1565-70.
14. Rehermann, B., Fowler, P., Sidney, J., Person, J., Redeker, A., Brown, M., Moss, B., Sette, A., Chisari, F. V. (1995) The cytotoxic T lymphocyte response to multiple hepatitis B virus polymerase epitopes during and after acute viral hepatitis. J Exp Med 181, 1047-58.
15. Bertoletti, A. and Ferrari, C. (2012) Innate and adaptive immune responses in chronic hepatitis B virus infections: towards restoration of immune control of viral infection. Gut 61, 1754-64.
16. Boonstra, A., Woltman, A. M., Janssen, H. L. (2008) Immunology of hepatitis B and hepatitis C virus infections. Best Pract Res Clin Gastroenterol 22, 1049-61.
17. Asabe, S., Wieland, S. F., Chattopadhyay, P. K., Roederer, M., Engle, R. E., Purcell, R. H., Chisari, F. V. (2009) The size of the viral inoculum contributes to the outcome of hepatitis B virus infection. J Virol 83, 9652-62.
18. Publicover, J., Gaggar, A., Nishimura, S., Van Horn, C. M., Goodsell, A., Muench, M. O., Reinhardt, R. L., van Rooijen, N., Wakil, A. E., Peters, M., Cyster, J. G., Erle, D. J., Rosenthal,

P., Cooper, S., Baron, J. L. (2013) Age-dependent hepatic lymphoid organization directs successful immunity to hepatitis B. J Clin Invest 123, 3728-39.

19. Kamatani, Y., Wattanapokayakit, S., Ochi, H., Kawaguchi, T., Takahashi, A., Hosono, N., Kubo, M., Tsunoda, T., Kamatani, N., Kumada, H., Puseenam, A., Sura, T., Daigo, Y., Chayama, K., Chantratita, W., Nakamura, Y., Matsuda, K. (2009) A genome-wide association study identifies variants in the HLA-DP locus associated with chronic hepatitis B in Asians. Nat Genet 41, 591-5.
20. Lok, A. S., McMahon, B. J., Practice Guidelines Committee, A. A. f. t. S. o. L. D. (2001) Chronic hepatitis B. Hepatology 34, 1225-41.
21. Trepo, C., Chan, H. L., Lok, A. (2014) Hepatitis B virus infection. Lancet 384, 2053-63.
22. European Association For The Study Of The, L. (2012) EASL clinical practice guidelines: Management of chronic hepatitis B virus infection. J Hepatol 57, 167-85.
23. Liang, T. J., Block, T. M., McMahon, B. J., Ghany, M. G., Urban, S., Guo, J. T., Locarnini, S., Zoulim, F., Chang, K. M., Lok, A. S. (2015) Present and future therapies of hepatitis B: From discovery to cure. Hepatology.
24. Akira, S., Uematsu, S., Takeuchi, O. (2006) Pathogen recognition and innate immunity. Cell 124, 783-801.
25. McNab, F., Mayer-Barber, K., Sher, A., Wack, A., O'Garra, A. (2015) Type I interferons in infectious disease. Nat Rev Immunol 15, 87-103.
26. Iwasaki, A. and Medzhitov, R. (2015) Control of adaptive immunity by the innate immune system. Nat Immunol 16, 343-53.
27. Banchereau, J., Briere, F., Caux, C., Davoust, J., Lebecque, S., Liu, Y. J., Pulendran, B., Palucka, K. (2000) Immunobiology of dendritic cells. Annu Rev Immunol 18, 767-811.
28. Steinman, R. M. (2008) Dendritic cells in vivo: a key target for a new vaccine science. Immunity 29, 319-24.
29. Merad, M., Sathe, P., Helft, J., Miller, J., Mortha, A. (2013) The dendritic cell lineage: ontogeny and function of dendritic cells and their subsets in the steady state and the inflamed setting. Annu Rev Immunol 31, 563-604.
30. Reis e Sousa, C. (2004) Toll-like receptors and dendritic cells: for whom the bug tolls. Semin Immunol 16, 27-34.
31. Joffre, O., Nolte, M. A., Sporri, R., Reis e Sousa, C. (2009) Inflammatory signals in dendritic cell activation and the induction of adaptive immunity. Immunol Rev 227, 234-47.
32. ten Broeke, T., Wubbolts, R., Stoorvogel, W. (2013) MHC class II antigen presentation by dendritic cells regulated through endosomal sorting. Cold Spring Harb Perspect Biol 5, a016873.
33. Schiavoni, G., Mattei, F., Gabriele, L. (2013) Type I Interferons as Stimulators of DC-Mediated Cross-Priming: Impact on Anti-Tumor Response. Front Immunol 4, 483.
34. O'Sullivan, B. and Thomas, R. (2003) CD40 and dendritic cell function. Crit Rev Immunol 23, 83-107.
35. Valenzuela, J., Schmidt, C., Mescher, M. (2002) The roles of IL-12 in providing a third signal for clonal expansion of naive CD8 T cells. J Immunol 169, 6842-9.
36. Curtsinger, J. M. and Mescher, M. F. (2010) Inflammatory cytokines as a third signal for T cell activation. Curr Opin Immunol 22, 333-40.
37. Castellino, F. and Germain, R. N. (2006) Cooperation between CD4+ and CD8+ T cells: when, where, and how. Annu Rev Immunol 24, 519-40.
38. Swain, S. L., McKinstry, K. K., Strutt, T. M. (2012) Expanding roles for CD4(+) T cells in immunity to viruses. Nat Rev Immunol 12, 136-48.
39. Curtsinger, J. M., Lins, D. C., Mescher, M. F. (2003) Signal 3 determines tolerance versus full activation of naive CD8 T cells: dissociating proliferation and development of effector function. J Exp Med 197, 1141-51.

40. Blanco, P., Palucka, A. K., Pascual, V., Banchereau, J. (2008) Dendritic cells and cytokines in human inflammatory and autoimmune diseases. Cytokine Growth Factor Rev 19, 41-52.

41. Reynolds, G. and Haniffa, M. (2015) Human and Mouse Mononuclear Phagocyte Networks: A Tale of Two Species? Front Immunol 6, 330.

42. Vu Manh, T. P., Bertho, N., Hosmalin, A., Schwartz-Cornil, I., Dalod, M. (2015) Investigating Evolutionary Conservation of Dendritic Cell Subset Identity and Functions. Front Immunol 6, 260.

43. Liu, Y. J. (2005) IPC: professional type 1 interferon-producing cells and plasmacytoid dendritic cell precursors. Annu Rev Immunol 23, 275-306.

44. Kadowaki, N., Ho, S., Antonenko, S., Malefyt, R. W., Kastelein, R. A., Bazan, F., Liu, Y. J. (2001) Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens. J Exp Med 194, 863-9.

45. Lauterbach, H., Bathke, B., Gilles, S., Traidl-Hoffmann, C., Luber, C. A., Fejer, G., Freudenberg, M. A., Davey, G. M., Vremec, D., Kallies, A., Wu, L., Shortman, K., Chaplin, P., Suter, M., O'Keeffe, M., Hochrein, H. (2010) Mouse CD8alpha+ DCs and human BDCA3+ DCs are major producers of IFN-lambda in response to poly IC. J Exp Med 207, 2703-17.

46. Nizzoli, G., Krietsch, J., Weick, A., Steinfelder, S., Facciotti, F., Gruarin, P., Bianco, A., Steckel, B., Moro, M., Crosti, M., Romagnani, C., Stolzel, K., Torretta, S., Pignataro, L., Scheibenbogen, C., Neddermann, P., De Francesco, R., Abrignani, S., Geginat, J. (2013) Human CD1c+ dendritic cells secrete high levels of IL-12 and potently prime cytotoxic T-cell responses. Blood 122, 932-42.

47. Zhang, S., Kodys, K., Li, K., Szabo, G. (2013) Human type 2 myeloid dendritic cells produce interferon-lambda and amplify interferon-alpha in response to hepatitis C virus infection. Gastroenterology 144, 414-425 e7.

48. Yoshio, S., Kanto, T., Kuroda, S., Matsubara, T., Higashitani, K., Kakita, N., Ishida, H., Hiramatsu, N., Nagano, H., Sugiyama, M., Murata, K., Fukuhara, T., Matsuura, Y., Hayashi, N., Mizokami, M., Takehara, T. (2013) Human blood dendritic cell antigen 3 (BDCA3)(+) dendritic cells are a potent producer of interferon-lambda in response to hepatitis C virus. Hepatology 57, 1705-15.

49. Jongbloed, S. L., Kassianos, A. J., McDonald, K. J., Clark, G. J., Ju, X., Angel, C. E., Chen, C. J., Dunbar, P. R., Wadley, R. B., Jeet, V., Vulink, A. J., Hart, D. N., Radford, K. J. (2010) Human CD141+ (BDCA-3)+ dendritic cells (DCs) represent a unique myeloid DC subset that cross-presents necrotic cell antigens. J Exp Med 207, 1247-60.

50. Poulin, L. F., Salio, M., Griessinger, E., Anjos-Afonso, F., Craciun, L., Chen, J. L., Keller, A. M., Joffre, O., Zelenay, S., Nye, E., Le Moine, A., Faure, F., Donckier, V., Sancho, D., Cerundolo, V., Bonnet, D., Reis e Sousa, C. (2010) Characterization of human DNGR-1+ BDCA3+ leukocytes as putative equivalents of mouse CD8alpha+ dendritic cells. J Exp Med 207, 1261-71.

51. Crozat, K., Guiton, R., Contreras, V., Feuillet, V., Dutertre, C. A., Ventre, E., Vu Manh, T. P., Baranek, T., Storset, A. K., Marvel, J., Boudinot, P., Hosmalin, A., Schwartz-Cornil, I., Dalod, M. (2010) The XC chemokine receptor 1 is a conserved selective marker of mammalian cells homologous to mouse CD8alpha+ dendritic cells. J Exp Med 207, 1283-92.

52. Bachem, A., Guttler, S., Hartung, E., Ebstein, F., Schaefer, M., Tannert, A., Salama, A., Movassaghi, K., Opitz, C., Mages, H. W., Henn, V., Kloetzel, P. M., Gurka, S., Kroczek, R. A. (2010) Superior antigen cross-presentation and XCR1 expression define human CD11c+CD141+ cells as homologues of mouse CD8+ dendritic cells. J Exp Med 207, 1273-81.

53. Chiang, M. C., Tullett, K. M., Lee, Y. S., Idris, A., Ding, Y., McDonald, K. J., Kassianos, A., Leal Rojas, I. M., Jeet, V., Lahoud, M. H., Radford, K. J. (2015) Differential uptake and cross-presentation of soluble and necrotic cell antigen by human DC subsets. Eur J Immunol.

54. den Haan, J. M., Lehar, S. M., Bevan, M. J. (2000) CD8(+) but not CD8(-) dendritic cells cross-prime cytotoxic T cells in vivo. J Exp Med 192, 1685-96.

55. Robbins, S. H., Walzer, T., Dembele, D., Thibault, C., Defays, A., Bessou, G., Xu, H., Vivier, E., Sellars, M., Pierre, P., Sharp, F. R., Chan, S., Kastner, P., Dalod, M. (2008) Novel insights into

the relationships between dendritic cell subsets in human and mouse revealed by genome-wide expression profiling. Genome Biol 9, R17.

56. Belz, G. T., Smith, C. M., Eichner, D., Shortman, K., Karupiah, G., Carbone, F. R., Heath, W. R. (2004) Cutting edge: conventional CD8 alpha+ dendritic cells are generally involved in priming CTL immunity to viruses. J Immunol 172, 1996-2000.
57. Allan, R. S., Smith, C. M., Belz, G. T., van Lint, A. L., Wakim, L. M., Heath, W. R., Carbone, F. R. (2003) Epidermal viral immunity induced by CD8alpha+ dendritic cells but not by Langerhans cells. Science 301, 1925-8.
58. Waithman, J., Zanker, D., Xiao, K., Oveissi, S., Wylie, B., Ng, R., Togel, L., Chen, W. (2013) Resident CD8(+) and migratory CD103(+) dendritic cells control CD8 T cell immunity during acute influenza infection. Plos One 8, e66136.
59. Hildner, K., Edelson, B. T., Purtha, W. E., Diamond, M., Matsushita, H., Kohyama, M., Calderon, B., Schraml, B. U., Unanue, E. R., Diamond, M. S., Schreiber, R. D., Murphy, T. L., Murphy, K. M. (2008) Batf3 deficiency reveals a critical role for CD8alpha+ dendritic cells in cytotoxic T cell immunity. Science 322, 1097-100.
60. Eickhoff, S., Brewitz, A., Gerner, M. Y., Klauschen, F., Komander, K., Hemmi, H., Garbi, N., Kaisho, T., Germain, R. N., Kastenmuller, W. (2015) Robust Anti-viral Immunity Requires Multiple Distinct T Cell-Dendritic Cell Interactions. Cell 162, 1322-37.
61. Hor, J. L., Whitney, P. G., Zaid, A., Brooks, A. G., Heath, W. R., Mueller, S. N. (2015) Spatiotemporally Distinct Interactions with Dendritic Cell Subsets Facilitates CD4+ and CD8+ T Cell Activation to Localized Viral Infection. Immunity 43, 554-65.
62. Bosma, B. M., Metselaar, H. J., Mancham, S., Boor, P. P., Kusters, J. G., Kazemier, G., Tilanus, H. W., Kuipers, E. J., Kwekkeboom, J. (2006) Characterization of human liver dendritic cells in liver grafts and perfusates. Liver Transpl 12, 384-93.
63. Hsu, W., Shu, S. A., Gershwin, E., Lian, Z. X. (2007) The current immune function of hepatic dendritic cells. Cell Mol Immunol 4, 321-8.
64. Kudo, S., Matsuno, K., Ezaki, T., Ogawa, M. (1997) A novel migration pathway for rat dendritic cells from the blood: hepatic sinusoids-lymph translocation. J Exp Med 185, 777-84.
65. Bamboat, Z. M., Stableford, J. A., Plitas, G., Burt, B. M., Nguyen, H. M., Welles, A. P., Gonen, M., Young, J. W., DeMatteo, R. P. (2009) Human liver dendritic cells promote T cell hyporesponsiveness. J Immunol 182, 1901-11.
66. Goddard, S., Youster, J., Morgan, E., Adams, D. H. (2004) Interleukin-10 secretion differentiates dendritic cells from human liver and skin. Am J Pathol 164, 511-9.
67. Gehring, A. J. and Ann D'Angelo, J. (2014) Dissecting the dendritic cell controversy in chronic hepatitis B virus infection. Cell Mol Immunol.
68. Duan, X. Z., Wang, M., Li, H. W., Zhuang, H., Xu, D., Wang, F. S. (2004) Decreased frequency and function of circulating plasmocytoid dendritic cells (pDC) in hepatitis B virus infected humans. J Clin Immunol 24, 637-46.
69. van der Molen, R. G., Sprengers, D., Biesta, P. J., Kusters, J. G., Janssen, H. L. (2006) Favorable effect of adefovir on the number and functionality of myeloid dendritic cells of patients with chronic HBV. Hepatology 44, 907-14.
70. van der Molen, R. G., Sprengers, D., Binda, R. S., de Jong, E. C., Niesters, H. G., Kusters, J. G., Kwekkeboom, J., Janssen, H. L. (2004) Functional impairment of myeloid and plasmacytoid dendritic cells of patients with chronic hepatitis B. Hepatology 40, 738-46.
71. Gehring, A. J., Haniffa, M., Kennedy, P. T., Ho, Z. Z., Boni, C., Shin, A., Banu, N., Chia, A., Lim, S. G., Ferrari, C., Ginhoux, F., Bertoletti, A. (2013) Mobilizing monocytes to cross-present circulating viral antigen in chronic infection. J Clin Invest 123, 3766-76.
72. Nattermann, J., Zimmermann, H., Iwan, A., von Lilienfeld-Toal, M., Leifeld, L., Nischalke, H. D., Langhans, B., Sauerbruch, T., Spengler, U. (2006) Hepatitis C virus E2 and CD81 interaction may be associated with altered trafficking of dendritic cells in chronic hepatitis C. Hepatology 44, 945-54.

73. Zhang, Z., Chen, D., Yao, J., Zhang, H., Jin, L., Shi, M., Zhang, H., Wang, F. S. (2007) Increased infiltration of intrahepatic DC subsets closely correlate with viral control and liver injury in immune active pediatric patients with chronic hepatitis B. Clin Immunol 122, 173-80.
74. Untergasser, A., Zedler, U., Langenkamp, A., Hosel, M., Quasdorff, M., Esser, K., Dienes, H. P., Tappertzhofen, B., Kolanus, W., Protzer, U. (2006) Dendritic cells take up viral antigens but do not support the early steps of hepatitis B virus infection. Hepatology 43, 539-47.
75. Tavakoli, S., Mederacke, I., Herzog-Hauff, S., Glebe, D., Grun, S., Strand, D., Urban, S., Gehring, A., Galle, P. R., Bocher, W. O. (2008) Peripheral blood dendritic cells are phenotypically and functionally intact in chronic hepatitis B virus (HBV) infection. Clin Exp Immunol 151, 61-70.
76. Op den Brouw, M. L., Binda, R. S., Geijtenbeek, T. B., Janssen, H. L., Woltman, A. M. (2009) The mannose receptor acts as hepatitis B virus surface antigen receptor mediating interaction with intrahepatic dendritic cells. Virology 393, 84-90.
77. Chen, L., Zhang, Z., Chen, W., Zhang, Z., Li, Y., Shi, M., Zhang, J., Chen, L., Wang, S., Wang, F. S. (2007) B7-H1 up-regulation on myeloid dendritic cells significantly suppresses T cell immune function in patients with chronic hepatitis B. J Immunol 178, 6634-41.
78. Zheng, B. J., Zhou, J., Qu, D., Siu, K. L., Lam, T. W., Lo, H. Y., Lee, S. S., Wen, Y. M. (2004) Selective functional deficit in dendritic cell--T cell interaction is a crucial mechanism in chronic hepatitis B virus infection. J Viral Hepat 11, 217-24.
79. Woltman, A. M., den Brouw, M. L. O., Biesta, P. J., Shi, C. C., Janssen, H. L. A. (2011) Hepatitis B Virus Lacks Immune Activating Capacity, but Actively Inhibits Plasmacytoid Dendritic Cell Function. Plos One 6.
80. Wang, K., Fan, X., Fan, Y., Wang, B., Han, L., Hou, Y. (2007) Study on the function of circulating plasmacytoid dendritic cells in the immunoactive phase of patients with chronic genotype B and C HBV infection. J Viral Hepat 14, 276-82.
81. Xu, Y., Hu, Y., Shi, B., Zhang, X., Wang, J., Zhang, Z., Shen, F., Zhang, Q., Sun, S., Yuan, Z. (2009) HBsAg inhibits TLR9-mediated activation and IFN-alpha production in plasmacytoid dendritic cells. Mol Immunol 46, 2640-6.
82. Shi, B., Ren, G., Hu, Y., Wang, S., Zhang, Z., Yuan, Z. (2012) HBsAg inhibits IFN-alpha production in plasmacytoid dendritic cells through TNF-alpha and IL-10 induction in monocytes. PLoS One 7, e44900.
83. Cao, W., Manicassamy, S., Tang, H., Kasturi, S. P., Pirani, A., Murthy, N., Pulendran, B. (2008) Toll-like receptor-mediated induction of type I interferon in plasmacytoid dendritic cells requires the rapamycin-sensitive PI(3)K-mTOR-p70S6K pathway. Nat Immunol 9, 1157-64.
84. Ank, N., West, H., Bartholdy, C., Eriksson, K., Thomsen, A. R., Paludan, S. R. (2006) Lambda interferon (IFN-lambda), a type III IFN, is induced by viruses and IFNs and displays potent antiviral activity against select virus infections in vivo. J Virol 80, 4501-9.
85. Doyle, S. E., Schreckhise, H., Khuu-Duong, K., Henderson, K., Rosler, R., Storey, H., Yao, L., Liu, H., Barahmand-pour, F., Sivakumar, P., Chan, C., Birks, C., Foster, D., Clegg, C. H., Wietzke-Braun, P., Mihm, S., Klucher, K. M. (2006) Interleukin-29 uses a type 1 interferon-like program to promote antiviral responses in human hepatocytes. Hepatology 44, 896-906.
86. Jordan, W. J., Eskdale, J., Srinivas, S., Pekarek, V., Kelner, D., Rodia, M., Gallagher, G. (2007) Human interferon lambda-1 (IFN-lambda1/IL-29) modulates the Th1/Th2 response. Genes Immun 8, 254-61.
87. Kelly, C., Klenerman, P., Barnes, E. (2011) Interferon lambdas: the next cytokine storm. Gut 60, 1284-93.
88. Robek, M. D., Boyd, B. S., Chisari, F. V. (2005) Lambda interferon inhibits hepatitis B and C virus replication. J Virol 79, 3851-4.
89. Tian, R. R., Guo, H. X., Wei, J. F., Yang, C. K., He, S. H., Wang, J. H. (2012) IFN-lambda inhibits HIV-1 integration and post-transcriptional events in vitro, but there is only limited in vivo repression of viral production. Antiviral Res 95, 57-65.
90. Zhou, L., Li, J., Wang, X., Ye, L., Hou, W., Ho, J., Li, H., Ho, W. (2011) IL-29/IL-28A suppress HSV-1 infection of human NT2-N neurons. J Neurovirol 17, 212-9.

91. Hernandez, P. P., Mahlakoiv, T., Yang, I., Schwierzeck, V., Nguyen, N., Guendel, F., Gronke, K., Ryffel, B., Holscher, C., Dumoutier, L., Renauld, J. C., Suerbaum, S., Staeheli, P., Diefenbach, A. (2015) Interferon-lambda and interleukin 22 act synergistically for the induction of interferon-stimulated genes and control of rotavirus infection. Nat Immunol 16, 698-707.

92. Lazear, H. M., Daniels, B. P., Pinto, A. K., Huang, A. C., Vick, S. C., Doyle, S. E., Gale, M., Jr., Klein, R. S., Diamond, M. S. (2015) Interferon-lambda restricts West Nile virus neuroinvasion by tightening the blood-brain barrier. Sci Transl Med 7, 284ra59.

93. Pott, J., Mahlakoiv, T., Mordstein, M., Duerr, C. U., Michiels, T., Stockinger, S., Staeheli, P., Hornef, M. W. (2011) IFN-lambda determines the intestinal epithelial antiviral host defense. Proc Natl Acad Sci U S A 108, 7944-9.

94. Jewell, N. A., Cline, T., Mertz, S. E., Smirnov, S. V., Flano, E., Schindler, C., Grieves, J. L., Durbin, R. K., Kotenko, S. V., Durbin, J. E. (2010) Lambda interferon is the predominant interferon induced by influenza A virus infection in vivo. J Virol 84, 11515-22.

95. Pagliaccetti, N. E., Chu, E. N., Bolen, C. R., Kleinstein, S. H., Robek, M. D. (2010) Lambda and alpha interferons inhibit hepatitis B virus replication through a common molecular mechanism but with different in vivo activities. Virology 401, 197-206.

96. Nakagawa, S., Hirata, Y., Kameyama, T., Tokunaga, Y., Nishito, Y., Hirabayashi, K., Yano, J., Ochiya, T., Tateno, C., Tanaka, Y., Mizokami, M., Tsukiyama-Kohara, K., Inoue, K., Yoshiba, M., Takaoka, A., Kohara, M. (2013) Targeted induction of interferon-lambda in humanized chimeric mouse liver abrogates hepatotropic virus infection. PLoS One 8, e59611.

97. Osterlund, P. I., Pietila, T. E., Veckman, V., Kotenko, S. V., Julkunen, I. (2007) IFN regulatory factor family members differentially regulate the expression of type III IFN (IFN-lambda) genes. J Immunol 179, 3434-42.

98. Onoguchi, K., Yoneyama, M., Takemura, A., Akira, S., Taniguchi, T., Namiki, H., Fujita, T. (2007) Viral infections activate types I and III interferon genes through a common mechanism. J Biol Chem 282, 7576-81.

99. Osterlund, P., Veckman, V., Siren, J., Klucher, K. M., Hiscott, J., Matikainen, S., Julkunen, I. (2005) Gene expression and antiviral activity of alpha/beta interferons and interleukin-29 in virus-infected human myeloid dendritic cells. J Virol 79, 9608-17.

100. Thomson, S. J., Goh, F. G., Banks, H., Krausgruber, T., Kotenko, S. V., Foxwell, B. M., Udalova, I. A. (2009) The role of transposable elements in the regulation of IFN-lambda1 gene expression. Proc Natl Acad Sci U S A 106, 11564-9.

101. Swider, A., Siegel, R., Eskdale, J., Gallagher, G. (2014) Regulation of interferon lambda-1 (IFNL1/IFN-lambda1/IL-29) expression in human colon epithelial cells. Cytokine 65, 17-23.

102. Lee, H. C., Narayanan, S., Park, S. J., Seong, S. Y., Hahn, Y. S. (2014) Transcriptional regulation of IFN-lambda genes in hepatitis C virus-infected hepatocytes via IRF-3.IRF-7.NF-kappaB complex. J Biol Chem 289, 5310-9.

103. Siegel, R., Eskdale, J., Gallagher, G. (2011) Regulation of IFN-lambda1 promoter activity (IFN-lambda1/IL-29) in human airway epithelial cells. J Immunol 187, 5636-44.

104. Kotenko, S. V., Gallagher, G., Baurin, V. V., Lewis-Antes, A., Shen, M., Shah, N. K., Langer, J. A., Sheikh, F., Dickensheets, H., Donnelly, R. P. (2003) IFN-lambdas mediate antiviral protection through a distinct class II cytokine receptor complex. Nat Immunol 4, 69-77.

105. Sheppard, P., Kindsvogel, W., Xu, W., Henderson, K., Schlutsmeyer, S., Whitmore, T. E., Kuestner, R., Garrigues, U., Birks, C., Roraback, J., Ostrander, C., Dong, D., Shin, J., Presnell, S., Fox, B., Haldeman, B., Cooper, E., Taft, D., Gilbert, T., Grant, F. J., Tackett, M., Krivan, W., McKnight, G., Clegg, C., Foster, D., Klucher, K. M. (2003) IL-28, IL-29 and their class II cytokine receptor IL-28R. Nat Immunol 4, 63-8.

106. Prokunina-Olsson, L., Muchmore, B., Tang, W., Pfeiffer, R. M., Park, H., Dickensheets, H., Hergott, D., Porter-Gill, P., Mumy, A., Kohaar, I., Chen, S., Brand, N., Tarway, M., Liu, L., Sheikh, F., Astemborski, J., Bonkovsky, H. L., Edlin, B. R., Howell, C. D., Morgan, T. R., Thomas, D. L., Rehermann, B., Donnelly, R. P., O'Brien, T. R. (2013) A variant upstream of IFNL3 (IL28B) creating a new interferon gene IFNL4 is associated with impaired clearance of hepatitis C

virus. Nat Genet 45, 164-71.

107. Egli, A., Santer, D. M., O'Shea, D., Tyrrell, D. L., Houghton, M. (2014) The impact of the interferon-lambda family on the innate and adaptive immune response to viral infections. Emerg Microbes Infect 3, e51.
108. Bolen, C. R., Ding, S., Robek, M. D., Kleinstein, S. H. (2014) Dynamic expression profiling of type I and type III interferon-stimulated hepatocytes reveals a stable hierarchy of gene expression. Hepatology 59, 1262-72.
109. Dellgren, C., Gad, H. H., Hamming, O. J., Melchjorsen, J., Hartmann, R. (2009) Human interferon-lambda3 is a potent member of the type III interferon family. Genes Immun 10, 125-31.
110. Liu, B. S., Janssen, H. L., Boonstra, A. (2011) IL-29 and IFNalpha differ in their ability to modulate IL-12 production by TLR-activated human macrophages and exhibit differential regulation of the IFNgamma receptor expression. Blood 117, 2385-95.
111. Novak, A. J., Grote, D. M., Ziesmer, S. C., Rajkumar, V., Doyle, S. E., Ansell, S. M. (2008) A role for IFN-lambda1 in multiple myeloma B cell growth. Leukemia 22, 2240-6.
112. Sommereyns, C., Paul, S., Staeheli, P., Michiels, T. (2008) IFN-lambda (IFN-lambda) is expressed in a tissue-dependent fashion and primarily acts on epithelial cells in vivo. PLoS Pathog 4, e1000017.
113. Megjugorac, N. J., Gallagher, G. E., Gallagher, G. (2009) Modulation of human plasmacytoid DC function by IFN-lambda1 (IL-29). J Leukoc Biol 86, 1359-63.
114. Lazear, H. M., Nice, T. J., Diamond, M. S. (2015) Interferon-lambda: Immune Functions at Barrier Surfaces and Beyond. Immunity 43, 15-28.
115. Wack, A., Terczynska-Dyla, E., Hartmann, R. (2015) Guarding the frontiers: the biology of type III interferons. Nat Immunol 16, 802-9.
116. Yin, Z., Dai, J., Deng, J., Sheikh, F., Natalia, M., Shih, T., Lewis-Antes, A., Amrute, S. B., Garrigues, U., Doyle, S., Donnelly, R. P., Kotenko, S. V., Fitzgerald-Bocarsly, P. (2012) Type III IFNs are produced by and stimulate human plasmacytoid dendritic cells. J Immunol 189, 2735-45.
117. de Groen, R. A., Boltjes, A., Hou, J., Liu, B. S., McPhee, F., Friborg, J., Janssen, H. L., Boonstra, A. (2015) IFN-lambda-mediated IL-12 production in macrophages induces IFN-gamma production in human NK cells. Eur J Immunol 45, 250-9.
118. de Groen, R. A., Groothuismink, Z. M., Liu, B. S., Boonstra, A. (2015) IFN-lambda is able to augment TLR-mediated activation and subsequent function of primary human B cells. J Leukoc Biol 98, 623-30.
119. Dai, J., Megjugorac, N. J., Gallagher, G. E., Yu, R. Y., Gallagher, G. (2009) IFN-lambda1 (IL-29) inhibits GATA3 expression and suppresses Th2 responses in human naive and memory T cells. Blood 113, 5829-38.
120. Sato, S., Li, K., Kameyama, T., Hayashi, T., Ishida, Y., Murakami, S., Watanabe, T., Iijima, S., Sakurai, Y., Watashi, K., Tsutsumi, S., Sato, Y., Akita, H., Wakita, T., Rice, C. M., Harashima, H., Kohara, M., Tanaka, Y., Takaoka, A. (2015) The RNA sensor RIG-I dually functions as an innate sensor and direct antiviral factor for hepatitis B virus. Immunity 42, 123-32.
121. Duong, F. H., Trincucci, G., Boldanova, T., Calabrese, D., Campana, B., Krol, I., Durand, S. C., Heydmann, L., Zeisel, M. B., Baumert, T. F., Heim, M. H. (2014) IFN-lambda receptor 1 expression is induced in chronic hepatitis C and correlates with the IFN-lambda3 genotype and with nonresponsiveness to IFN-alpha therapies. J Exp Med 211, 857-68.
122. de Groen, R. A., McPhee, F., Friborg, J., Janssen, H. L., Boonstra, A. (2014) Endogenous IFNlambda in viral hepatitis patients. J Interferon Cytokine Res 34, 552-6.
123. Mihm, S., Frese, M., Meier, V., Wietzke-Braun, P., Scharf, J. G., Bartenschlager, R., Ramadori, G. (2004) Interferon type I gene expression in chronic hepatitis C. Lab Invest 84, 1148-59.
124. Park, H., Serti, E., Eke, O., Muchmore, B., Prokunina-Olsson, L., Capone, S., Folgori, A., Rehermann, B. (2012) IL-29 is the dominant type III interferon produced by hepatocytes

during acute hepatitis C virus infection. Hepatology 56, 2060-70.

125. Dunn, C., Peppa, D., Khanna, P., Nebbia, G., Jones, M., Brendish, N., Lascar, R. M., Brown, D., Gilson, R. J., Tedder, R. J., Dusheiko, G. M., Jacobs, M., Klenerman, P., Maini, M. K. (2009) Temporal analysis of early immune responses in patients with acute hepatitis B virus infection. Gastroenterology 137, 1289-300.
126. Wieland, S., Thimme, R., Purcell, R. H., Chisari, F. V. (2004) Genomic analysis of the host response to hepatitis B virus infection. Proc Natl Acad Sci U S A 101, 6669-74.
127. Luangsay, S., Gruffaz, M., Isorce, N., Testoni, B., Michelet, M., Faure-Dupuy, S., Maadadi, S., Ait-Goughoulte, M., Parent, R., Rivoire, M., Javanbakht, H., Lucifora, J., Durantel, D., Zoulim, F. (2015) Early inhibition of hepatocyte innate responses by hepatitis B virus. J Hepatol 63, 1314-22.
128. Sonneveld, M. J., Wong, V. W., Woltman, A. M., Wong, G. L., Cakaloglu, Y., Zeuzem, S., Buster, E. H., Uitterlinden, A. G., Hansen, B. E., Chan, H. L., Janssen, H. L. (2012) Polymorphisms near IL28B and serologic response to peginterferon in HBeAg-positive patients with chronic hepatitis B. Gastroenterology 142, 513-520 e1.
129. Chan, H. L., Ahn, S. H., Chang, T. T., Peng, C. Y., Wong, D., Coffin, C. S., Lim, S. G., Chen, P. J., Janssen, H. L., Marcellin, P., Serfaty, L., Zeuzem, S., Cohen, D., Critelli, L., Xu, D., Wind-Rotolo, M., Cooney, E., Team, L.-B. S. (2016) Peginterferon lambda for the treatment of HBeAg-positive chronic hepatitis B: A randomized phase 2b study (LIRA-B). J Hepatol 64, 1011-9.
130. Petersen, J., Dandri, M., Mier, W., Lutgehetmann, M., Volz, T., von Weizsacker, F., Haberkorn, U., Fischer, L., Pollok, J. M., Erbes, B., Seitz, S., Urban, S. (2008) Prevention of hepatitis B virus infection in vivo by entry inhibitors derived from the large envelope protein. Nat Biotechnol 26, 335-41.
131. Volz, T., Allweiss, L., Ben, M. M., Warlich, M., Lohse, A. W., Pollok, J. M., Alexandrov, A., Urban, S., Petersen, J., Lutgehetmann, M., Dandri, M. (2013) The entry inhibitor Myrcludex-B efficiently blocks intrahepatic virus spreading in humanized mice previously infected with hepatitis B virus. J Hepatol 58, 861-7.
132. Lucifora, J., Xia, Y., Reisinger, F., Zhang, K., Stadler, D., Cheng, X., Sprinzl, M. F., Koppensteiner, H., Makowska, Z., Volz, T., Remouchamps, C., Chou, W. M., Thasler, W. E., Huser, N., Durantel, D., Liang, T. J., Munk, C., Heim, M. H., Browning, J. L., Dejardin, E., Dandri, M., Schindler, M., Heikenwalder, M., Protzer, U. (2014) Specific and nonhepatotoxic degradation of nuclear hepatitis B virus cccDNA. Science 343, 1221-8.
133. Seeger, C. and Sohn, J. A. (2014) Targeting Hepatitis B Virus With CRISPR/Cas9. Mol Ther Nucleic Acids 3, e216.
134. Wooddell, C. I., Rozema, D. B., Hossbach, M., John, M., Hamilton, H. L., Chu, Q., Hegge, J. O., Klein, J. J., Wakefield, D. H., Oropeza, C. E., Deckert, J., Roehl, I., Jahn-Hofmann, K., Hadwiger, P., Vornlocher, H. P., McLachlan, A., Lewis, D. L. (2013) Hepatocyte-targeted RNAi therapeutics for the treatment of chronic hepatitis B virus infection. Mol Ther 21, 973-85.
135. Michel, M. L., Bourgine, M., Fontaine, H., Pol, S. (2015) Therapeutic vaccines in treating chronic hepatitis B: the end of the beginning or the beginning of the end? Med Microbiol Immunol 204, 121-9.
136. Koh, S. and Bertoletti, A. (2015) Circumventing failed antiviral immunity in chronic hepatitis B virus infection: triggering virus-specific or innate-like T cell response? Med Microbiol Immunol 204, 87-94.
137. Yang, N. and Bertoletti, A. (2015) Advances in therapeutics for chronic hepatitis B. Hepatol Int.
138. Ilan, Y., Nagler, A., Adler, R., Tur-Kaspa, R., Slavin, S., Shouval, D. (1993) Ablation of persistent hepatitis B by bone marrow transplantation from a hepatitis B-immune donor. Gastroenterology 104, 1818-21.
139. Lau, G. K., Suri, D., Liang, R., Rigopoulou, E. I., Thomas, M. G., Mullerova, I., Nanji, A., Yuen, S. T., Williams, R., Naoumov, N. V. (2002) Resolution of chronic hepatitis B and anti-HBs seroconversion in humans by adoptive transfer of immunity to hepatitis B core antigen.

Gastroenterology 122, 614-24.

140. Lau, G. K., Lok, A. S., Liang, R. H., Lai, C. L., Chiu, E. K., Lau, Y. L., Lam, S. K. (1997) Clearance of hepatitis B surface antigen after bone marrow transplantation: role of adoptive immunity transfer. Hepatology 25, 1497-501.
141. Boni, C., Fisicaro, P., Valdatta, C., Amadei, B., Di Vincenzo, P., Giuberti, T., Laccabue, D., Zerbini, A., Cavalli, A., Missale, G., Bertoletti, A., Ferrari, C. (2007) Characterization of hepatitis B virus (HBV)-specific T-cell dysfunction in chronic HBV infection. J Virol 81, 4215-25.
142. Fisicaro, P., Valdatta, C., Massari, M., Loggi, E., Biasini, E., Sacchelli, L., Cavallo, M. C., Silini, E. M., Andreone, P., Missale, G., Ferrari, C. (2010) Antiviral intrahepatic T-cell responses can be restored by blocking programmed death-1 pathway in chronic hepatitis B. Gastroenterology 138, 682-93, 693 e1-4.
143. Nebbia, G., Peppa, D., Schurich, A., Khanna, P., Singh, H. D., Cheng, Y., Rosenberg, W., Dusheiko, G., Gilson, R., ChinAleong, J., Kennedy, P., Maini, M. K. (2012) Upregulation of the Tim-3/galectin-9 pathway of T cell exhaustion in chronic hepatitis B virus infection. PLoS One 7, e47648.
144. Raziorrouh, B., Schraut, W., Gerlach, T., Nowack, D., Gruner, N. H., Ulsenheimer, A., Zachoval, R., Wachtler, M., Spannagl, M., Haas, J., Diepolder, H. M., Jung, M. C. (2010) The immunoregulatory role of CD244 in chronic hepatitis B infection and its inhibitory potential on virus-specific CD8+ T-cell function. Hepatology 52, 1934-47.
145. Bengsch, B., Martin, B., Thimme, R. (2014) Restoration of HBV-specific CD8+ T cell function by PD-1 blockade in inactive carrier patients is linked to T cell differentiation. J Hepatol 61, 1212-9.
146. Gehring, A. J., Xue, S. A., Ho, Z. Z., Teoh, D., Ruedl, C., Chia, A., Koh, S., Lim, S. G., Maini, M. K., Stauss, H., Bertoletti, A. (2011) Engineering virus-specific T cells that target HBV infected hepatocytes and hepatocellular carcinoma cell lines. J Hepatol 55, 103-10.
147. Koh, S., Shimasaki, N., Suwanarusk, R., Ho, Z. Z., Chia, A., Banu, N., Howland, S. W., Ong, A. S., Gehring, A. J., Stauss, H., Renia, L., Sallberg, M., Campana, D., Bertoletti, A. (2013) A practical approach to immunotherapy of hepatocellular carcinoma using T cells redirected against hepatitis B virus. Mol Ther Nucleic Acids 2, e114.
148. Krebs, K., Bottinger, N., Huang, L. R., Chmielewski, M., Arzberger, S., Gasteiger, G., Jager, C., Schmitt, E., Bohne, F., Aichler, M., Uckert, W., Abken, H., Heikenwalder, M., Knolle, P., Protzer, U. (2013) T cells expressing a chimeric antigen receptor that binds hepatitis B virus envelope proteins control virus replication in mice. Gastroenterology 145, 456-65.
149. Qasim, W., Brunetto, M., Gehring, A. J., Xue, S. A., Schurich, A., Khakpoor, A., Zhan, H., Ciccorossi, P., Gilmour, K., Cavallone, D., Moriconi, F., Farzhenah, F., Mazzoni, A., Chan, L., Morris, E., Thrasher, A., Maini, M. K., Bonino, F., Stauss, H., Bertoletti, A. (2015) Immunotherapy of HCC metastases with autologous T cell receptor redirected T cells, targeting HBsAg in a liver transplant patient. J Hepatol 62, 486-91.
150. Lanford, R. E., Guerra, B., Chavez, D., Giavedoni, L., Hodara, V. L., Brasky, K. M., Fosdick, A., Frey, C. R., Zheng, J., Wolfgang, G., Halcomb, R. L., Tumas, D. B. (2013) GS-9620, an oral agonist of Toll-like receptor-7, induces prolonged suppression of hepatitis B virus in chronically infected chimpanzees. Gastroenterology 144, 1508-17, 1517 e1-10.
151. Menne, S., Tumas, D. B., Liu, K. H., Thampi, L., AlDeghaither, D., Baldwin, B. H., Bellezza, C. A., Cote, P. J., Zheng, J., Halcomb, R., Fosdick, A., Fletcher, S. P., Daffis, S., Li, L., Yue, P., Wolfgang, G. H., Tennant, B. C. (2015) Sustained efficacy and seroconversion with the Toll-like receptor 7 agonist GS-9620 in the Woodchuck model of chronic hepatitis B. J Hepatol 62, 1237-45.
152. Gane, E. J., Lim, Y. S., Gordon, S. C., Visvanathan, K., Sicard, E., Fedorak, R. N., Roberts, S., Massetto, B., Ye, Z., Pflanz, S., Garrison, K. L., Gaggar, A., Mani Subramanian, G., McHutchison, J. G., Kottilil, S., Freilich, B., Coffin, C. S., Cheng, W., Kim, Y. J. (2015) The oral toll-like receptor-7 agonist GS-9620 in patients with chronic hepatitis B virus infection. J Hepatol 63, 320-8.
153. Mancini-Bourgine, M., Fontaine, H., Scott-Algara, D., Pol, S., Brechot, C., Michel, M. L. (2004) Induction or expansion of T-cell responses by a hepatitis B DNA vaccine administered to chronic HBV carriers. Hepatology 40, 874-82.

154. Mancini-Bourgine, M., Fontaine, H., Brechot, C., Pol, S., Michel, M. L. (2006) Immunogenicity of a hepatitis B DNA vaccine administered to chronic HBV carriers. Vaccine 24, 4482-9.

155. Boni, C., Penna, A., Ogg, G. S., Bertoletti, A., Pilli, M., Cavallo, C., Cavalli, A., Urbani, S., Boehme, R., Panebianco, R., Fiaccadori, F., Ferrari, C. (2001) Lamivudine treatment can overcome cytotoxic T-cell hyporesponsiveness in chronic hepatitis B: new perspectives for immune therapy. Hepatology 33, 963-71.

156. Kosinska, A. D., Zhang, E., Johrden, L., Liu, J., Seiz, P. L., Zhang, X., Ma, Z., Kemper, T., Fiedler, M., Glebe, D., Wildner, O., Dittmer, U., Lu, M., Roggendorf, M. (2013) Combination of DNA prime--adenovirus boost immunization with entecavir elicits sustained control of chronic hepatitis B in the woodchuck model. PLoS Pathog 9, e1003391.

157. Yang, S. H., Lee, C. G., Park, S. H., Im, S. J., Kim, Y. M., Son, J. M., Wang, J. S., Yoon, S. K., Song, M. K., Ambrozaitis, A., Kharchenko, N., Yun, Y. D., Kim, C. M., Kim, C. Y., Lee, S. H., Kim, B. M., Kim, W. B., Sung, Y. C. (2006) Correlation of antiviral T-cell responses with suppression of viral rebound in chronic hepatitis B carriers: a proof-of-concept study. Gene Ther 13, 1110-7.

158. Huang, L. R., Wohlleber, D., Reisinger, F., Jenne, C. N., Cheng, R. L., Abdullah, Z., Schildberg, F. A., Odenthal, M., Dienes, H. P., van Rooijen, N., Schmitt, E., Garbi, N., Croft, M., Kurts, C., Kubes, P., Protzer, U., Heikenwalder, M., Knolle, P. A. (2013) Intrahepatic myeloid-cell aggregates enable local proliferation of CD8(+) T cells and successful immunotherapy against chronic viral liver infection. Nat Immunol 14, 574-83.

159. Isogawa, M., Chung, J., Murata, Y., Kakimi, K., Chisari, F. V. (2013) CD40 activation rescues antiviral CD8(+) T cells from PD-1-mediated exhaustion. PLoS Pathog 9, e1003490.

160. Couillin, I., Pol, S., Mancini, M., Driss, F., Brechot, C., Tiollais, P., Michel, M. L. (1999) Specific vaccine therapy in chronic hepatitis B: induction of T cell proliferative responses specific for envelope antigens. J Infect Dis 180, 15-26.

161. Pol, S., Nalpas, B., Driss, F., Michel, M. L., Tiollais, P., Denis, J., Brecho, C., Multicenter study, g. (2001) Efficacy and limitations of a specific immunotherapy in chronic hepatitis B. J Hepatol 34, 917-21.

162. Yalcin, K., Acar, M., Degertekin, H. (2003) Specific hepatitis B vaccine therapy in inactive HBsAg carriers: a randomized controlled trial. Infection 31, 221-5.

163. Jung, M. C., Gruner, N., Zachoval, R., Schraut, W., Gerlach, T., Diepolder, H., Schirren, C. A., Page, M., Bailey, J., Birtles, E., Whitehead, E., Trojan, J., Zeuzem, S., Pape, G. R. (2002) Immunological monitoring during therapeutic vaccination as a prerequisite for the design of new effective therapies: induction of a vaccine-specific CD4+ T-cell proliferative response in chronic hepatitis B carriers. Vaccine 20, 3598-612.

164. Wei, M. J., Pan, X. N., Wei, K. P., Li, X. H., Liu, X. L., Zhang, X. M., Jiang, Y. L., Zhang, C. Y., Shen, J. K. (2015) Efficacy of HBV-pulsed DCs in combination with entecavir in patients with chronic hepatitis B infection. Int Immunopharmacol 27, 238-43.

165. Chen, M., Li, Y. G., Zhang, D. Z., Wang, Z. Y., Zeng, W. Q., Shi, X. F., Guo, Y., Guo, S. H., Ren, H. (2005) Therapeutic effect of autologous dendritic cell vaccine on patients with chronic hepatitis B: a clinical study. World J Gastroenterol 11, 1806-8.

166. Luo, J., Li, J., Chen, R. L., Nie, L., Huang, J., Liu, Z. W., Luo, L., Yan, X. J. (2010) Autologus dendritic cell vaccine for chronic hepatitis B carriers: a pilot, open label, clinical trial in human volunteers. Vaccine 28, 2497-504.

167. Kastenmuller, W., Kastenmuller, K., Kurts, C., Seder, R. A. (2014) Dendritic cell-targeted vaccines--hope or hype? Nat Rev Immunol 14, 705-11.

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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Chapter 2

Abstract

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 allows 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 DC subsets involved, however, these results cannot be readily translated towards 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.

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]. Dendritic cells (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 towards 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].

DC 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 antiviral 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 other hand, can produce high levels of type III IFN (IFNλ) [8], which possess direct antiviral 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 [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 pathogen-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 interstitial DC and Langerhans cells (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 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 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 [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 surrogate 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 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.

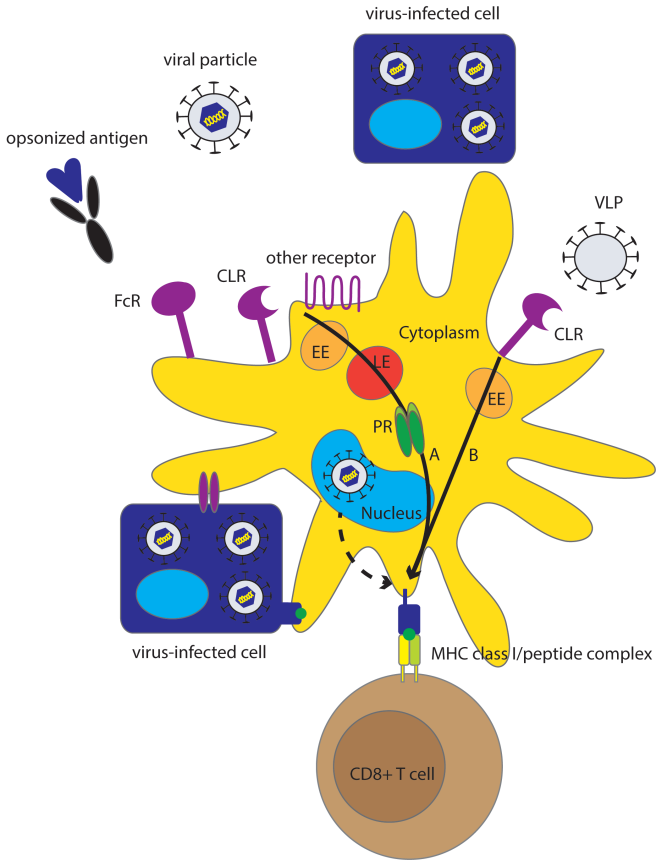

Figure 1: Overview of different pathways underlying MHC class I presentation of viral antigens by human DC.
Although direct MHC class I presentation may contribute to virus-specific CTL induction (dashed arrow), cross-presentation is an effective mechanism for MHC class I presentation of viruses that 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, virus-like particles (VLP) and virus-infected cells, also referred to as cell-associated Ag. Endocytic receptors including C-type lectin receptors (CLR), Fc receptors (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, facilitates cross-presentation of viral particles, infected cells and opsonized viral proteins (A). The relatively fast vacuolar pathway is independent of proteasomal degradation and facilitates cross-presentation of VLP (B), as well as other forms of viral antigen by pDC. Alternatively, DC can obtain viral peptides or MHC class I-peptide complexes by interaction with virus-infected cells.
Abbreviations: EE: early endosome, LE: late endosome, PR: proteasome

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 [76]. 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 [77]. 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 [78], DCIR [79], DC-SIGN [80] and CLEC9A [81] (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 [82]. Binding of immune complexes to Fcγ receptor (FcγR) on DC leads to efficient cross-presentation in MHC class I [82]. 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 [83], 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 [77]. 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 [84]. A similar role for extracellular heat-shock proteins (HSP) has been proposed (reviewed by [85], 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. In addition, cross-presentation requires access of incoming antigen to the MHC class I pathway that is mechanistically separated from the uptake vesicles by membranes [86].
DC 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 [20]. These pathways are not mutually exclusive and may operate together in one cell [87]. 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 [88–90]. 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 heat-shock proteins and p97 and sec61, which belong to the endoplasmatic 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 have totally different kinetics, which can be used to determine which pathway is involved [91]. Whereas cross-presentation via the vacuolar pathway is fast and can be detected after 20 minutes [92], cross-presentation via the cytosolic pathway is much slower and formation of MHC class I – peptide complexes via this pathway may take at least eight hours [88], probably because it relies on MHC class I neosynthesis [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 [93].

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	References
C-type lectin receptors	DEC205 (CD205)	+	+	+	-	+	+	[146] [147]
	DCIR	+	-	+	+	+	+	[148] [149] [150] [79]
	MMR (CD206)	+/-	+	-	-	+	+	[78] [146] [149]
	DC-SIGN (CD209)	-	-	-	-	+	+	[151] [146]
	CLEC9A (DNGR1)	-	+	-	-	-	-	[129]
	Langerin (CD207)	-	-	-	+	-	-	[151] [146]
Toll-like receptors	1	+	+	+	+	+	+	[5]
	2	+	+	-	+	+	+	[9]
	3	+	+	-	+	+	+	[149]
	4	+	-	-	-	+	+	
	5	+	-	-	-	+	+	
	6	+	+	+	+	+	+	
	7	-	-	+	+	+	-	
	8	+	+	-	+	+	+	
	9	-	-	+	-	-	-	
	10	+	+	+	-	-	+	
Fcγreceptors	FcγRI (CD64)	+	-	nf	nf	nf	+/-	[82]
	FcγRIIA (CD32)	+	+	+	nf	nf	+	[82] [132]
	FcγRIII (CD16)	-	-	-	nf	nf	-	[82]
Complement receptors	CR4, (CD11c)	+	+	-	+	+	+	[146] [149]
	CR3, (CD11b)	+/-	-	-	+/-	+	+	[152] [134] [21]
Heparan sulfate proteoglycan	Syndecan 3	nf	nf	nf	nf	nf	+	[74]
Chemokine receptor	XCR1	-	+	-	-	-	-	[126] [153]

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

The viral road to cross-presentation

The cytosolic and the vacuolar pathways were largely established based on model antigens and mouse studies. It 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 [91]. 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 [94]. IAV is efficiently cross-presented, at least when its fusogenic activity is intact [43,95]. 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 abovementioned work suggests a role for the cytosolic pathway in cross-presentation of fusion-competent viral particles, at least by myeloid DC. 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 [96], 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 [97]. Cross-presentation of Ag-Ig immune complexes also requires both proteasomal and endosomal antigen processing [82]. 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 [91].

Interestingly, several lines of evidence suggest that virus-like particles 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 [51,84,98]. Furthermore, it was shown that cross-presentation of HBV VLP by both mouse DC [98] 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 [78]. 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 [99]. 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 [100]. 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 [80]. 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 [101].
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 abovementioned 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 myeloid DC, thus the physiological role of this pathway remains to be further understood. However, since this pathway is highly efficient, as was demonstrated in pDC [102], 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 pattern-recognition receptors (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 [103] and reduced lysosomal antigen degradation [104] due to lower expression of lysosomal proteases [95]. It is well accepted that matured human DC have an enhanced capacity to activate virus-specific CTL

[25,42,56,60,105,106]. 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 [34,39,42,107,108]. 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 [109]. 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 [110] and damage-associated molecular patterns (DAMP) released by damaged or dying cells [111]. During interaction of DC with cell-associated Ag, DC can encounter both virus-derived danger signals and host-derived maturation signals [27,112,113] or host cell-derived DAMP, such as TLR4 ligand high-mobility group box 1 (HMGB1) [114] or CLEC9A ligand F-actin [115].

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 [42,105]. 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 [9,56,116,117]. Also TLR7/8 agonists have been shown to enhance DC-induced CTL expansion and effector function in vitro [79]. In contrast, cross-presentation of cell-associated antigen was inhibited when poly I: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 [118]. IFNα, a widely studied representative of virus-induced signals, can exert multiple effects on human DC that promote CTL cross-priming (reviewed by [4]. For example, moDC differentiated in the presence of IFNα, so called IFNα-DC, have superior cross-presentation capacity compared to classical moDC [52,119]. 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 [120], VV, via the production of cytokine receptor homologues [33], HSV, via destabilization of host mRNA [35,121] and HCMV, which prevents up-regulation of co-stimulatory molecules and production of cytokines [122] and induces TGFβ production by its target cells [112]. 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 [123,124].
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 type of 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 monocyte-derived DC (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 [9,21,125,126]. Comparison of transcriptional profiles revealed that BDCA3$^+$ DC represent the human equivalent of murine CD8α$^+$ and CD103$^+$ DC [56,127], which have a superior intrinsic cross-presentation capacity compared to other DC subsets [128]. BDCA3$^+$ DC were believed to have a superior capacity to cross-present cell-associated antigen based on the selective expression of CLEC9A [129], a receptor that senses dead cells [130] and has been shown to facilitate cross-presentation by mouse [131] and human DC [81]. Superior capacity to cross-present cell-associated antigen by BDCA3$^+$ DC was demonstrated by several independent studies [9,21,90,125,126], however, not observed in all studies [106]. Also for other types of antigen, among human DC subsets, cross-presentation capacity is not restricted to the BDCA3$^+$ DC subset. Cross-presentation of protein antigen was shown for peripheral blood and tissue-derived BDCA1$^+$ DC [9,116], BDCA2$^+$ pDC [90,116] and BDCA3$^+$ DC [9,56,90,116,125], as well as for in vitro-generated CD34$^+$-derived DC [90] and monocyte-derived DC, 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 [90], pDC [106,126] and moDC, as discussed above.
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 [90]. 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 [95]. 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 [95]. 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 [82].

pDC contribute to antiviral 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 [132]. 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 [90]. However, pDC express a broad repertoire of antigen-uptake receptors on their cell surface such as Fc receptors and CLR BDCA-2, DEC-205, DCIR that can facilitate the uptake and cross-presentation of viral antigens [104] (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 [90]. Numerous functional studies showed that human pDC can cross-present recombinant protein antigens, long peptide antigens, IAV-derived antigens and cell-associated antigens (Hoeffel et al., 2007; Tel et al., 2013a; Fonteneau et al., 2003b; Lui et al., 2009). 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 [102]. 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 [102], 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 [106,107,133].

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 [56,90,135]. In addition to BDCA1$^+$ and BDCA3$^+$ DC, skin contains Langerin$^+$ Langerhans cells (LC) and dermal interstitial DC (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 [136]. 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 [10,90,137], 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 [138,139]. 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 hundred millions of patients worldwide. Viral persistence is associated with a failure of the patient's immune response to eradicate the virus [124]. In addition to chronic persistent infections, reactivation of latent infections including HCMV, EBV, and HPVis 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 [140]. 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 [140].
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 [79,81,82,104,141]. 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 [142] or CLEC9A [81]. 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, at least in mice [143]. However, it was recently shown that FcγR-dependent DC maturation in human DC is less pronounced [82]. Other interesting approaches that combine antigen targeting to DC and DC maturation in one cargo include TLR-ligand-peptide conjugates [144] and nanoparticles that contain both antigen and adjuvant [104].

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 [145].

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. However, although these studies were very convincing, these viruses only represent a small number of viruses that can productively infect human DC and, thus the contribution of direct presentation by human DC infected with other viruses in vivo 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.

References

[1] K.R. Demers, M.A. Reuter, M.R. Betts, CD8(+) T-cell effector function and transcriptional regulation during HIV pathogenesis., Immunological Reviews. 254 (2013) 190–206. doi:10.1111/imr.12069.

[2] J. Banchereau, F. Briere, C. Caux, J. Davoust, S. Lebecque, Y.J. Liu, et al., Immunobiology of dendritic cells., Annual Review of Immunology. 18 (2000) 767–811. doi:10.1146/annurev.immunol.18.1.767.

[3] P. Kaliński, C.M. Hilkens, E.A. Wierenga, M.L. Kapsenberg, T-cell priming by type-1 and type-2 polarized dendritic cells: the concept of a third signal., Immunology Today. 20 (1999) 561–7.

[4] G. Schiavoni, F. Mattei, L. Gabriele, Type I Interferons as Stimulators of DC-Mediated Cross-Priming: Impact on Anti-Tumor Response., Frontiers in Immunology. 4 (2013) 483. doi:10.3389/fimmu.2013.00483.

[5] N. Kadowaki, S. Ho, S. Antonenko, R.W. Malefyt, R.A. Kastelein, F. Bazan, et al., Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens., The Journal of Experimental Medicine. 194 (2001) 863–9.

[6] A. Dzionek, A. Fuchs, P. Schmidt, S. Cremer, M. Zysk, S. Miltenyi, et al., BDCA-2, BDCA-3, and BDCA-4: three markers for distinct subsets of dendritic cells in human peripheral blood., Journal of Immunology (Baltimore, Md. : 1950). 165 (2000) 6037–46.

[7] M. Gilliet, W. Cao, Y.-J. Liu, Plasmacytoid dendritic cells: sensing nucleic acids in viral infection and autoimmune diseases., Nature Reviews. Immunology. 8 (2008) 594–606. doi:10.1038/nri2358.

[8] H. Lauterbach, B. Bathke, S. Gilles, C. Traidl-Hoffmann, C.A. Luber, G. Fejer, et al., Mouse CD8alpha+ DCs and human BDCA3+ DCs are major producers of IFN-lambda in response to poly IC., The Journal of Experimental Medicine. 207 (2010) 2703–17. doi:10.1084/jem.20092720.

[9] S.L. Jongbloed, A.J. Kassianos, K.J. McDonald, G.J. Clark, X. Ju, C.E. Angel, et al., Human CD141+ (BDCA-3)+ dendritic cells (DCs) represent a unique myeloid DC subset that cross-presents necrotic cell antigens., The Journal of Experimental Medicine. 207 (2010) 1247–60. doi:10.1084/jem.20092140.

[10] E. Klechevsky, R. Morita, M. Liu, Y. Cao, L. Thompson-snipes, F. Briere, et al., Functional Specializations of Human Epidermal Langerhans Cells and CD14+ Dermal Dendritic Cells, Immunity. 29 (2008) 497–510. doi:10.1016/j.immuni.2008.07.013.

[11] F. Sallusto, A. Lanzavecchia, Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha., The Journal of Experimental Medicine. 179 (1994) 1109–18.

[12] C. Caux, C. Massacrier, C. Dezutter-Dambuyant, B. Vanbervliet, C. Jacquet, D. Schmitt, et al., Human dendritic Langerhans cells generated in vitro from CD34+ progenitors can prime naive CD4+ T cells and process soluble antigen., Journal of Immunology (Baltimore, Md. : 1950). 155 (1995) 5427–35.

[13] S.E. Macatonia, S. Patterson, S.C. Knight, Primary proliferative and cytotoxic T-cell responses to HIV induced in vitro by human dendritic cells, Immunology. 74 (1991) 399–406.

[14] N. Bhardwaj, A. Bender, N. Gonzalez, L.K. Bui, M.C. Garrett, R.M. Steinman, Influenza Virus-infected Dendritic Cells Stimulate Strong Proliferative and Cytolytic Responses from Human CD8 + T Cells, Journal of Clinical Investigation. 94 (1994) 797–807.

[15] C. Kurts, B.W.S. Robinson, P.A. Knolle, Cross-priming in health and disease., Nature Reviews. Immunology. 10 (2010) 403–14. doi:10.1038/nri2780.

[16] L.J. Sigal, S. Crotty, R. Andino, K.L. Rock, Cytotoxic T-cell immunity to virus-infected non-haematopoietic cells requires presentation of exogenous antigen., Nature. 398 (1999) 77–80.

doi:10.1038/18038.

[17] C.M. Snyder, J.E. Allan, E.L. Bonnett, C.M. Doom, A.B. Hill, Cross-presentation of a spread-defective MCMV is sufficient to prime the majority of virus-specific CD8+ T cells., PloS One. 5 (2010) e9681. doi:10.1371/journal.pone.0009681.

[18] A. Busche, A.C. Jirmo, S.P.M. Welten, J. Zischke, J. Noack, H. Constabel, et al., Priming of CD8+ T cells against cytomegalovirus-encoded antigens is dominated by cross-presentation., Journal of Immunology (Baltimore, Md. : 1950). 190 (2013) 2767–77. doi:10.4049/jimmunol.1200966.

[19] S. Burgdorf, C. Kurts, Endocytosis mechanisms and the cell biology of antigen presentation., Current Opinion in Immunology. 20 (2008) 89–95. doi:10.1016/j.coi.2007.12.002.

[20] O.P. Joffre, E. Segura, A. Savina, S. Amigorena, Cross-presentation by dendritic cells., Nature Reviews. Immunology. 12 (2012) 557–69. doi:10.1038/nri3254.

[21] L.F. Poulin, M. Salio, E. Griessinger, F. Anjos-Afonso, L. Craciun, J.-L. Chen, et al., Characterization of human DNGR-1+ BDCA3+ leukocytes as putative equivalents of mouse CD8alpha+ dendritic cells., The Journal of Experimental Medicine. 207 (2010) 1261–71. doi:10.1084/jem.20092618.

[22] S. Thordardottir, B.N. Hangalapura, T. Hutten, M. Cossu, J. Spanholtz, N. Schaap, et al., The Aryl Hydrocarbon Receptor Antagonist StemRegenin 1 Promotes Human Plasmacytoid and Myeloid Dendritic Cell Development from CD34(+) Hematopoietic Progenitor Cells., Stem Cells and Development. (2014). doi:10.1089/scd.2013.0521.

[23] L. van de Laar, P.J. Coffer, A.M. Woltman, Regulation of dendritic cell development by GM-CSF: molecular control and implications for immune homeostasis and therapy., Blood. 119 (2012) 3383–93. doi:10.1182/blood-2011-11-370130.

[24] I. Fugier-Vivier, C. Servet-delprat, P. Rivailler, M.-C. Rissoan, Y.-J. Liu, C. Rabourdin-Combe, Measles Virus Suppresses Cell-mediated Immunity by Interfering with the Survival and Functions of Dendritic and T Cells, Journal of Experimental Medicine. 186 (1997) 813–823.

[25] M. van der Vlist, L. de Witte, R.D. De Vries, M. Litjens, M.A.W.P. de Jong, D. Fluitsma, et al., Human Langerhans cells capture measles virus through Langerin and present viral antigens to CD4+ T cells but are incapable of cross-presentation, European Journal of Immunology. 41 (2011) 2619–2631. doi:10.1002/eji.201041305.

[26] S. Riegler, H. Hebart, H. Einsele, P. Brossart, G. Jahn, C. Sinzger, Monocyte-derived dendritic cells are permissive to the complete replicative cycle of human cytomegalovirus, Journal of General Virology. 81 (2000) 393–399.

[27] Z. Tabi, M. Moutaftsi, L.K. Borysiewicz, Human Cytomegalovirus pp65- and Immediate Early 1 Antigen-Specific HLA Class I-restricted Cytotoxic T Cell Responses Induced by Cross-Presentation of Viral Antigens, Journal of Immunology. 166 (2001) 5695–5703.

[28] F. Halary, A. Amara, H. Lortat-Jacob, M. Messerle, T. Delaunay, C. Houles, et al., Human Cytomegalovirus Binding to DC-SIGN Is Required for Dendritic Cell Infection and Target Cell trans -Infection, Immunity. 17 (2002) 653–664.

[29] A. Bender, M. Albert, A. Reddy, M. Feldman, B. Sauter, G. Kaplan, et al., The distinctive features of influenza virus infection of dendritic cells., Immunobiology. 198 (1998) 552–67. doi:10.1016/S0171-2985(98)80078-8.

[30] K.S. Jones, C. Petrow-Sadowski, Y.K. Huang, D.C. Bertolette, F.W. Ruscetti, Cell-free HTLV-1 infects dendritic cells leading to transmission and transformation of CD4(+) T cells., Nature Medicine. 14 (2008) 429–36. doi:10.1038/nm1745.

[31] L. Manuel, T.D. Schell, E. Acheampong, S. Rahman, S.L. Manuel, Presentation of human T cell leukemia virus type 1 (HTLV-1) Tax protein by dendritic cells : the underlying mechanism of HTLV-1- associated neuroinflammatory disease, Journal of Leucocyte Biology. 86 (2009) 1205–1216. doi:10.1189/jlb.0309172.

[32] B. Tassaneetrithep, T.H. Burgess, A. Granelli-piperno, C. Trumpfheller, J. Finke, W. Sun, et al., DC-SIGN (CD209) mediates dengue virus infection of human dendritic cells., Journal of Experimental Medicine. 197 (2003) 823–9. doi:10.1084/jem.20021840.

[33] J. Engelmayer, M. Larsson, M. Subklewe, A. Chahroudi, W.I. Cox, R.M. Steinman, et al., Vaccinia Virus Inhibits the Maturation of Human Dendritic Cells: A Novel Mechanism of Immune Evasion, Journal of Immunology. 163 (1999) 6762–6768.

[34] P.M.A. de Graaff, E.C. de Jong, T.M. van Capel, M.E.A. van Dijk, P.J.M. Roholl, J. Boes, et al., Respiratory Syncytial Virus Infection of Monocyte-Derived Dendritic Cells Decreases Their Capacity to Activate CD4 T Cells, Journal of Immunology. 175 (2005) 5904–5911.

[35] M. Salio, M. Cella, M. Suter, A. Lanzavecchia, Inhibition of dendritic cell maturation by herpes simplex virus, European Journal of Immunology. 29 (1999) 3245–3253.

[36] M.C. Tan, L. Battini, A.C. Tuyama, S. Macip, G. a Melendi, M.-A. Horga, et al., Characterization of human metapneumovirus infection of myeloid dendritic cells., Virology. 357 (2007) 1–9. doi:10.1016/j.virol.2006.08.004.

[37] C. St Gelais, S. de Silva, S.M. Amie, C.M. Coleman, H. Hoy, J. a Hollenbaugh, et al., SAMHD1 restricts HIV-1 infection in dendritic cells (DCs) by dNTP depletion, but its expression in DCs and primary CD4+ T-lymphocytes cannot be upregulated by interferons., Retrovirology. 9 (2012) 105. doi:10.1186/1742-4690-9-105.

[38] A. Smed-Sörensen, K. Loré, J. Vasudevan, M.K. Louder, J. Andersson, J.R. Mascola, et al., Differential susceptibility to human immunodeficiency virus type 1 infection of myeloid and plasmacytoid dendritic cells., Journal of Virology. 79 (2005) 8861–9. doi:10.1128/JVI.79.14.8861-8869.2005.

[39] M.R. Gupta, D. Kolli, R.P. Garofalo, Differential response of BDCA-1 + and BDCA-3 + myeloid dendritic cells to respiratory syncytial virus infection, Respiratory Research. 14 (2013) 1. doi:10.1186/1465-9921-14-71.

[40] A. Smed-Sörensen, C. Chalouni, B. Chatterjee, L. Cohn, P. Blattmann, N. Nakamura, et al., Influenza A virus infection of human primary dendritic cells impairs their ability to cross-present antigen to CD8 T cells., PLoS Pathogens. 8 (2012) e1002572. doi:10.1371/journal.ppat.1002572.

[41] L. de Witte, A. Nabatov, M. Pion, D. Fluitsma, M.A.W.P. de Jong, T. de Gruijl, et al., Langerin is a natural barrier to HIV-1 transmission by Langerhans cells., Nature Medicine. 13 (2007) 367–71. doi:10.1038/nm1541.

[42] M. Cella, M. Salio, Y. Sakakibara, H. Langen, I. Julkunen, A. Lanzavecchia, Maturation, Activation, and Protection of Dendritic Cells Induced by Double-stranded RNA, Journal of Experimental Medicine. 189 (1999) 821 – 829.

[43] A. Bender, L.K. Bui, M.A.X. Feldman, M. Larsson, N. Bhardwaj, Inactivated Influenza Virus, when Presented on Dendritic Cells, Elicits Human CD8+ Cytolytic T Cell Responses, Journal of Experimental Medicine. 182 (1995) 1663–1671.

[44] F. Buseyne, S. Le Gall, C. Boccaccio, J.-P. Abastado, J.D. Lifson, L.O. Arthur, et al., MHC-I – restricted presentation of HIV-1 virion antigens without viral replication, Nature Medicine. 7 (2001) 344–349.

[45] M. Larsson, J. Fonteneau, M. Lirvall, P. Haslett, J.D. Lifson, N. Bhardwaj, Activation of HIV-1 specific CD4 and CD8 T cells by human dendritic cells : roles for cross-presentation and non-infectious HIV-1 virus, AIDS. 16 (2002) 1319–1329.

[46] R.L. Sabado, E. Babcock, D.G. Kavanagh, V. Tjomsland, B.D. Walker, J.D. Lifson, et al., Pathways utilized by dendritic cells for binding , uptake , processing and presentation of antigens derived from HIV-1, European Journal of Immunology. 37 (2007) 1752–1763. doi:10.1002/eji.200636981.

[47] T.H. Hansen, M. Bouvier, MHC class I antigen presentation: learning from viral evasion strategies., Nature Reviews. Immunology. 9 (2009) 503–13. doi:10.1038/nri2575.

[48] R.L. Sabado, M. O'Brien, A. Subedi, L. Qin, N. Hu, E. Taylor, et al., Evidence of dysregulation of dendritic cells in primary HIV infection., Blood. 116 (2010) 3839–52. doi:10.1182/blood-2010-03-273763.

[49] H. Barth, A. Ulsenheimer, G.R. Pape, H.M. Diepolder, M. Hoffmann, R. Thimme, et al., Uptake and presentation of hepatitis C virus – like particles by human dendritic cells, Blood. 105

(2005) 3605–3614. doi:10.1182/blood-2004-05-1952.

[50] M.P. Rudolf, S.C. Fausch, D.M. Da Silva, W.M. Kast, Human Dendritic Cells are activated by Chimeric Human Papillomavirus Type-16 Virus-Like Particles and induce epitope-specific human T cell responses in vitro, Journal of Immunology. 166 (2001) 5917–5924.

[51] D. Leclerc, D. Beauseigle, J. Denis, H. Morin, C. Paré, A. Lamarre, et al., Proteasome-Independent Major Histocompatibility Complex Class I Cross-Presentation Mediated by Papaya Mosaic Virus-Like Particles Leads to Expansion of Specific Human T Cells, Journal of Virology. 81 (2007) 1319. doi:10.1128/JVI.01720-06.

[52] F. Spadaro, C. Lapenta, S. Donati, L. Abalsamo, V. Barnaba, F. Belardelli, et al., IFN-α enhances cross-presentation in human dendritic cells by modulating antigen survival, endocytic routing, and processing., Blood. 119 (2012) 1407–17. doi:10.1182/blood-2011-06-363564.

[53] D. Accapezzato, V. Visco, V. Francavilla, C. Molette, T. Donato, M. Paroli, et al., Chloroquine enhances human CD8+ T cell responses against soluble antigens in vivo, Journal of Experimental Medicine. 202 (2005) 817–828. doi:10.1084/jem.20051106.

[54] H.T. Maecker, S. a Ghanekar, M. a Suni, X.S. He, L.J. Picker, V.C. Maino, Factors affecting the efficiency of CD8+ T cell cross-priming with exogenous antigens., Journal of Immunology (Baltimore, Md. : 1950). 166 (2001) 7268–75.

[55] Y. Jin, S.J. Wai-kuo, I. Berkhower, Human T cell response to the surface antigen of hepatitis B virus (HBsAg), Journal of Experimental Medicine. 168 (1988) 293–306.

[56] M. Haniffa, A. Shin, V. Bigley, N. McGovern, P. Teo, P. See, et al., Human Tissues Contain CD141(hi) Cross-Presenting Dendritic Cells with Functional Homology to Mouse CD103(+) Nonlymphoid Dendritic Cells., Immunity. 37 (2012) 1–14. doi:10.1016/j.immuni.2012.04.012.

[57] M.L. Albert, B. Sauter, N. Bhardwaj, Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs., Nature. 392 (1998) 86–9. doi:10.1038/32183.

[58] M. Larsson, J.-F. Fonteneau, S. Somersan, C. Sanders, K. Bickham, E.K. Thomas, et al., Efficiency of cross presentation of vaccinia virus- derived antigens by human dendritic cells, European Journal of Immunology. 31 (2001) 3432–3442.

[59] G. Arrode, C. Boccaccio, J. Lulé, S. Allart, N. Moinard, J. Abastado, et al., Incoming Human Cytomegalovirus pp65 (UL83) contained in Apoptotic Infected Fibroblasts is Cross-Presented to CD8 + T Cells by Dendritic Cells, Journal of Virology. 74 (2000) 10018 – 10024. doi:10.1128/JVI.74.21.10018-10024.

[60] W. Herr, E. Ranieri, W. Olson, H. Zarour, L. Gesualdo, W.J. Storkus, Mature dendritic cells pulsed with freeze – thaw cell lysates define an effective in vitro vaccine designed to elicit EBV-specific CD4+ and CD8+ T lymphocyte responses, Blood. 96 (2000) 1857–1864.

[61] M. Subklewe, C. Paludan, M.L. Tsang, K. Mahnke, R.M. Steinman, C. Münz, Dendritic Cells Cross-present Latency Gene Products from Epstein-Barr Virus – transformed B Cells and Expand Tumor-reactive CD8+ Killer T Cells, Journal of Experimental Medicine. 193 (2001) 405–411.

[62] J. Neijssen, C. Herberts, J.W. Drijfhout, E. Reits, L. Janssen, J. Neefjes, Cross-presentation by intercellular peptide transfer through gap junctions., Nature. 434 (2005) 83–8. doi:10.1038/nature03290.

[63] B.P. Dolan, K.D. Gibbs, S. Ostrand-Rosenberg, Dendritic cells cross-dressed with peptide MHC class I complexes prime CD8+ T cells., Journal of Immunology (Baltimore, Md. : 1950). 177 (2006) 6018–24.

[64] C. Qu, V.A. Nguyen, M. Merad, G.J. Randolph, MHC class I/peptide transfer between dendritic cells overcomes poor cross-presentation by monocyte-derived APCs that engulf dying cells., Journal of Immunology (Baltimore, Md. : 1950). 182 (2009) 3650–9. doi:10.4049/jimmunol.0801532.

[65] P.-Y. Lozach, L. Burleigh, I. Staropoli, E. Navarro-Sanchez, J. Harriague, J.-L. Virelizier, et al., Dendritic cell-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN)-mediated enhancement of dengue virus infection is independent of DC-SIGN internalization signals., The Journal of Biological Chemistry. 280 (2005) 23698–708. doi:10.1074/jbc.

M504337200.

[66] M.A.W.P. de Jong, L. de Witte, A. Bolmstedt, Y. van Kooyk, T.B.H. Geijtenbeek, Dendritic cells mediate herpes simplex virus infection and transmission through the C-type lectin, Journal of General Virology. 89 (2008) 2398–2409. doi:10.1099/vir.0.2008/003129-0.

[67] L. de Witte, M. Abt, S. Schneider-Schaulies, Y. van Kooyk, T.B.H. Geijtenbeek, Measles virus targets DC-SIGN to enhance dendritic cell infection., Journal of Virology. 80 (2006) 3477–86. doi:10.1128/JVI.80.7.3477-3486.2006.

[68] M.L.B. Hillaire, N.J. Nieuwkoop, A.C.M. Boon, G.F. Rimmelzwaan, Binding of DC-SIGN to the Hemagglutinin of Influenza A Viruses Supports Virus Replication in DC-SIGN Expressing Cells, 8 (2013) 1–10. doi:10.1371/journal.pone.0056164.

[69] T.B.H. Geijtenbeek, D.S. Kwon, R. Torensma, S.J. van Vliet, G.C.F. van Duijnhoven, J. Middel, et al., DC-SIGN , a Dendritic Cell – Specific HIV-1-Binding Protein that Enhances trans -Infection of T Cells, Cell. 100 (2000) 587–597.

[70] P. Jain, S.L. Manuel, Z.K. Khan, J. Ahuja, K. Quann, B. Wigdahl, DC-SIGN mediates cell-free infection and transmission of human T-cell lymphotropic virus type 1 by dendritic cells., Journal of Virology. 83 (2009) 10908–21. doi:10.1128/JVI.01054-09.

[71] W.W.J. Unger, Y. van Kooyk, “Dressed for success” C-type lectin receptors for the delivery of glyco-vaccines to dendritic cells., Current Opinion in Immunology. 23 (2011) 131–7. doi:10.1016/j.coi.2010.11.011.

[72] A.A. Lambert, C. Gilbert, M. Richard, A.D. Beaulieu, M.J. Tremblay, The C-type lectin surface receptor DCIR acts as a new attachment factor for HIV-1 in dendritic cells and contributes to trans- and cis-infection pathways., Blood. 112 (2008) 1299–307. doi:10.1182/blood-2008-01-136473.

[73] M.L. Op den Brouw, R.S. Binda, T.B.H. Geijtenbeek, H.L.A. Janssen, A.M. Woltman, The mannose receptor acts as hepatitis B virus surface antigen receptor mediating interaction with intrahepatic dendritic cells., Virology. 393 (2009) 84–90. doi:10.1016/j.virol.2009.07.015.

[74] L. de Witte, M. Bobardt, U. Chatterji, G. Degeest, G. David, T.B.H. Geijtenbeek, et al., Syndecan-3 is a dendritic cell-specific attachment receptor for HIV-1., Proceedings of the National Academy of Sciences of the United States of America. 104 (2007) 19464–9. doi:10.1073/pnas.0703747104.

[75] L. de Witte, Y. Zoughlami, B. Aengeneyndt, G. David, Y. van Kooyk, L. Gissmann, et al., Binding of human papilloma virus L1 virus-like particles to dendritic cells is mediated through heparan sulfates and induces immune activation., Immunobiology. 212 (2007) 679–91. doi:10.1016/j.imbio.2007.09.006.

[76] A. Moris, C. Nobile, F. Buseyne, F. Porrot, J.-P. Abastado, O. Schwartz, DC-SIGN promotes exogenous MHC-I-restricted HIV-1 antigen presentation., Blood. 103 (2004) 2648–54. doi:10.1182/blood-2003-07-2532.

[77] V. Tjomsland, R. Elleg, K.F. Che, G. Westmacott, J. Hinkula, J.D. Lifson, et al., Complement opsonization of HIV-1 results in a different intracellular processing pattern and enhanced MHC class I presentation by dendritic cells, European Journal of Immunology. 43 (2013) 1–14. doi:10.1002/eji.201242935.

[78] B. Chatterjee, A. Smed-sörensen, L. Cohn, C. Chalouni, R. Vandlen, J. Widger, et al., Internalization and endosomal degradation of receptor-bound antigens regulate the efficiency of cross presentation by human dendritic cells, Blood. 120 (2012) 2011–2020. doi:10.1182/blood-2012-01-402370.

[79] E. Klechevsky, A.-L. Flamar, Y. Cao, J.-P. Blanck, M. Liu, A. O’Bar, et al., Cross-priming CD8+ T cells by targeting antigens to human dendritic cells through DCIR., Blood. 116 (2010) 1685–97. doi:10.1182/blood-2010-01-264960.

[80] P.J. Tacken, W. Ginter, L. Berod, L.J. Cruz, B. Joosten, T. Sparwasser, et al., Targeting DC-SIGN via its neck region leads to prolonged antigen residence in early endosomes, delayed lysosomal degradation, and cross-presentation., Blood. 118 (2011) 4111–9. doi:10.1182/blood-2011-04-346957.

[81] G. Schreibelt, L.J.J. Klinkenberg, L.J. Cruz, P.J. Tacken, J. Tel, M. Kreutz, et al., The C-type lectin receptor CLEC9A mediates antigen uptake and (cross-)presentation by human blood BDCA3+ myeloid dendritic cells., Blood. 119 (2012) 2284–92. doi:10.1182/blood-2011-08-373944.

[82] T.W.H. Flinsenberg, E.B. Compeer, D. Koning, M. Klein, F.J. Amelung, D. van Baarle, et al., Fcγ receptor antigen targeting potentiates cross-presentation by human blood and lymphoid tissue BDCA-3+ dendritic cells., Blood. 120 (2012) 5163–5172. doi:10.1182/blood-2012-06-434498.

[83] E. Celis, V.R. Zurawski, T.W. Chang, Regulation of T-cell function by antibodies: enhancement of the response of human T-cell clones to hepatitis B surface antigen by antigen-specific monoclonal antibodies., Proceedings of the National Academy of Sciences of the United States of America. 81 (1984) 6846–50.

[84] H. Barth, E.K. Schnober, C. Neumann-Haefelin, C. Thumann, M.B. Zeisel, H.M. Diepolder, et al., Scavenger receptor class B is required for hepatitis C virus uptake and cross-presentation by human dendritic cells., Journal of Virology. 82 (2008) 3466–79. doi:10.1128/JVI.02478-07.

[85] A. Murshid, J. Gong, S.K. Calderwood, The role of heat shock proteins in antigen cross presentation., Frontiers in Immunology. 3 (2012) 63. doi:10.3389/fimmu.2012.00063.

[86] S. Burgdorf, C. Schölz, A. Kautz, R. Tampé, C. Kurts, Spatial and mechanistic separation of cross-presentation and endogenous antigen presentation., Nature Immunology. 9 (2008) 558–66. doi:10.1038/ni.1601.

[87] E. Segura, J.A. Villadangos, A modular and combinatorial view of the antigen cross-presentation pathway in dendritic cells., Traffic (Copenhagen, Denmark). 12 (2011) 1677–85. doi:10.1111/j.1600-0854.2011.01254.x.

[88] N. van Montfoort, M.G. Camps, S. Khan, D. V Filippov, J.J. Weterings, J.M. Griffith, et al., Antigen storage compartments in mature dendritic cells facilitate prolonged cytotoxic T lymphocyte cross-priming capacity., Proceedings of the National Academy of Sciences of the United States of America. 106 (2009) 6730–5. doi:10.1073/pnas.0900969106.

[89] F. Faure, A. Mantegazza, C. Sadaka, C. Sedlik, F. Jotereau, S. Amigorena, Long-lasting cross-presentation of tumor antigen in human DC., European Journal of Immunology. 39 (2009) 380–90. doi:10.1002/eji.200838669.

[90] E. Segura, M. Durand, S. Amigorena, Similar antigen cross-presentation capacity and phagocytic functions in all freshly isolated human lymphoid organ-resident dendritic cells., The Journal of Experimental Medicine. 210 (2013) 1035–47. doi:10.1084/jem.20121103.

[91] J. Neefjes, C. Sadaka, Into the intracellular logistics of cross-presentation., Frontiers in Immunology. 3 (2012) 31. doi:10.3389/fimmu.2012.00031.

[92] S. Burgdorf, A. Kautz, V. Böhnert, P. a Knolle, C. Kurts, Distinct pathways of antigen uptake and intracellular routing in CD4 and CD8 T cell activation., Science (New York, N.Y.). 316 (2007) 612–6. doi:10.1126/science.1137971.

[93] E.B. Compeer, T.W.H. Flinsenberg, S.G. van der Grein, M. Boes, Antigen processing and remodeling of the endosomal pathway: requirements for antigen cross-presentation., Frontiers in Immunology. 3 (2012) 37. doi:10.3389/fimmu.2012.00037.

[94] J.J. Skehel, D.C. Wiley, Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin., Annual Review of Biochemistry. 69 (2000) 531–69. doi:10.1146/annurev.biochem.69.1.531.

[95] L. Cohn, B. Chatterjee, F. Esselborn, A. Smed-sörensen, N. Nakamura, C. Chalouni, et al., Antigen delivery to early endosomes eliminates the superiority of human blood BDCA3 + dendritic cells at cross presentation, Journal of Experimental Medicine. 210 (2013) 1049–1063. doi:10.1084/jem.20121251.

[96] M.L. Albert, S.F. Pearce, L.M. Francisco, B. Sauter, P. Roy, R.L. Silverstein, et al., Immature dendritic cells phagocytose apoptotic cells via alphavbeta5 and CD36, and cross-present antigens to cytotoxic T lymphocytes., The Journal of Experimental Medicine. 188 (1998) 1359–68.

[97] J.F. Fonteneau, D.G. Kavanagh, M. Lirvall, C. Sanders, T.L. Cover, N. Bhardwaj, et al., Characterization of the MHC class I cross-presentation pathway for cell-associated antigens

by human dendritic cells., Blood. 102 (2003) 4448–55. doi:10.1182/blood-2003-06-1801.

[98] D. Stober, Z. Trobonjaca, J. Reimann, R. Schirmbeck, Dendritic cells pulsed with exogenous hepatitis B surface antigen particles efficiently present epitopes to MHC class I-restricted cytotoxic T cells., European Journal of Immunology. 32 (2002) 1099–108. doi:10.1002/1521-4141(200204)32:4<1099::AID-IMMU1099>3.0.CO;2-8.

[99] M. Lakadamyali, M.J. Rust, X. Zhuang, Ligands for clathrin-mediated endocytosis are differentially sorted into distinct populations of early endosomes., Cell. 124 (2006) 997–1009. doi:10.1016/j.cell.2005.12.038.

[100] I.S. Ludwig, A.N. Lekkerkerker, E. Depla, F. Bosman, J.P. Musters, S. Depraetere, et al., Hepatitis C Virus Targets DC-SIGN and L-SIGN To Escape Lysosomal Degradation, Journal of Virology. 78 (2004) 8322–8332. doi:10.1128/JVI.78.15.8322.

[101] M. Zehner, A.I. Chasan, V. Schuette, M. Embgenbroich, T. Quast, W. Kolanus, et al., Mannose receptor polyubiquitination regulates endosomal recruitment of p97 and cytosolic antigen translocation for cross-presentation., Proceedings of the National Academy of Sciences of the United States of America. 108 (2011) 9933–8. doi:10.1073/pnas.1102397108.

[102] T. Di Pucchio, B. Chatterjee, A. Smed-Sörensen, S. Clayton, A. Palazzo, M. Montes, et al., Direct proteasome-independent cross-presentation of viral antigen by plasmacytoid dendritic cells on major histocompatibility complex class I., Nature Immunology. 9 (2008) 551–7. doi:10.1038/ni.1602.

[103] F. Ossendorp, N. Fu, M. Camps, F. Granucci, S.J.P. Gobin, P.J. van den Elsen, et al., Differential expression regulation of the alpha and beta subunits of the PA28 proteasome activator in mature dendritic cells., Journal of Immunology. 174 (2005) 7815–22.

[104] J. Tel, S.P. Sittig, R.A.M. Blom, J. Luis, G. Schreibelt, C.G. Figdor, et al., Targeting Uptake Receptors on Human Plasmacytoid Dendritic Cells Triggers Antigen Cross-Presentation and Robust Type I IFN Secretion, Journal of Immunology. 191 (2013) 5005–5012. doi:10.4049/jimmunol.1300787.

[105] M. Larsson, D. Messmer, S. Somersan, J. Fonteneau, S.M. Donahoe, M. Lee, et al., Requirement of Mature Dendritic Cells for Efficient Activation of Influenza A-Specific Memory CD8 + T Cells, Journal of Immunology. 165 (2000) 1182–1190.

[106] J. Tel, G. Schreibelt, S.P. Sittig, T.S.M. Mathan, S.I. Buschow, L.J. Cruz, et al., Human plasmacytoid dendritic cells efficiently cross-present exogenous Ags to CD8+ T cells despite lower Ag uptake than myeloid dendritic cell subsets, Blood. 121 (2013) 459–467. doi:10.1182/blood-2012-06-435644.

[107] J.-F. Fonteneau, M. Gilliet, M. Larsson, I. Dasilva, C. Münz, Y.-J. Liu, et al., Activation of influenza virus-specific CD4+ and CD8+ T cells: a new role for plasmacytoid dendritic cells in adaptive immunity., Blood. 101 (2003) 3520–6. doi:10.1182/blood-2002-10-3063.

[108] A.M. Woltman, M.L. Op den Brouw, P.J. Biesta, C.C. Shi, H.L.A. Janssen, Hepatitis B virus lacks immune activating capacity, but actively inhibits plasmacytoid dendritic cell function., PloS One. 6 (2011) e15324. doi:10.1371/journal.pone.0015324.

[109] C.K. Holm, S.B. Jensen, M.R. Jakobsen, N. Cheshenko, K.A. Horan, H.B. Moeller, et al., Virus-cell fusion as a trigger of innate immunity dependent on the adaptor STING., Nature Immunology. 13 (2012) 737–43. doi:10.1038/ni.2350.

[110] O. Joffre, M.A. Nolte, R. Spörri, C. Reis e Sousa, Inflammatory signals in dendritic cell activation and the induction of adaptive immunity., Immunological Reviews. 227 (2009) 234–47. doi:10.1111/j.1600-065X.2008.00718.x.

[111] G.Y. Chen, G. Nuñez, Sterile inflammation: sensing and reacting to damage., Nature Reviews. Immunology. 10 (2010) 826–37. doi:10.1038/nri2873.

[112] G. Arrode, C. Boccaccio, J. Abastado, C. Davrinche, Cross-Presentation of Human Cytomegalovirus pp65 (UL83) to CD8+ T Cells Is Regulated by Virus-Induced , Soluble-Mediator-Dependendent Maturation of Dendritic Cells, Journal of Virology. 76 (2002) 142 – 150. doi:10.1128/JVI.76.1.142.

[113] T. Ebihara, M. Shingai, M. Matsumoto, T. Wakita, T. Seya, Hepatitis C Virus–Infected

Hepatocytes Extrinsically Modulate Dendritic Cell Maturation To Activate T Cells and Natural Killer Cells, Hepatology. 48 (2008) 48–58. doi:10.1002/hep.22337.

[114] C. Locher, R. Conforti, L. Aymeric, Y. Ma, T. Yamazaki, S. Rusakiewicz, et al., Desirable cell death during anticancer chemotherapy., Annals of the New York Academy of Sciences. 1209 (2010) 99–108. doi:10.1111/j.1749-6632.2010.05763.x.

[115] S. Ahrens, S. Zelenay, D. Sancho, P. Hanč, S. Kjær, C. Feest, et al., F-Actin Is an Evolutionarily Conserved Damage-Associated Molecular Pattern Recognized by DNGR-1, a Receptor for Dead Cells, Immunity. (2012) 1–11. doi:10.1016/j.immuni.2012.03.008.

[116] D. Mittag, A.I. Proietto, T. Loudovaris, S.I. Mannering, D. Vremec, K. Shortman, et al., Human dendritic cell subsets from spleen and blood are similar in phenotype and function but modified by donor health status., Journal of Immunology (Baltimore, Md. : 1950). 186 (2011) 6207–17. doi:10.4049/jimmunol.1002632.

[117] G. Nizzoli, J. Krietsch, A. Weick, S. Steinfelder, F. Facciotti, P. Gruarin, et al., Human CD1c+ dendritic cells secrete high levels of IL-12 and potently prime cytotoxic T-cell responses., Blood. 122 (2013) 932–42. doi:10.1182/blood-2013-04-495424.

[118] D. Frleta, C.I. Yu, E. Klechevsky, A.-L. Flamar, G. Zurawski, J. Banchereau, et al., Influenza virus and poly(I:C) inhibit MHC class I-restricted presentation of cell-associated antigens derived from infected dead cells captured by human dendritic cells., Journal of Immunology (Baltimore, Md. : 1950). 182 (2009) 2766–76. doi:10.4049/jimmunol.0801720.

[119] C. Lapenta, S.M. Santini, M. Spada, S. Donati, F. Urbani, D. Accapezzato, et al., IFN-alpha-conditioned dendritic cells are highly efficient in inducing cross-priming CD8(+) T cells against exogenous viral antigens., European Journal of Immunology. 36 (2006) 2046–60. doi:10.1002/eji.200535579.

[120] I. Grosjean, C. Caux, C. Bella, I. Berger, F. Wild, J. Banchereau, et al., Measles virus infects human dendritic cells and blocks their allostimulatory properties for CD4+ T cells., The Journal of Experimental Medicine. 186 (1997) 801–12.

[121] L. Samady, E. Costigliola, L. MacCormac, Y. McGrath, S. Cleverley, C.E. Lilley, et al., Deletion of the Virion Host Shutoff Protein (vhs) from Herpes Simplex Virus (HSV) Relieves the Viral Block to Dendritic Cell Activation : Potential of vhs − HSV Vectors for Dendritic Cell-Mediated Immunotherapy, Journal of Virology. 77 (2003) 3768–3776. doi:10.1128/JVI.77.6.3768.

[122] M. Moutaftsi, A.M. Mehl, L.K. Borysiewicz, Z. Tabi, Human cytomegalovirus inhibits maturation and impairs function of monocyte-derived dendritic cells, Blood. 99 (2002) 2913–2921. doi:10.1182/blood.V99.8.2913.

[123] B. Liu, A.M. Woltman, H.L.A. Janssen, A. Boonstra, Modulation of dendritic cell function by persistent viruses., Journal of Leukocyte Biology. 85 (2009) 205–14. doi:10.1189/jlb.0408241.

[124] A.M. Woltman, A. Boonstra, H.L.A. Janssen, Dendritic cells in chronic viral hepatitis B and C: victims or guardian angels?, Gut. 59 (2010) 115–25. doi:10.1136/gut.2009.181040.

[125] A. Bachem, S. Güttler, E. Hartung, F. Ebstein, M. Schaefer, A. Tannert, et al., Superior antigen cross-presentation and XCR1 expression define human CD11c+CD141+ cells as homologues of mouse CD8+ dendritic cells., The Journal of Experimental Medicine. 207 (2010) 1273–81. doi:10.1084/jem.20100348.

[126] K. Crozat, R. Guiton, V. Contreras, V. Feuillet, C.-A. Dutertre, E. Ventre, et al., The XC chemokine receptor 1 is a conserved selective marker of mammalian cells homologous to mouse CD8alpha+ dendritic cells., The Journal of Experimental Medicine. 207 (2010) 1283–92. doi:10.1084/jem.20100223.

[127] S.H. Robbins, T. Walzer, D. Dembélé, C. Thibault, A. Defays, G. Bessou, et al., Novel insights into the relationships between dendritic cell subsets in human and mouse revealed by genome-wide expression profiling., Genome Biology. 9 (2008) R17. doi:10.1186/gb-2008-9-1-r17.

[128] A. Savina, A. Peres, I. Cebrian, N. Carmo, C. Moita, N. Hacohen, et al., The small GTPase Rac2 controls phagosomal alkalinization and antigen crosspresentation selectively in CD8(+) dendritic cells., Immunity. 30 (2009) 544–55. doi:10.1016/j.immuni.2009.01.013.

[129] C. Huysamen, J.A. Willment, K.M. Dennehy, G.D. Brown, CLEC9A is a novel activation C-type

lectin-like receptor expressed on BDCA3+ dendritic cells and a subset of monocytes., The Journal of Biological Chemistry. 283 (2008) 16693–701. doi:10.1074/jbc.M709923200.

[130] D. Sancho, C. Reis e Sousa, Sensing of cell death by myeloid C-type lectin receptors., Current Opinion in Immunology. 25 (2013) 46–52. doi:10.1016/j.coi.2012.12.007.

[131] S. Zelenay, A.M. Keller, P.G. Whitney, B.U. Schraml, S. Deddouche, N.C. Rogers, et al., The dendritic cell receptor DNGR-1 controls endocytic handling of necrotic cell antigens to favor cross-priming of CTLs in virus-infected mice., The Journal of Clinical Investigation. 122 (2012) 1615–27. doi:10.1172/JCI60644.

[132] J. Tel, A.M. van der Leun, C.M. Figdor, R. Torensma, I.J.M. de Vries, Harnessing human plasmacytoid dendritic cells as professional APCs, Cancer Immunology Immunotherapy. 61 (2012) 1279–1288. doi:10.1007/s00262-012-1210-z.

[133] G. Hoeffel, A. Ripoche, D. Matheoud, M. Nascimbeni, M. Gannage, N. Escriou, et al., Antigen Crosspresentation by Human Plasmacytoid Dendritic Cells, (2007) 481–492. doi:10.1016/j.immuni.2007.07.021.

[134] G. Lui, O. Manches, J. Angel, J.-P. Molens, L. Chaperot, J. Plumas, Plasmacytoid dendritic cells capture and cross-present viral antigens from influenza-virus exposed cells., PloS One. 4 (2009) e7111. doi:10.1371/journal.pone.0007111.

[135] E. Segura, J. Valladeau-Guilemond, M.-H. Donnadieu, X. Sastre-Garau, V. Soumelis, S. Amigorena, Characterization of resident and migratory dendritic cells in human lymph nodes, Journal of Experimental Medicine. 209 (2012) 653–660. doi:10.1084/jem.20111457.

[136] J. Banchereau, S. Zurawski, L. Thompson-Snipes, J.-P. Blanck, S. Clayton, A. Munk, et al., Immunoglobulin-like transcript receptors on human dermal CD14+ dendritic cells act as a CD8-antagonist to control cytotoxic T cell priming., Proceedings of the National Academy of Sciences of the United States of America. 109 (2012) 18885–90. doi:10.1073/pnas.1205785109.

[137] M. Matsuo, Y. Nagata, E. Sato, D. Atanackovic, D. Valmori, Y.-T. Chen, et al., IFN-gamma enables cross-presentation of exogenous protein antigen in human Langerhans cells by potentiating maturation., Proceedings of the National Academy of Sciences of the United States of America. 101 (2004) 14467–72. doi:10.1073/pnas.0405947101.

[138] Y. Zhang, Y. Liu, K.M. Moxley, L. Golden-Mason, M.G. Hughes, T. Liu, et al., Transduction of human T cells with a novel T-cell receptor confers anti-HCV reactivity., PLoS Pathogens. 6 (2010) e1001018. doi:10.1371/journal.ppat.1001018.

[139] A.J. Gehring, S.-A. Xue, Z.Z. Ho, D. Teoh, C. Ruedl, A. Chia, et al., Engineering virus-specific T cells that target HBV infected hepatocytes and hepatocellular carcinoma cell lines., Journal of Hepatology. 55 (2011) 103–10. doi:10.1016/j.jhep.2010.10.025.

[140] S.H. van der Burg, R. Arens, C.J.M. Melief, Immunotherapy for persistent viral infections and associated disease., Trends in Immunology. 32 (2011) 97–103. doi:10.1016/j.it.2010.12.006.

[141] J. Tel, D. Benitez-ribas, S. Hoosemans, A. Cambi, G.J. Adema, C.G. Figdor, et al., DEC-205 mediates antigen uptake and presentation by both resting and activated human plasmacytoid dendritic cells, European Journal of Immunology. 41 (2011) 1014–1023. doi:10.1002/eji.201040790.

[142] R. a Kroczek, V. Henn, The Role of XCR1 and its Ligand XCL1 in Antigen Cross-Presentation by Murine and Human Dendritic Cells., Frontiers in Immunology. 3 (2012) 14. doi:10.3389/fimmu.2012.00014.

[143] D.H. Schuurhuis, A. Ioan-Facsinay, B. Nagelkerken, J.J. van Schip, C. Sedlik, C.J.M. Melief, et al., Antigen-antibody immune complexes empower dendritic cells to efficiently prime specific CD8+ CTL responses in vivo., Journal of Immunology (Baltimore, Md. : 1950). 168 (2002) 2240–6.

[144] G.G.P. Zom, S. Khan, D. V Filippov, F. Ossendorp, TLR ligand-peptide conjugate vaccines: toward clinical application., Advances in Immunology. 114 (2012) 177–201. doi:10.1016/B978-0-12-396548-6.00007-X.

[145] A.J. Gehring, M. Haniffa, P.T. Kennedy, Z.Z. Ho, C. Boni, A. Shin, et al., Mobilizing monocytes to

cross-present circulating viral antigen in chronic infection., The Journal of Clinical Investigation. 123 (2013) 3766–76. doi:10.1172/JCI66043.

[146] K.P.A. MacDonald, D.J. Munster, G.J. Clark, A. Dzionek, J. Schmitz, D.N.J. Hart, Characterization of human blood dendritic cell subsets., Blood. 100 (2002) 4512–20. doi:10.1182/blood-2001-11-0097.

[147] S. Ebner, Z. Ehammer, S. Holzmann, P. Schwingshackl, M. Forstner, P. Stoitzner, et al., Expression of C-type lectin receptors by subsets of dendritic cells in human skin., International Immunology. 16 (2004) 877–87. doi:10.1093/intimm/dxh088.

[148] E.E. Bates, N. Fournier, E. Garcia, J. Valladeau, I. Durand, J.J. Pin, et al., APCs express DCIR, a novel C-type lectin surface receptor containing an immunoreceptor tyrosine-based inhibitory motif., Journal of Immunology (Baltimore, Md. : 1950). 163 (1999) 1973–83.

[149] M. Lambotin, S. Raghuraman, F. Stoll-Keller, T.F. Baumert, H. Barth, A look behind closed doors: interaction of persistent viruses with dendritic cells., Nature Reviews. Microbiology. 8 (2010) 350–60. doi:10.1038/nrmicro2332.

[150] C. Eklöw, D. Makrygiannakis, L. Bäckdahl, L. Padyukov, A.-K. Ulfgren, J.C. Lorentzen, et al., Cellular distribution of the C-type II lectin dendritic cell immunoreceptor (DCIR) and its expression in the rheumatic joint: identification of a subpopulation of DCIR+ T cells., Annals of the Rheumatic Diseases. 67 (2008) 1742–9. doi:10.1136/ard.2007.076976.

[151] S.G. Turville, P.U. Cameron, A. Handley, G. Lin, S. Pöhlmann, R.W. Doms, et al., Diversity of receptors binding HIV on dendritic cell subsets., Nature Immunology. 3 (2002) 975–83. doi:10.1038/ni841.

[152] H. Donaghy, J. Wilkinson, A.L. Cunningham, HIV interactions with dendritic cells: has our focus been too narrow?, Journal of Leukocyte Biology. 80 (2006) 1001–12. doi:10.1189/jlb.0306158.

[153] A. Bachem, E. Hartung, S. Güttler, A. Mora, X. Zhou, A. Hegemann, et al., Expression of XCR1 Characterizes the Batf3-Dependent Lineage of Dendritic Cells Capable of Antigen Cross-Presentation., Frontiers in Immunology. 3 (2012) 214. doi:10.3389/fimmu.2012.00214.

BDCA3^{+}CLEC9A^{+} human dendritic cell function and development

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Chapter 3

Abstract

Dendritic cells (DC) are the most potent antigen presenting cells (APC). They comprise a family of different subsets and play an essential role in the induction and regulation of immune responses. Recently, gene expression profiling identified BDCA3$^+$CLEC9A$^+$ DC as a separate human DC subset. This subset was identified in blood, where they represent the smallest population of human DC, as well as in lymphoid and peripheral tissues. This review summarizes the phenotypic, functional and developmental characteristics of BDCA3$^+$CLEC9A$^+$ DC in relation to their mouse equivalents CD8α$^+$ DC and CD103$^+$ DC and other human DC subsets. Apart from being potent antigen presenting cells, their specialized functional capacities compared to other human DC subsets, indicate that these BDCA3$^+$CLEC9A$^+$ DC cells are of major importance in the induction of anti-viral and anti-tumor immunity. Further characterization of their functional properties, developmental pathways and underlying molecular mechanisms may identify target molecules to fully exploit the immune modulatory function of BDCA3$^+$CLEC9A$^+$ DC and potential use of these cells in immunotherapy.

BDCA3+CLEC9A+ DC as a unique human DC subset

DC represent a heterogeneous population of professional APC that are found in almost all tissues. Due to their unique ability to stimulate naive T cells, DC serve as a major link between innate and adaptive immunity [1, 2]. Additionally, they interact with other cells of the innate and adaptive immune system. DC comprise a family of different subsets that all share the capacity to activate naive T cell responses, but vary in ontogeny, localization, phenotype and specialized immune functions [3, 4].

Most of the present knowledge on DC has been obtained from studies of DC in mice. In mice, DC are divided into plasmacytoid DC (pDC), conventional lymphoid-resident DC and migratory DC. The lymphoid-resident DC can be further divided into CD8α+ and CD8α- DC, whereas migratory DC include CD103+ DC, CD11b+ DC and Langerhans cells [5-7]. CD8α+ lymphoid-resident DC are more efficient than CD8α- DC at cross-presentation of exogenous antigen (Ag) and priming of CD8+ T cells, due to different intrinsic molecular properties [7]. CD103+ migratory DC were identified as the equivalent of CD8α+ DC in peripheral tissues since they share a common gene signature, developmental pathway, functional capacities, and several phenotypic characteristics [7-11].

Direct correlation of human and mouse DC subsets has been hampered by a lack of common surface markers. Human DC, which are characterized by expression of HLA-DR and lack of lineage markers, can be divided into two main categories: pDC and myeloid DC (mDC). mDC, which in contrast to pDC express CD11c+, can be further divided into BDCA1+ DC and BDCA3+ DC [12]. Comparative genomic analysis indicated that the gene expression profile of human blood BDCA3+ DC is comparable to that of mouse CD8α+ and CD103+ DC, suggesting that BDCA3+ DC might also be functionally related to this lineage [13].

Phenotypic characteristics

The finding of the equivalence between CD8α+ and BDCA3+ DC was confirmed by the identification of markers that were expressed on both DC subsets, including CLEC9A (c-type lectin domain family 9A), also known as DNGR-1, XCR1 (XC chemokine receptor 1), Necl2 (Nectin-like molecule 2), also known as CADM1 (cell adhesion molecule 1), and TLR3 (Toll-like receptor 3) [14-21]. These markers will be discussed in more detail below.

BDCA3/CD141/Thrombomodulin

BDCA3 is a cell surface-expressed transmembrane glycoprotein, also known as Thrombomodulin (TM) or CD141. Together with BDCA1, BDCA2 and BDCA4, BDCA3 is generally used to classify human DC. However, BDCA3 expression is not restricted to the typical BDCA3+ DC subset as discussed here, since intermediate expression of BDCA3 has also been found on monocytes and other DC, such as blood pDC, pulmonary Langerhans-type and interstitial DC, and skin interstitial DC [11, 22-26]. In addition, *in vitro* maturation of blood DC is associated with upregulation of BDCA3 on pDC and BDCA1+ mDC [12]. In many studies which analyze the function of BDCA3+ DC, these DC were isolated by magnetic separation using MACS beads directed against BDCA3. The typical BDCA3+ DC subset expresses higher levels of BDCA3 than other DC, so optimal use of antibodies will result in enrichment of these BDCA3hi DC. However, since BDCA3 expression is not selectively expressed by the BDCA3hi DC subset, this type of isolation should be performed with caution, since it may

also result in isolation of a heterogeneous BDCA3$^+$ cell population that includes other DC subtypes and/or monocytes, as mentioned above. Therefore, it is important to use BDCA3 in combination with discriminating markers that are unique to the BDCA3^{hi} DC subset to assure selection of BDCA3$^+$ DC that belong to the CD8α$^+$-like lineage.
The function of BDCA3 expression on DC is thus far unknown. Besides its expression on DC, BDCA3 is predominantly expressed on vascular endothelial cells, where it is generally denominated as TM and well known for its anticoagulant activity. By binding thrombin, which is a pro-coagulant playing an important role in the coagulation cascade, TM/BDCA3 reduces thrombin's pro-coagulant activity. However, the expression of TM/BDCA3 on other cells than endothelial cells suggests an additional function of TM/BDCA3. Indeed, more recently TM/BDCA3 was described to have also a potent anti-inflammatory function through several direct and indirect mechanisms, which are reviewed by Li *et al.* [8]. In brief, these mechanisms include blocking of the pro-inflammatory factors thrombin, lipopolysaccharide (LPS) and necrotic or inflammatory cell-derived high mobility group box 1 (HMGB1) protein, activation of the anti-inflammatory proteins activated protein C (aPC) and activated thrombin-activatable fibrinolysis inhibitor (TAFI) and activation of inhibitors of the complement system [8].
Although high expression of this marker on BDCA3$^+$ DC might suggests an anti-inflammatory function of this DC subset, the exact function of BDCA3 expression on these cells remains to be elucidated.

CLEC9A
Although low levels of CLEC9A are expressed on mouse pDC and a subset of human monocytes, high CLEC9A expression is restricted to mouse CD8α$^+$ and CD103$^+$ DC and human BDCA3^{hi} DC, which share a common transcriptomic profile and set of phenotypic characteristics [15, 16, 27]. This indicates that CLEC9A, in combination with BDCA3, can be used as a distinctive marker to identify DC of this lineage and therefore, the human BDCA3^{hi} DC subset will be further referred to as BDCA3$^+$CLEC9A$^+$ DC in the present review. CLEC9A is a damage-associated molecular pattern (DAMP) receptor that senses necrotic cells [27]. Ligation of CLEC9A does not activate DC, but regulates cross-presentation of necrotic cell-associated Ag via recruitment and activation of the tyrosine kinase Syk [27, 28]. However, until recently the ligand for CLEC9A was not known. Two different research groups reported filamentous actin (F-actin), which is exposed on cells upon cellular damage or necrosis, as a CLEC9A ligand [29, 30].

XCR1
XCR1 is a chemokine receptor which binds to its unique ligand chemokine (C motif) ligand 1 (XCL1), selectively expressed by CD8$^+$ T cells, Th1 cells and NK cells [17]. In both human and mice, XCR1 was shown to play an important role in the interaction between XCL1-producing CD8$^+$ T cells and XCR1$^+$ DC and subsequent activation of CD8$^+$ T cells [31-33]. Upon interaction with XCR1$^+$ DC, CD8$^+$ T cells secreted high levels of XCL1, which enhanced the survival and effector functions of CD8$^+$ T cells [33].
XCR1 is selectively expressed on human BDCA3$^+$ DC, mouse CD8α$^+$ DC, mouse CD103$^+$ DC, and sheep CD26$^+$ DC. This selective expression was confirmed by the observation that BDCA3$^+$CLEC9A$^+$ DC were the only human DC subset that migrates to XCL1 [18]. Since low CLEC9A expression can also be found on other cells, whereas XCR1 expression is highly restricted to BDCA3$^+$CLEC9A$^+$ DC in human, XCR1 seems to be the ideal marker to identify

DC belonging to the BDCA3+ CD8α+-like DC subset [17]. However, the specificity of currently available antibodies against human XCR1 is debatable, and therefore, thorough research on this marker has been hampered.

Necl2

Another marker which is characteristic for the CD8α+-like DC lineage is Necl2. Necl2 is a ubiquitously expressed adhesion molecule that is involved in wound healing and can also act as a tumor suppressor [34, 35]. Although Necl2 can be expressed by various celltypes, among blood leukocytes its expression is restricted to BDCA3+CLEC9A+ DC. Necl2 interacts with Class-I-restricted T-cell-associated molecule (CRTAM). Since CRTAM is primarily expressed on activated cytotoxic T lymphocytes (CTL), the Necl2-CRTAM pair seems to be involved in the cross-talk between DC and T cells [19]. However, the specific function of this adhesion molecule on BDCA3+CLEC9A+ DC thus far remains elusive.

TLR3

Toll-like receptor 3 is a pattern recognition receptor (PRR) that recognizes viral double-stranded RNA (dsRNA) and induces production of anti-viral interferons (IFN) through activation of interferon regulatory factor 3 (IRF3) and pro-inflammatory cytokines through NFκB. Hence, TLR3 is thought to play an important role in anti-viral defense [36]. Although TLR3 is also expressed by many other non-hematopoietic as well as hematopoietic cells, expression is significantly higher in BDCA3+CLEC9A+ DC compared to BDCA1+ DC and monocyte-derived DC (moDC) [21]. Because of this high expression, TLR3 ligation by polyinosine-polycytidylic acid (polyI:C), which is a synthetic analog of dsRNA, is commonly used for *in vitro* stimulation of BDCA3+CLEC9A+ DC.

Other markers expressed by BDCA3+CLEC9A+ DC are depicted in table 1. Elucidation of all phenotypic markers expressed by BDCA3+CLEC9A+ DC will be of great importance for the identification of this subset in different organs. In addition, knowledge of these phenotypic markers will help to obtain insight into the functional properties of this DC subset. Currently, the BDCA3+CLEC9A+ DC subset is widely denominated as the 'BDCA3+ DC subset'. However, it will be essential to define a better designation for these cells which will distinguish this specific BDCA3hi subset from other DC expressing BDCA3. We conclude that CLEC9A is currently the optimal marker to use in combination with BDCA3 for identification of the BDCA3hi DC subset belonging to the CD8α+ DC lineage, due to its specific high-level expression on this subset and the feasibility of detection both at RNA and protein level.

Localization

BDCA3+CLEC9A+ DC were originally identified in blood. Compared to pDC and BDCA1+ DC, BDCA3+CLEC9A+ DC represent a very rare subset of circulating DC, comprising only about 0.04% of peripheral blood mononuclear cells (PBMC) [12]. In the following years, BDCA3+CLEC9A+ DC were discovered in various lymphoid and peripheral tissues, including tonsil, spleen, skin, lung, liver, and kidney [22, 37-43]. However, at that time BDCA3+CLEC9A+ DC were not yet identified as the homologue of lymphoid-resident CD8α+CLEC9A+ mouse DC. Therefore, it was unclear whether the BDCA3+ cells described in lymphoid tissues belong to this CLEC9A+ lineage. Jongbloed *et al.* and Poulin *et al.* were the first to show BDCA3+CLEC9A+ DC in blood, lymph nodes, tonsil, bone marrow and spleen and proved that

these cells belonged to the CLEC9A$^+$ lineage by phenotypic and functional analysis [21, 44]. Next, mouse migratory CD103$^+$ DC were identified as the major cross-presenting subset in peripheral tissues and found to be related to CD8α$^+$ DC [8, 45-47]. This suggested the existence of a human homologue of cross-presenting CD103$^+$ mouse DC in peripheral tissues [5]. Indeed, CLEC9A$^+$BDCA3$^+$ DC were discovered in peripheral tissues, including skin, liver and lung [24, 25, 48]. Gene expression analysis of different blood and skin DC subsets indicated that blood BDCA3$^+$CLEC9A$^+$ DC were most closely related to skin BDCA3$^+$CLEC9A$^+$. In addition, the transcriptional profile of skin BDCA3$^+$CLEC9A$^+$ DC appeared to be equivalent to that of mouse peripheral tissue CD103$^+$ DC [24].

Table 1. Phenotypic markers of BDCA3$^+$CLEC9A$^+$ DC.

	BDCA3$^+$CLEC9A$^+$ DC			
Antigen	**Blood**	**Lymphoid-tissue**	**Peripheral-tissue**	**References**
CD4	+	+		[37, 108]
CD5	+/-			[12]
CD11b (CR3)	-	+	+ (low)	[24, 37]
CD11c (CR4)	+	+		[37, 108]
CD16 (FcγRIII)	-			[4, 109]
CD18	+			[12]
CD23 (FcεRI)	-			[109]
CD32a (FcγRIIa)	+			[4, 109]
CD32b (FcγRIIb)	+			[4, 109]
CD33	+			[12]
CD36	+/-			[12]
CD38	+			[12]
CD40	+	+		[37, 108]
CD43	+			[12]
CD44	+	+		[12, 37]
CD45RA		+		[37]
CD45RO	+	+		[12, 37]
CD52	+			[108]
CD54	+			[12]
CD56	+/-			[108]
CD58	+			[12]
CD62L	+			[12]
CD64 (FcγRI)	-			[4, 109]
CD80	-	-	+	[24, 37]
CD83	-		+	[24, 109]
CD85k (ILT3)	+			[108]
CD86	+	+ (low)	+	[24, 37, 108]
CD89 (FcαR)	-			[109]
CD98	+			[12]
CD116	+			[12]
CD162	+			[19]
CD184 (CXCR4)	-	+		[37]
CD197 (CCR7)	-		+	[24]
CD205 (DEC205)	+			[108]
CD206 (MR)	-	-		[25, 26, 106]
CD207 (Langerin)			-	[24]
CD209 (DC-SIGN)	-	-		[26, 106]
CD274 (PDL1)	-		+	[24]
CLA	+			[108]
CLEC9A	+	+	+	[15, 21, 24]
HLA-A,B,C	+	+	+	[12, 24-26, 108]
HLA-DR	+	+	+	[24, 37, 108]
HLA-DQ	+			[12]
Necl2 (CADM1)		+	+	[19, 24]
TLR	1, 2, 3, 6, 8, 10		3	[21]
XCR1	+	+	+	[18, 24]

Quantitative analysis revealed that BDCA3+CLEC9A+ DC are more frequently present in peripheral tissues, including lung, skin and liver, than in blood [24, 48, 49]. Remarkably, liver and lungs contain comparable or even higher frequencies of BDCA3+CLEC9A+ DC than of BDCA1+ DC and pDC, suggesting a preferential role for BDCA3+CLEC9A+ DC in regulation of immunity in these organs [22, 40, 50]. The relative high frequency of BDCA3+CLEC9A+ DC seems to be organ-specific and has not been observed in lymphoid tissues [21].
Although in mice the phenotype of cross-presenting DC subsets clearly differs between lymphoid tissue DC and peripheral tissue DC, in human the distinction between peripheral blood DC, lymphoid tissue DC and peripheral tissue DC is less evident due to a lack of tissue-discriminating markers.
Compared to blood DC, peripheral tissue BDCA3+CLEC9A+ DC in skin express higher levels of C-C chemokine receptor 7 (CCR7), which regulates migration of DC from the periphery to lymph nodes, and lower levels of the skin-homing molecule cutaneous lymphocyte antigen (CLA) [24]. In addition, BDCA3+CLEC9A+ DC derived from both peripheral and lymphoid tissues, including lung, liver, skin, spleen, bone marrow and tonsil, express higher levels of activation markers such as CD40, CD80, CD83, CD86 and PD-L1 than those derived from blood, indicating that tissue DC have a more activated phenotype than blood DC [24, 48, 49]. This is in line with the observation that blood BDCA3+CLEC9A+ DC require stimulation with TLR ligands, such as polyI:C, for the cross-presentation of Ag, whereas tissue-derived BDCA3+CLEC9A+ DC can cross-present Ag even in the absence of stimulation [24, 26]. A more in-depth analysis of blood and spleen BDCA3+CLEC9A+ DC showed comparable patterns of TLR and transcription factor expression, except for TLR3 expression, which was lower in spleen DC. In line, the higher intrinsic cross-presenting capacity of blood BDCA3+CLEC9A+ DC compared to spleen DC was boosted by polyI:C, whereas spleen DC did not respond to polyI:C. The cytokine production upon stimulation with a cocktail of CD40L, IFNγ, IL-4 and GM-CSF was comparable between these blood and spleen DC [51].
Thus, BDCA3+CLEC9A+ DC are present in peripheral blood as well as in lymphoid and peripheral organs, where they, except for their activation status and expression of chemokine receptors, do not show major differences in phenotype or function. The increased frequencies of BDCA3+CLEC9A+ DC in peripheral tissues compared to blood indicate a preferential localization of BDCA3+CLEC9A+ DC to these tissues.

Developmental pathway

Investigation of the developmental pathway of BDCA3+CLEC9A+ DC has been hampered by their low abundance in blood and tissues and hence difficult isolation. Therefore, the developmental pathway of BDCA3+CLEC9A+ DC is poorly defined. Nevertheless, transcriptional profiling and the possibility to generate BDCA3+CLEC9A+ DCs in vitro have recently boosted research into the developmental pathways of this subset [44, 52-54].
In mice, all different types of DC subsets have a common origin, the common DC precursor (CDP). From that point differential transcription factor expression results in the differentiation of distinct DC subsets [55]. However, the equivalent of CDP has not yet been described in humans. Since BDCA3+CLEC9A+ and BDCA1+ DC cluster together in transcription profiling and hierarchical clustering analysis, these DC subsets were suggested to represent different maturational stages of the same cell type [12, 37]. However, comparative genome-wide expression profiling of mouse and human DC subsets revealed that BDCA3+CLEC9A+ DC have

a unique expression profile compared to the other DC subsets and, in contrast to BDCA1$^+$ DC, resemble mouse CD8α$^+$ DC [13, 52].
The differentiation of mouse CD8α$^+$ and CD103$^+$ DC is regulated by a conserved set of transcription factors, including Batf3, Irf8, Id2 and Nfil3 (nuclear factor interleukin 3-regulated, also known as E4BP4) [56, 57].
In line with this, development of human BDCA3$^+$CLEC9A$^+$ DC from cord blood-derived hematopoietic progenitor cells (HPC) *in vitro* is BATF3-dependent, as indicated by a significant decrease in BDCA3$^+$CLEC9A$^+$ DC frequencies upon BATF3 silencing [53]. However, this BATF3-dependence could not be proven *in vivo* since silencing of BATF3 in humanized mice did not result in reduction of BDCA3$^+$CLEC9A$^+$ DC. Since bypassing of BATF3-dependence has previously been observed for CD8α$^+$ DC development in mice, it might be that the same occurs for BDCA3$^+$CLEC9A$^+$ DC in humanized mice. In patients with IRF8 deficiency, a loss of peripheral blood BDCA3$^+$CLEC9A$^+$ DC was observed [58]. Since this loss was also observed for monocytes, pDC and BDCA1$^+$ DC, this observation indicates that IRF8 might be essential for the development of a certain CDP that also gives rise to BDCA3$^+$CLEC9A$^+$ DC. However, further research is required to define the exact role of BATF3, IRF8 and other transcription factors in the development of BDCA3$^+$CLEC9A$^+$ DC.
An essential growth factor for the development of both murine and human DC *in vitro* and *in vivo* is Flt3L [59]. Since frequencies of peripheral blood BDCA3$^+$CLEC9A$^+$ DC, but not other DC subsets, were reported to be increased in malaria patients who contain elevated levels of Flt3L in their plasma, it seems that Flt3L has a direct effect on homeostasis of BDCA3$^+$CLEC9A$^+$ DC or can enhance proliferation of BDCA3$^+$CLEC9A$^+$ DC precursors [60]. In addition, BDCA3$^+$CLEC9A$^+$ DC frequencies were increased in blood of Flt3L-treated human subjects and in blood and spleen of Flt3L-treated humanized mice [19, 60, 61]. However, this effect of Flt3L was not specific to BDCA3$^+$CLEC9A$^+$ DC, since also BDCA1$^+$ DC and pDC frequencies were increased in humanized mice, indicating that Flt3L can enhance the development of most DC subsets.
In addition to these humanized mouse models, also *in vitro* systems have been described that generate DC of the CLEC9A$^+$ lineage from HPC [44, 54]. In the system described by Poulin *et al.* human lineage-negative cord blood cells, including HPC, were cultured in the presence of stem cell factor (SCF), Flt3L, IL-3 and IL-6 and subsequently differentiated with Flt3L, GM-CSF and IL-4, which resulted in the development of a small population of BDCA3$^+$CLEC9A$^+$ DC [44]. The system described by Proietto *et al.* on the other hand, generated CLEC9A$^+$ DC, simultaneously with BDCA1$^+$ DC and pDC, from CD34$^+$ HSC of G-CSF-mobilized blood in the presence of Flt3L and thrombopoietin (TPO) [54]. However, these CLEC9A$^+$ DC did not co-express BDCA3. Of note, both culture systems generated relatively low numbers of DC. A third system, described by Thordardottir and colleagues, involved TPO, SCF, Flt3L, IL-6, and StemRegenin 1 (SR1) and yielded much higher numbers of BDCA3$^+$CLEC9A$^+$ DC. This system also simultaneously generated BDCA1$^+$ DC and pDC [62]. Although these reported culture systems all involve Flt3L, the requirement of Flt3L in BDCA3$^+$CLEC9A$^+$ DC development and the underlying molecular mechanisms remain to be elucidated.
Based on comparative gene expression analysis and previously published functional studies, Crozat *et al.* proposed that BDCA3$^+$CLEC9A$^+$ DC development is promoted by Flt3L, which activates signal transducer and activator of transcription 3 (STAT3) signaling and subsequent induction of IRF8 [52]. In the same model, the authors predicted that GM-CSF will inhibit BDCA3$^+$CLEC9A$^+$ DC development by activating STAT5, which on its turn will inhibit induction of IRF8 by STAT3 signaling. However, from our own studies we know that the level of STAT5

activation and its effect on DC development depends on the stage of DC development and could be either positive or negative [63]. Since GM-CSF is a cytokine involved in steady-state DC homeostasis and essential for development of peripheral-tissue DC, low levels of GM-CSF may therefore even contribute to development of BDCA3+ DC [64-66]. This was indeed observed in the system described by Poulin *et al.*, which contains both Flt3L and GM-CSF [44].

The generation of BDCA3+CLEC9A+ DC *in vitro* will be instrumental to further study the developmental pathways and to define the precursor cells of this specific DC subset. With regard to BDCA3+CLEC9A+ DC *in vivo*, it is likely that both peripheral tissue and lymphoid tissue BDCA3+CLEC9A+ DC derive from blood BDCA3+CLEC9A+ DC. This hypothesis is supported by several studies including (a) studies of Segura *et al.* and Nizzoli *et al.*, which reported that blood BDCA3+CLEC9A+ DC still proliferate, as shown by expression of the proliferation marker Ki67, whereas the proliferation of lymphoid tissue BDCA3+CLEC9A+ DC was found to be very low [26, 49] and (b) the study of Haniffa *et al.* which demonstrated that in contrast to blood BDCA3+CLEC9A+ DC skin BDCA3+CLEC9A+ DC partially co-express BDCA1 and CD1a, and that blood BDCA3+CLEC9A+ DC upregulate CD1a and BDCA1 when added to skin preparations [24]. Together this indicates that blood BDCA3+CLEC9A+ DC are not fully differentiated and may represent the precursors of fully differentiated DC in tissue.

Thus, although roles for BATF3, IRF8 and Flt3L in development of BDCA3+CLEC9A+ DC have been indicated, their exact contribution in this process remains to be determined. Additionally, the nature of precursors of BDCA3+CLEC9A+ DC in human needs to be further characterized. Further analysis of these factors will help to elucidate the BDCA3+CLEC9A+ DC differentiation pathway during steady state and inflammation.

Specialized immune functions

Although all DC share the unique capacity to initiate naive T cell responses, they possess different specialized immune functions [3, 4]. Here, we summarize the specialized functions that have been reported for BDCA3+CLEC9A+ DC.

IFNλ production

Type III IFN (IFNλ) are antiviral cytokines including three subtypes, IFNλ1 (IL-29), IFNλ2 (IL-28A) and IFNλ3 (IL-28B). IFNλ signals through the heterodimeric IFNλ receptor which consists of an IL-28 receptor α (IL-28Rα) chain and IL-10Rβ chain and activates JAK-STAT and MAPK pathways that are involved in the regulation of immunity [67]. Expression of the IFNλ receptor is mainly found on hepatocytes, epithelial cells, B cells, pDC and macrophages, however, the expression of the IFNλ receptor on immune cells is still under debate since contradictory results were obtained between receptor expression on RNA level and protein level due to a lack of appropriate antibodies [68-71].

IFNλ has direct antiviral effects by inhibiting replication of multiple viruses, including influenza virus, hepatitis C virus (HCV), hepatitis B virus (HBV), human immunodeficiency virus (HIV), rotavirus, encephalomyocarditis virus, vesicular stomatitis virus (VSV), cytomegalovirus (CMV), West Nile virus (WNV) and herplex simplex virus (HSV) [72-80], but may also support the skewing towards Th1 responses by downregulating Th-2 cytokine production, that will favor clearance of virus-infected cells [81-83].

Like mouse CD8α+ DC, blood BDCA3+CLEC9A+ DC were found to be major producers of all

three IFNλ subtypes upon polyI:C stimulation [48, 84, 85]. The same results were reported for tonsil and liver-derived BDCA3$^+$CLEC9A$^+$ DC, indicating that production of high levels of IFNλ is a specific characteristic of BDCA3$^+$CLEC9A$^+$ DC in blood as well as in lymphoid and peripheral tissues [48, 49].
Although production of IFNλ is also observed for pDC upon ligation of TLR9 and for BDCA1$^+$ DC and macrophages upon ligation of TLR3, IFNλ levels produced by BDCA3$^+$CLEC9A$^+$ DC are significantly higher [49, 71, 86]. However, it remains to be elucidated whether this superior IFNλ-producing capacity is fully restricted to BDCA3^{hi} DC that belong to the CLEC9A$^+$ lineage, or if it is also shared by other BDCA3-expressing immune cells.
As known for TLR3-induced type I IFN production by DC, polyI:C-induced IFNλ3 production by BDCA3$^+$CLEC9A$^+$ DC was shown to occur via a TLR3-TRIF-dependent pathway, as indicated by decreased IFNλ3 production upon inhibition of endosomal TLRs or TRIF. Although the mechanism of type I IFN production is well characterized, the regulation of IFNλ gene expression is incompletely understood. The IFNλ1, -2 and -3 promoters contain binding sites for IRFs and NFκB, indicating that these factors are involved in the induction of IFNλ expression [87-89]. Indeed, activation of NFκB and IRF3/7 was shown to induce gene transcription of IFNλ1, -2 and -3 in human embryonic kidney (HEK)293 cells [88]. Since stimulation of TLR3, MDA5, retinoic acid-inducible gene I (RIG-I), TLR7, TLR8 and TLR9 can all lead to activation of IRF3, IRF7 and even more receptors are known to trigger NFκB, these findings suggest that also other stimuli than polyI:C may induce IFNλ production by BDCA3$^+$CLEC9A$^+$ DC. However, BDCA3$^+$CLEC9A$^+$ DC lack expression of TLR7 and TLR9 and, although it is known that murine CD8α$^+$ DC lack RIG-I expression, it is unknown whether BDCA3$^+$CLEC9A$^+$ DC express RIG-I or MDA5 [21]. BDCA3$^+$CLEC9A$^+$ DC do express TLR8, but to the best of our knowledge, no studies have been published in which IFNλ production by BDCA3$^+$CLEC9A$^+$ DC upon TLR8 ligation was analyzed. Stimulation with the TLR8 ligand R848 in combination with polyI:C did not enhance IFNλ production by BDCA3$^+$CLEC9A$^+$ DC compared to stimulation with polyI:C alone [49]. Thus, BDCA3$^+$CLEC9A$^+$ DC produce IFNλ upon stimulation of TLR3, but involvement of other pattern recognition receptors in the induction of IFNλ expression remains to be investigated.
Since BDCA3$^+$ DC produce high levels of anti-viral IFNλ in response to TLR3 ligation by viral dsRNA, these cells are likely to play an important role in the host defense against DNA and RNA viruses that produce dsRNA intermediates [48, 84]. The role of IFNλ in the host defense against HCV infection has drawn a lot of attention, since single-nucleotide polymorphisms (SNPs) near the IL-28B (IFNλ3) gene are associated with spontaneous and therapy-induced clearance of HCV infection [90-93]. Both blood and hepatic BDCA3$^+$CLEC9A$^+$ DC, of which frequencies were increased in HCV-infected livers, were shown to produce IFNλ3 in response to HCV [48, 85, 94]. Interestingly, blood BDCA3$^+$CLEC9A$^+$ DC from subjects with minor IL-28B genotype produced lower levels of IFNλ3 in response to HCV than those from subjects with major genotype [48]. Although the exact mechanism by which IL-28B SNPs affect the anti-viral responses in HCV patients needs to be investigated, these findings indicate that IFNλ production by BDCA3$^+$CLEC9A$^+$ DC may play an important role.

Production of other cytokines

One of the first studies on the function of BDCA3$^+$CLEC9A$^+$ indicated that blood BDCA3$^+$CLEC9A$^+$ DC are, like their murine counterpart, superior at the production of IL-12p70 in response to stimulation with a cytokine cocktail of polyI:C, IFNγ, TNF, IFNα and IL-1β compared to BDCA1$^+$ DC [21]. In line with this, Poulin *et al.* reported production of IL-12p70

by spleen BDCA3+CLEC9A+ DC upon stimulation with a cocktail containing TLR1-9 agonists, IL-4, IFNγ, and T cells [44]. In contrast, a recent paper identified tonsil and blood BDCA1+ DC, but not BDCA3+CLEC9A+ DC, as the most potent IL-12-producing DC upon stimulation with polyI:C [49]. Only in response to polyI:C together with R848 and IFNγ, BDCA3+CLEC9A+ DC produced low levels of IL-12p70, which corresponded to the levels detected by Jongbloed *et al.* and Poulin *et al.* [21, 44]. Superior IL-12p70 production by BDCA1+ DC compared to BDCA3+CLEC9A+ DC was also observed for spleen-derived DC upon stimulation with CD40L, IFNγ, IL-4 and granulocyte macrophage-colony stimulating factor (GM-CSF) [51]. Direct comparison of different skin DC subsets, including BDCA3+CLEC9A+ DC, indicated that moDC, but not any of the skin DC subsets, produced IL-12p70 in response to polyI:C alone or combined with a cocktail of TNFα, IL-1β, IFNα, IFNγ and LPS [24]. Together, these results indicate that BDCA3+CLEC9A+ DC are able to produce IL-12p70 upon combinational TLR stimulation, but in contrast to BDCA1+ DC, don't exhibit strong IL-12-producing capacity.

In addition, blood-derived BDCA3+CLEC9A+ DC were shown to produce IFNα, IFNβ, IL-6, IL-8, TNFα and CXCL10 upon polyI:C stimulation [21, 95]. Skin-derived BDCA3+CLEC9A+ DC also produced TNFα, IL-8 and CXCL10 upon polyI:C stimulation, although CXCL10 levels were much lower than those produced by blood-derived BDCA3+CLEC9A+ DC, but no IL-6 or IL-1β were detected [24]. The production of CXCL10, together with XCR1 expression, is suggestive for interaction between BDCA3+CLEC9A+ DC and Th1 cells or NK cells, since Th1 cells and NK cells express the CXCL10 receptor CXCR3 and are major producers of XCL1 [33, 96]. However, to the best of our knowledge, no studies on the interaction between NK cells and BDCA3+CLEC9A+ DC have been published.

Whereas Chu *et al.* demonstrated that skin-derived BDCA3+ DC are potent producers of IL-10, Haniffa *et al.* could not detect any IL-10 production by skin BDCA3+CLEC9A+ DC [24, 25]. Since the BDCA3+ cell population analyzed by Chu *et al.* co-expressed CD14, this population probably includes not only DC of the BDCA3+CLEC9A+ lineage but also IL-10-producing CD14+ DC.

Induction of Th1/Th2 responses

The production of IFNβ, CXCL10 and IL-12p70 by blood-derived BDCA3+CLEC9A+ DC upon stimulation suggests that these cells are potent inducers of Th1 responses, which together with type I and III IFN production, play a key role in anti-viral immune responses. Co-culture of blood BDCA3+CLEC9A+ DC with allogeneic naïve CD4+ T cells indeed induced high production of the Th1 cytokines IFNγ and IL-2, whereas secretion of the Th2 cytokines IL-4, IL-5 or IL-10 was low or absent [21, 26]. Induction of Th17 or regulatory T cells could not be observed. Lymph node-derived BDCA3+CLEC9A+ DC on the other hand, induced production of both Th1 and Th2 cytokines [26]. Direct comparison of BDCA3+CLEC9A+ DC with BDCA1+ DC and pDC for their capacity to stimulate allogeneic naïve CD4+ T cell proliferation showed that, although no major differences were observed, BDCA3+CLEC9A+ DC induced the highest T cell proliferation levels [97].

The preferential polarization towards Th1 responses, suggests a contribution of BDCA3+CLEC9A+ DC to typical Th-1 mediated immunity seen in auto-immune diseases or transplant rejection. In contrast, Yerkovich *et al.* showed that BDCA3-expressing cells induced a Th2-skewed response upon co-culture with PBMC and that atopic asthmatic patients had higher frequencies of BDCA3+CLEC9A+ DC in peripheral blood [98]. However, DC-PBMC co-culture was performed with BDCA3-expressing moDC instead of BDCA3+CLEC9A+ DC, which may have a different function. Several other studies showed that BDCA3 expression is

associated with a tolerogenic function of DC [25, 99, 100]. However, in these studies it is not clear whether BDCA3$^+$CLEC9A$^+$ DC or other BDCA3-expressing DC were used for analysis, which also illustrates the importance of using the combination of BDCA3 and CLEC9A to identify these cells. Thus, the plasticity of these BDCA3$^+$CLEC9A$^+$ DC with regard to Th2-skewing and regulatory capacities in addition to their Th1-promoting ability remains to be established. Like for other DC subsets, plasticity of DC function enables BDCA3$^+$CLEC9A$^+$ DC to respond to environmental factors and adjust their function in order to induce the appropriate immune response. Therefore, the role of BDCA3$^+$CLEC9A$^+$ DC may vary among different types of disease and should be studied for each setting.

Cross-presentation

mDC are known for their ability to efficiently capture and present Ag to T cells. Activation of CD8$^+$ T cells, which plays an essential role in the immune control of tumors and viral infections, occurs via presentation of Ag in major histocompatibility complex class I (MHC class I). Although the majority of Ag presented by DC in MHC class I derive from endogenously synthesized proteins, DC have also the capacity to cross-present exogenous-derived Ag in MHC class I molecules [101].

BDCA3$^+$CLEC9A$^+$ DC were initially described to be more efficient at cross-presentation of both soluble and cell-associated Ag in MHC class I than other DC subsets, such as BDCA1$^+$ DC, moDC or pDC [4, 17, 18, 21, 44]. These findings suggested that BDCA3$^+$CLEC9A$^+$ DC exhibit a specialized cross-presenting capacity, as has previously been shown for mouse CD8α$^+$ DC [7, 10, 102]. In contrast, recent papers showed that other DC are able to cross-present soluble Ag as efficient as BDCA3$^+$CLEC9A$^+$ DC [26, 49, 51, 97, 103]. Additionally, features associated with efficient cross-presenting capacities, including high phagosomal pH, production of ROS within endocytic compartments, and efficient antigen transfer from endocytic compartments into the cytosol, which is required for entrance of exogenous-derived proteins into the MHC class I pathway, were not significantly different between BDCA1$^+$ and BDCA3$^+$CLEC9A$^+$ DC derived from blood and tonsil [97]. Therefore, it is not likely that BDCA3$^+$CLEC9A$^+$ DC are equipped with an intrinsic specialization for cross-presentation. In line, Cohn *et al*. found that BDCA1$^+$ and BDCA3$^+$CLEC9A$^+$ DC had similar efficiency of cross-presentation of Ag delivered to early endosomes by antibody (Ab)-mediated targeting to CD40 and CD11c [104]. Furthermore, MHC class I presentation of endogenously generated Ag after transfection was comparable between the subsets, suggesting that the general efficiency of MHC class I presentation is similar. However, delivery of Ag to late endosomes and lysosomes by DEC205 targeting resulted in enhanced cross-presentation by BDCA3$^+$CLEC9A$^+$ DC compared to BDCA1$^+$ DC. Although BDCA3$^+$CLEC9A$^+$ DC expressed higher levels of DEC205, enhanced cross-presentation was not due to increased Ag internalization. Together, these studies indicate that differences in cross-presentation of soluble antigen between DC subsets may not be dependent on intrinsic cross-presentation capacities, but are highly dependent on the type of Ag used in each experiment and differential expression of Ag receptors, which varies on each DC subset. Nevertheless, BDCA3$^+$CLEC9A$^+$ DC may exhibit a specialized machinery to transfer Ag from late endosomes and lysosomes to the cytosol [104].

Although conflicting results were reported regarding the superiority of BDCA3$^+$CLEC9A$^+$ DC at cross-presentation of soluble Ag, this was not the case for their superiority at the uptake and cross-presentation of necrotic cell-associated Ag [17, 21, 44, 97]. This enhanced capacity might be due to CLEC9A, which is selectively expressed by BDCA3^{hi} DC and recognizes F-actin

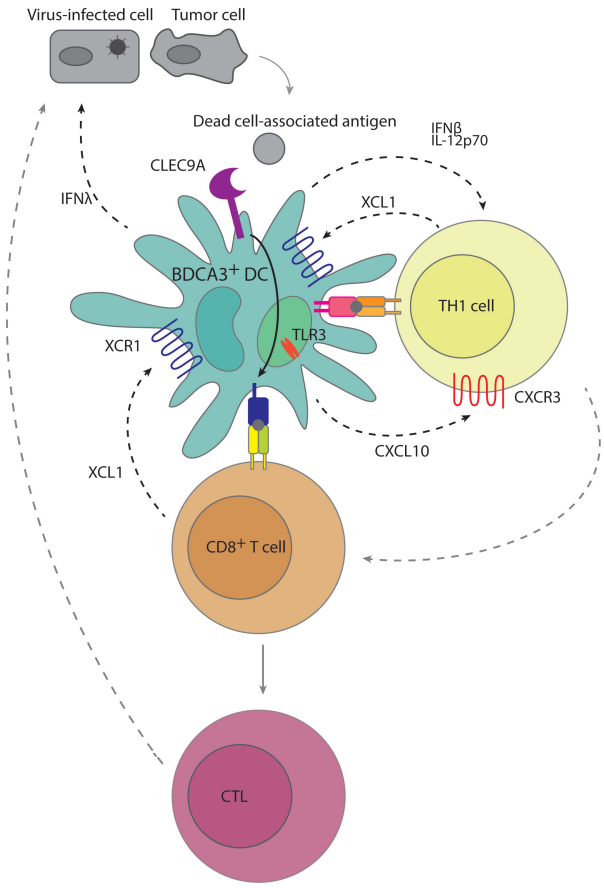

Figure 1. Functional specializations of BDCA3⁺CLEC9A⁺ DC suggest a key role in virus-specific and tumor-specific immunity. BDCA3⁺CLEC9A⁺DC are superior at the uptake and cross-presentation of dead cell-associated Ag by CLEC9A. Cross-presentation of Ag from tumor or virus-infected cells results in the induction of tumor- or virus-specific CTL, respectively. The XCR1-XCL1 receptor-ligand pair enhances survival and effector functions of CD8⁺ T and Th1 cells. Upon TLR3 ligation, BDCA3⁺CLEC9A⁺ DC produce high levels of IFN-λ, which can directly inhibit viral replication. In addition, BDCA3⁺CLEC9A⁺ DC produce IFN-β and IL-12p70 upon activation, and thereby induce polarization toward Th1 responses. Secretion of CXCL10, together with XCR1 expression, contributes to the interaction betweenBDCA3⁺CLEC9A⁺ DC and Th1 cells, which express the CXCL10 receptor CXCR3 and are major producers of XCL1.

exposed on necrotic cells [27, 29, 30]. CLEC9A was shown to mediate cross-presentation by coordinating the delivery of necrotic cell-associated Ag into early endosomes, which is associated with efficient cross-presentation [27, 28, 104, 105]. Indeed, Ag delivered to CLEC9A by anti-CLEC9A-coated nanoparticles were cross-presented by BDCA3⁺CLEC9A⁺ DC to CD8⁺ T cells [106]. Cross-presentation of dead cell-associated Ag might also be enhanced through stimulation of TLR3 by dsRNA released from infected dying cells or by other DAMPs. Moreover, as previously suggested by M. Dalod, CLEC9A and TLR3 may act together in the optimization of the cross-presentation capacity of BDCA3⁺CLEC9A⁺ DC, since CLEC9A may coordinate Ag into endosomes containing TLR3 [107]. However, the exact mechanism by which CLEC9A regulates the trafficking of internalized Ag and promotes cross-presentation remains to be elucidated.

In addition, enhanced activation of CD8⁺ T cells upon presentation of exogenous Ag in MHC class I by BDCA3⁺CLEC9A⁺ DC might be due to their unique expression of XCR1, which probably has no direct effect on the intracellular cross-presentation process, but can enhance cellular interaction with CD8⁺ T cells by the XCR1-XCL1 axis [17, 18]. If XCR1 indeed has this effect, also the capacity to activate CD8⁺ T cells via direct presentation of endogenously synthesized Ag in MHC class I may be enhanced for BDCA3⁺CLEC9A⁺ DC. However, no studies on direct

Ag presentation by BDCA3$^+$CLEC9A$^+$ DC could be found. To confirm the superior capacity of BDCA3$^+$CLEC9A$^+$ DC at cross-presentation of dead cell-associated Ag, further research on the intrinsic cellular processes that enable efficient cross-presentation, and the involvement of BDCA3$^+$CLEC9A$^+$ DC-specific receptors in these processes is required.
In summary, BDCA3$^+$CLEC9A$^+$ DC produce high levels of IFNλ upon polyI:C stimulation, preferentially induce Th1 polarization of allogeneic naive CD4$^+$ T cells, facilitate efficient cross-presentation of soluble Ag and exhibit superior capacity to cross-present dead cell-associated Ag. Given these functional characteristics, BDCA3$^+$CLEC9A$^+$ DC are likely to play an important role in initiation of adaptive immunity, including CTL, which are essential in the host defense against viral infections and tumors (Figure 1).

Concluding remarks

BDCA3$^+$CLEC9A$^+$ DC represent a unique DC subset with a gene expression profile distinct from other DC. So far, reliable, easily accessible tools are lacking to identify this DC subset based on a single marker, such as XCR1. Therefore, identification based on simultaneous expression of BDCA3 and CLEC9A is recommended since low-to-intermediate expression of these markers can also be found on other immune cells. An important area for future research on BDCA3$^+$CLEC9A$^+$ DC is the developmental pathway of these cells and its precursors. Development of BDCA3$^+$CLEC9A$^+$ DC seems to specifically depend on transcription factor BATF3, as was also previously shown for their CD8α$^+$ murine counterpart, but signaling pathways, precursor cells and growth factors involved in the differentiation of these cells remain to be elucidated. Recent development of protocols to generate BDCA3$^+$CLEC9A$^+$ DC *in vitro* will contribute to elucidation of molecular mechanisms underlying BDCA3$^+$CLEC9A$^+$ DC development.
Although BDCA3$^+$CLEC9A$^+$ DC represent the smallest population of human DC subset in peripheral blood, a different distribution, including relatively higher BDCA3$^+$CLEC9A$^+$ DC frequencies, in peripheral tissues and lymphoid organs together with several unique functions indicate that BDCA3$^+$CLEC9A$^+$ DC play a significant role in the induction and regulation of immunity. BDCA3$^+$CLEC9A$^+$ DC have functional specializations that include the capacity to produce high levels of IFNλ, elicit Th1 responses and activate CTL by internalizing and cross-presenting dead cell-associated Ag. These characteristics suggest that BDCA3$^+$CLEC9A$^+$ DC may be key players in the induction of anti-viral and anti-tumor immunity. Therefore, this DC subset might be a potential tool or target for immunotherapy against chronic viral infections or cancer. Nevertheless, a better phenotypical and functional characterization of these cells as well as the underlying molecular mechanisms are required to fully understand their role in immune regulation and to identify target molecules to exploit the potential of these cells for immunotherapy.

References

1. Banchereau J, Briere F, Caux C, Davoust J, Lebecque S, Liu YJ, et al. Immunobiology of dendritic cells. Annu Rev Immunol 2000;18:767-811.
2. Steinman RM. Dendritic cells in vivo: a key target for a new vaccine science. Immunity 2008;29:319-24.
3. Allan RS, Waithman J, Bedoui S, Jones CM, Villadangos JA, Zhan Y, et al. Migratory dendritic cells transfer antigen to a lymph node-resident dendritic cell population for efficient CTL priming. Immunity 2006;25:153-62.
4. Flinsenberg TW, Compeer EB, Koning D, Klein M, Amelung FJ, van Baarle D, et al. Fcgamma receptor antigen targeting potentiates cross-presentation by human blood and lymphoid tissue BDCA-3+ dendritic cells. Blood 2012;120:5163-72.
5. Guilliams M, Henri S, Tamoutounour S, Ardouin L, Schwartz-Cornil I, Dalod M, et al. From skin dendritic cells to a simplified classification of human and mouse dendritic cell subsets. Eur J Immunol 2010;40:2089-94.
6. Shortman K, Liu YJ. Mouse and human dendritic cell subtypes. Nat Rev Immunol 2002;2:151-61.
7. Shortman K, Heath WR. The CD8+ dendritic cell subset. Immunol Rev 2010;234:18-31.
8. Li YH, Kuo CH, Shi GY, Wu HL. The role of thrombomodulin lectin-like domain in inflammation. J Biomed Sci 2012;19:34.
9. Edelson BT, Kc W, Juang R, Kohyama M, Benoit LA, Klekotka PA, et al. Peripheral CD103+ dendritic cells form a unified subset developmentally related to CD8alpha+ conventional dendritic cells. J Exp Med 2010;207:823-36.
10. Hildner K, Edelson BT, Purtha WE, Diamond M, Matsushita H, Kohyama M, et al. Batf3 deficiency reveals a critical role for CD8alpha+ dendritic cells in cytotoxic T cell immunity. Science 2008;322:1097-100.
11. Van Pottelberge GR, Bracke KR, Demedts IK, De Rijck K, Reinartz SM, van Drunen CM, et al. Selective accumulation of langerhans-type dendritic cells in small airways of patients with COPD. Respir Res 2010;11:35.
12. Dzionek A, Fuchs A, Schmidt P, Cremer S, Zysk M, Miltenyi S, et al. BDCA-2, BDCA-3, and BDCA-4: three markers for distinct subsets of dendritic cells in human peripheral blood. J Immunol 2000;165:6037-46.
13. Robbins SH, Walzer T, Dembele D, Thibault C, Defays A, Bessou G, et al. Novel insights into the relationships between dendritic cell subsets in human and mouse revealed by genome-wide expression profiling. Genome Biol 2008;9:R17.
14. Contreras V, Urien C, Guiton R, Alexandre Y, Vu Manh TP, Andrieu T, et al. Existence of CD8alpha-like dendritic cells with a conserved functional specialization and a common molecular signature in distant mammalian species. J Immunol 2010;185:3313-25.
15. Huysamen C, Willment JA, Dennehy KM, Brown GD. CLEC9A is a novel activation C-type lectin-like receptor expressed on BDCA3+ dendritic cells and a subset of monocytes. J Biol Chem 2008;283:16693-701.
16. Caminschi I, Proietto AI, Ahmet F, Kitsoulis S, Shin Teh J, Lo JC, et al. The dendritic cell subtype-restricted C-type lectin Clec9A is a target for vaccine enhancement. Blood 2008;112:3264-73.
17. Crozat K, Guiton R, Contreras V, Feuillet V, Dutertre CA, Ventre E, et al. The XC chemokine receptor 1 is a conserved selective marker of mammalian cells homologous to mouse CD8alpha+ dendritic cells. J Exp Med 2010;207:1283-92.
18. Bachem A, Guttler S, Hartung E, Ebstein F, Schaefer M, Tannert A, et al. Superior antigen cross-presentation and XCR1 expression define human CD11c+CD141+ cells as homologues of mouse CD8+ dendritic cells. J Exp Med 2010;207:1273-81.
19. Galibert L, Diemer GS, Liu Z, Johnson RS, Smith JL, Walzer T, et al. Nectin-like protein 2 defines

a subset of T-cell zone dendritic cells and is a ligand for class-I-restricted T-cell-associated molecule. J Biol Chem 2005;280:21955-64.

20. Edwards AD, Diebold SS, Slack EM, Tomizawa H, Hemmi H, Kaisho T, et al. Toll-like receptor expression in murine DC subsets: lack of TLR7 expression by CD8 alpha+ DC correlates with unresponsiveness to imidazoquinolines. Eur J Immunol 2003;33:827-33.
21. Jongbloed SL, Kassianos AJ, McDonald KJ, Clark GJ, Ju X, Angel CE, et al. Human CD141+ (BDCA-3)+ dendritic cells (DCs) represent a unique myeloid DC subset that cross-presents necrotic cell antigens. J Exp Med 2010;207:1247-60.
22. Demedts IK, Brusselle GG, Vermaelen KY, Pauwels RA. Identification and characterization of human pulmonary dendritic cells. Am J Respir Cell Mol Biol 2005;32:177-84.
23. Dzionek A, Inagaki Y, Okawa K, Nagafune J, Rock J, Sohma Y, et al. Plasmacytoid dendritic cells: from specific surface markers to specific cellular functions. Hum Immunol 2002;63:1133-48.
24. Haniffa M, Shin A, Bigley V, McGovern N, Teo P, See P, et al. Human Tissues Contain CD141(hi) Cross-Presenting Dendritic Cells with Functional Homology to Mouse CD103(+) Nonlymphoid Dendritic Cells. Immunity 2012.
25. Chu CC, Ali N, Karagiannis P, Di Meglio P, Skowera A, Napolitano L, et al. Resident CD141 (BDCA3)+ dendritic cells in human skin produce IL-10 and induce regulatory T cells that suppress skin inflammation. J Exp Med 2012;209:935-45.
26. Segura E, Valladeau-Guilemond J, Donnadieu MH, Sastre-Garau X, Soumelis V, Amigorena S. Characterization of resident and migratory dendritic cells in human lymph nodes. J Exp Med 2012;209:653-60.
27. Sancho D, Joffre OP, Keller AM, Rogers NC, Martinez D, Hernanz-Falcon P, et al. Identification of a dendritic cell receptor that couples sensing of necrosis to immunity. Nature 2009;458:899-903.
28. Zelenay S, Keller AM, Whitney PG, Schraml BU, Deddouche S, Rogers NC, et al. The dendritic cell receptor DNGR-1 controls endocytic handling of necrotic cell antigens to favor cross-priming of CTLs in virus-infected mice. J Clin Invest 2012;122:1615-27.
29. Ahrens S, Zelenay S, Sancho D, Hanc P, Kjaer S, Feest C, et al. F-actin is an evolutionarily conserved damage-associated molecular pattern recognized by DNGR-1, a receptor for dead cells. Immunity 2012;36:635-45.
30. [30] Zhang JG, Czabotar PE, Policheni AN, Caminschi I, Wan SS, Kitsoulis S, et al. The dendritic cell receptor Clec9A binds damaged cells via exposed actin filaments. Immunity 2012;36:646-57.
31. Lei Y, Takahama Y. XCL1 and XCR1 in the immune system. Microbes Infect 2012;14:262-7.
32. Yamazaki C, Miyamoto R, Hoshino K, Fukuda Y, Sasaki I, Saito M, et al. Conservation of a chemokine system, XCR1 and its ligand, XCL1, between human and mice. Biochem Biophys Res Commun 2010;397:756-61.
33. Dorner BG, Dorner MB, Zhou X, Opitz C, Mora A, Guttler S, et al. Selective expression of the chemokine receptor XCR1 on cross-presenting dendritic cells determines cooperation with CD8+ T cells. Immunity 2009;31:823-33.
34. Giangreco A, Jensen KB, Takai Y, Miyoshi J, Watt FM. Necl2 regulates epidermal adhesion and wound repair. Development 2009;136:3505-14.
35. Murakami Y. Involvement of a cell adhesion molecule, TSLC1/IGSF4, in human oncogenesis. Cancer Sci 2005;96:543-52.
36. O'Neill LA, Golenbock D, Bowie AG. The history of Toll-like receptors - redefining innate immunity. Nat Rev Immunol 2013;13:453-60.
37. Lindstedt M, Lundberg K, Borrebaeck CA. Gene family clustering identifies functionally associated subsets of human in vivo blood and tonsillar dendritic cells. J Immunol 2005;175:4839-46.
38. Velasquez-Lopera MM, Correa LA, Garcia LF. Human spleen contains different subsets of dendritic cells and regulatory T lymphocytes. Clin Exp Immunol 2008;154:107-14.

39. Narbutt J, Lesiak A, Sysa-Jedrzejowska A, Smolewski P, Robak T, Zalewska A. The number and distribution of blood dendritic cells in the epidermis and dermis of healthy human subjects. Folia Histochem Cytobiol 2006;44:61-3.

40. Tsoumakidou M, Tzanakis N, Papadaki HA, Koutala H, Siafakas NM. Isolation of myeloid and plasmacytoid dendritic cells from human bronchoalveolar lavage fluid. Immunol Cell Biol 2006;84:267-73.

41. McCarthy NE, Jones HA, Marks NA, Shiner RJ, Ind PW, Al-Hassi HO, et al. Inhaled allergen-driven CD1c up-regulation and enhanced antigen uptake by activated human respiratory-tract dendritic cells in atopic asthma. Clin Exp Allergy 2007;37:72-82.

42. Bamboat ZM, Stableford JA, Plitas G, Burt BM, Nguyen HM, Welles AP, et al. Human liver dendritic cells promote T cell hyporesponsiveness. J Immunol 2009;182:1901-11.

43. Fiore N, Castellano G, Blasi A, Capobianco C, Loverre A, Montinaro V, et al. Immature myeloid and plasmacytoid dendritic cells infiltrate renal tubulointerstitium in patients with lupus nephritis. Mol Immunol 2008;45:259-65.

44. Poulin LF, Salio M, Griessinger E, Anjos-Afonso F, Craciun L, Chen JL, et al. Characterization of human DNGR-1+ BDCA3+ leukocytes as putative equivalents of mouse CD8alpha+ dendritic cells. J Exp Med 2010;207:1261-71.

45. Laffont S, Siddiqui KR, Powrie F. Intestinal inflammation abrogates the tolerogenic properties of MLN CD103+ dendritic cells. Eur J Immunol 2010;40:1877-83.

46. Bedoui S, Whitney PG, Waithman J, Eidsmo L, Wakim L, Caminschi I, et al. Cross-presentation of viral and self antigens by skin-derived CD103+ dendritic cells. Nat Immunol 2009;10:488-95.

47. Henri S, Poulin LF, Tamoutounour S, Ardouin L, Guilliams M, de Bovis B, et al. CD207+ CD103+ dermal dendritic cells cross-present keratinocyte-derived antigens irrespective of the presence of Langerhans cells. J Exp Med 2010;207:189-206.

48. Yoshio S, Kanto T, Kuroda S, Matsubara T, Higashitani K, Kakita N, et al. Human blood dendritic cell antigen 3 (BDCA3)(+) dendritic cells are a potent producer of interferon-lambda in response to hepatitis C virus. Hepatology 2013;57:1705-15.

49. Nizzoli G, Krietsch J, Weick A, Steinfelder S, Facciotti F, Gruarin P, et al. Human CD1c+ dendritic cells secrete high levels of IL-12 and potently prime cytotoxic T-cell responses. Blood 2013;122:932-42.

50. Kelly A, Fahey R, Fletcher JM, Keogh C, Carroll AG, Siddachari R, et al. CD141 myeloid dendritic cells are enriched in healthy human liver. J Hepatol 2013.

51. Mittag D, Proietto AI, Loudovaris T, Mannering SI, Vremec D, Shortman K, et al. Human dendritic cell subsets from spleen and blood are similar in phenotype and function but modified by donor health status. J Immunol 2011;186:6207-17.

52. Crozat K, Guiton R, Guilliams M, Henri S, Baranek T, Schwartz-Cornil I, et al. Comparative genomics as a tool to reveal functional equivalences between human and mouse dendritic cell subsets. Immunol Rev 2010;234:177-98.

53. Poulin LF, Reyal Y, Uronen-Hansson H, Schraml BU, Sancho D, Murphy KM, et al. DNGR-1 is a specific and universal marker of mouse and human Batf3-dependent dendritic cells in lymphoid and nonlymphoid tissues. Blood 2012;119:6052-62.

54. Proietto A, Mittag D, Roberts A, Sprigg N, Wu L. The equivalents of human blood and spleen dendritic cell subtypes can be generated in vitro from human CD34(+) stem cells in the presence of fms-like tyrosine kinase 3 ligand and thrombopoietin. Cell Mol Immunol 2012;9:446-54.

55. Moore AJ, Anderson MK. Dendritic cell development: a choose-your-own-adventure story. Adv Hematol 2013;2013:949513.

56. Belz GT, Nutt SL. Transcriptional programming of the dendritic cell network. Nat Rev Immunol 2012;12:101-13.

57. Miller JC, Brown BD, Shay T, Gautier EL, Jojic V, Cohain A, et al. Deciphering the transcriptional

network of the dendritic cell lineage. Nat Immunol 2012;13:888-99.

58. Hambleton S, Salem S, Bustamante J, Bigley V, Boisson-Dupuis S, Azevedo J, et al. IRF8 mutations and human dendritic-cell immunodeficiency. N Engl J Med 2011;365:127-38.
59. Merad M, Manz MG. Dendritic cell homeostasis. Blood 2009;113:3418-27.
60. Guermonprez P, Helft J, Claser C, Deroubaix S, Karanje H, Gazumyan A, et al. Inflammatory Flt3l is essential to mobilize dendritic cells and for T cell responses during Plasmodium infection. Nat Med 2013;19:730-8.
61. Ding Y, Wilkinson A, Idris A, Fancke B, O'Keeffe M, Khalil D, et al. FLT3-ligand treatment of humanized mice results in the generation of large numbers of CD141+ and CD1c+ dendritic cells in vivo. J Immunol 2014;192:1982-9.
62. Thordardottir S, Hangalapura BN, Hutten T, Cossu M, Spanholtz J, Schaap N, et al. The Aryl Hydrocarbon Receptor Antagonist StemRegenin 1 Promotes Human Plasmacytoid and Myeloid Dendritic Cell Development from CD34 Hematopoietic Progenitor Cells. Stem Cells Dev 2014.
63. van de Laar L, van den Bosch A, Wierenga AT, Janssen HL, Coffer PJ, Woltman AM. Tight control of STAT5 activity determines human CD34-derived interstitial dendritic cell and langerhans cell development. J Immunol 2011;186:7016-24.
64. van de Laar L, Coffer PJ, Woltman AM. Regulation of dendritic cell development by GM-CSF: molecular control and implications for immune homeostasis and therapy. Blood 2012;119:3383-93.
65. Greter M, Helft J, Chow A, Hashimoto D, Mortha A, Agudo-Cantero J, et al. GM-CSF controls nonlymphoid tissue dendritic cell homeostasis but is dispensable for the differentiation of inflammatory dendritic cells. Immunity 2012;36:1031-46.
66. Kingston D, Schmid MA, Onai N, Obata-Onai A, Baumjohann D, Manz MG. The concerted action of GM-CSF and Flt3-ligand on in vivo dendritic cell homeostasis. Blood 2009;114:835-43.
67. Li M, Liu X, Zhou Y, Su SB. Interferon-lambdas: the modulators of antivirus, antitumor, and immune responses. J Leukoc Biol 2009;86:23-32.
68. Sheppard P, Kindsvogel W, Xu W, Henderson K, Schlutsmeyer S, Whitmore TE, et al. IL-28, IL-29 and their class II cytokine receptor IL-28R. Nat Immunol 2003;4:63-8.
69. Novak AJ, Grote DM, Ziesmer SC, Rajkumar V, Doyle SE, Ansell SM. A role for IFN-lambda1 in multiple myeloma B cell growth. Leukemia 2008;22:2240-6.
70. Sommereyns C, Paul S, Staeheli P, Michiels T. IFN-lambda (IFN-lambda) is expressed in a tissue-dependent fashion and primarily acts on epithelial cells in vivo. PLoS Pathog 2008;4:e1000017.
71. Megjugorac NJ, Gallagher GE, Gallagher G. Modulation of human plasmacytoid DC function by IFN-lambda1 (IL-29). J Leukoc Biol 2009;86:1359-63.
72. Wang J, Oberley-Deegan R, Wang S, Nikrad M, Funk CJ, Hartshorn KL, et al. Differentiated human alveolar type II cells secrete antiviral IL-29 (IFN-lambda 1) in response to influenza A infection. J Immunol 2009;182:1296-304.
73. Robek MD, Boyd BS, Chisari FV. Lambda interferon inhibits hepatitis B and C virus replication. J Virol 2005;79:3851-4.
74. Tian RR, Guo HX, Wei JF, Yang CK, He SH, Wang JH. IFN-lambda inhibits HIV-1 integration and post-transcriptional events in vitro, but there is only limited in vivo repression of viral production. Antiviral Res 2012;95:57-65.
75. Pott J, Mahlakoiv T, Mordstein M, Duerr CU, Michiels T, Stockinger S, et al. IFN-lambda determines the intestinal epithelial antiviral host defense. Proc Natl Acad Sci U S A 2011;108:7944-9.
76. Ank N, Iversen MB, Bartholdy C, Staeheli P, Hartmann R, Jensen UB, et al. An important role for type III interferon (IFN-lambda/IL-28) in TLR-induced antiviral activity. J Immunol 2008;180:2474-85.

77. Ank N, West H, Bartholdy C, Eriksson K, Thomsen AR, Paludan SR. Lambda interferon (IFN-lambda), a type III IFN, is induced by viruses and IFNs and displays potent antiviral activity against select virus infections in vivo. J Virol 2006;80:4501-9.
78. Doyle SE, Schreckhise H, Khuu-Duong K, Henderson K, Rosler R, Storey H, et al. Interleukin-29 uses a type 1 interferon-like program to promote antiviral responses in human hepatocytes. Hepatology 2006;44:896-906.
79. Ma D, Jiang D, Qing M, Weidner JM, Qu X, Guo H, et al. Antiviral effect of interferon lambda against West Nile virus. Antiviral Res 2009;83:53-60.
80. Zhou L, Li J, Wang X, Ye L, Hou W, Ho J, et al. IL-29/IL-28A suppress HSV-1 infection of human NT2-N neurons. J Neurovirol 2011;17:212-9.
81. Kotenko SV. IFN-lambdas. Curr Opin Immunol 2011;23:583-90.
82. Kelly C, Klenerman P, Barnes E. Interferon lambdas: the next cytokine storm. Gut 2011;60:1284-93.
83. Jordan WJ, Eskdale J, Srinivas S, Pekarek V, Kelner D, Rodia M, et al. Human interferon lambda-1 (IFN-lambda1/IL-29) modulates the Th1/Th2 response. Genes Immun 2007;8:254-61.
84. Lauterbach H, Bathke B, Gilles S, Traidl-Hoffmann C, Luber CA, Fejer G, et al. Mouse CD8alpha+ DCs and human BDCA3+ DCs are major producers of IFN-lambda in response to poly IC. J Exp Med 2010;207:2703-17.
85. Zhang S, Kodys K, Li K, Szabo G. Human type 2 myeloid dendritic cells produce interferon-lambda and amplify interferon-alpha in response to hepatitis C virus infection. Gastroenterology 2013;144:414-25 e7.
86. Coccia EM, Severa M, Giacomini E, Monneron D, Remoli ME, Julkunen I, et al. Viral infection and Toll-like receptor agonists induce a differential expression of type I and lambda interferons in human plasmacytoid and monocyte-derived dendritic cells. Eur J Immunol 2004;34:796-805.
87. Onoguchi K, Yoneyama M, Takemura A, Akira S, Taniguchi T, Namiki H, et al. Viral infections activate types I and III interferon genes through a common mechanism. J Biol Chem 2007;282:7576-81.
88. Osterlund PI, Pietila TE, Veckman V, Kotenko SV, Julkunen I. IFN regulatory factor family members differentially regulate the expression of type III IFN (IFN-lambda) genes. J Immunol 2007;179:3434-42.
89. Iversen MB, Paludan SR. Mechanisms of type III interferon expression. J Interferon Cytokine Res 2010;30:573-8.
90. Suppiah V, Moldovan M, Ahlenstiel G, Berg T, Weltman M, Abate ML, et al. IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. Nat Genet 2009;41:1100-4.
91. Tanaka Y, Nishida N, Sugiyama M, Kurosaki M, Matsuura K, Sakamoto N, et al. Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. Nat Genet 2009;41:1105-9.
92. Ge D, Fellay J, Thompson AJ, Simon JS, Shianna KV, Urban TJ, et al. Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. Nature 2009;461:399-401.
93. Thomas DL, Thio CL, Martin MP, Qi Y, Ge D, O'Huigin C, et al. Genetic variation in IL28B and spontaneous clearance of hepatitis C virus. Nature 2009;461:798-801.
94. Velazquez VM, Hon H, Ibegbu C, Knechtle SJ, Kirk AD, Grakoui A. Hepatic enrichment and activation of myeloid dendritic cells during chronic hepatitis C virus infection. Hepatology 2012;56:2071-81.
95. Meixlsperger S, Leung CS, Ramer PC, Pack M, Vanoaica LD, Breton G, et al. CD141+ dendritic cells produce prominent amounts of IFN-alpha after dsRNA recognition and can be targeted via DEC-205 in humanized mice. Blood 2013;121:5034-44.
96. Acosta-Rodriguez EV, Rivino L, Geginat J, Jarrossay D, Gattorno M, Lanzavecchia A, et al.

Surface phenotype and antigenic specificity of human interleukin 17-producing T helper memory cells. Nat Immunol 2007;8:639-46.

97. Segura E, Durand M, Amigorena S. Similar antigen cross-presentation capacity and phagocytic functions in all freshly isolated human lymphoid organ-resident dendritic cells. J Exp Med 2013;210:1035-47.
98. Yerkovich ST, Roponen M, Smith ME, McKenna K, Bosco A, Subrata LS, et al. Allergen-enhanced thrombomodulin (blood dendritic cell antigen 3, CD141) expression on dendritic cells is associated with a TH2-skewed immune response. J Allergy Clin Immunol 2009;123:209-16 e4.
99. Takagi T, Taguchi O, Toda M, Ruiz DB, Bernabe PG, D'Alessandro-Gabazza CN, et al. Inhibition of allergic bronchial asthma by thrombomodulin is mediated by dendritic cells. Am J Respir Crit Care Med 2011;183:31-42.
100. Velten FW, Duperrier K, Bohlender J, Metharom P, Goerdt S. A gene signature of inhibitory MHC receptors identifies a BDCA3(+) subset of IL-10-induced dendritic cells with reduced allostimulatory capacity in vitro. Eur J Immunol 2004;34:2800-11.
101. Joffre OP, Segura E, Savina A, Amigorena S. Cross-presentation by dendritic cells. Nat Rev Immunol 2012;12:557-69.
102. Dudziak D, Kamphorst AO, Heidkamp GF, Buchholz VR, Trumpfheller C, Yamazaki S, et al. Differential antigen processing by dendritic cell subsets in vivo. Science 2007;315:107-11.
103. Tel J, Schreibelt G, Sittig SP, Mathan TS, Buschow SI, Cruz LJ, et al. Human plasmacytoid dendritic cells efficiently cross-present exogenous Ags to CD8+ T cells despite lower Ag uptake than myeloid dendritic cell subsets. Blood 2013;121:459-67.
104. Cohn L, Chatterjee B, Esselborn F, Smed-Sorensen A, Nakamura N, Chalouni C, et al. Antigen delivery to early endosomes eliminates the superiority of human blood BDCA3+ dendritic cells at cross presentation. J Exp Med 2013;210:1049-63.
105. Iborra S, Izquierdo HM, Martinez-Lopez M, Blanco-Menendez N, Reis e Sousa C, Sancho D. The DC receptor DNGR-1 mediates cross-priming of CTLs during vaccinia virus infection in mice. J Clin Invest 2012;122:1628-43.
106. Schreibelt G, Klinkenberg LJ, Cruz LJ, Tacken PJ, Tel J, Kreutz M, et al. The C-type lectin receptor CLEC9A mediates antigen uptake and (cross-)presentation by human blood BDCA3+ myeloid dendritic cells. Blood 2012;119:2284-92.
107. Dalod M. Professional cross-presenting CD8alpha-type CD141(hi) dendritic cells: we have got you in our skin! Immunity 2012;37:3-5.
108. MacDonald KP, Munster DJ, Clark GJ, Dzionek A, Schmitz J, Hart DN. Characterization of human blood dendritic cell subsets. Blood 2002;100:4512-20.
109. Tel J, van der Leun AM, Figdor CG, Torensma R, de Vries IJ. Harnessing human plasmacytoid dendritic cells as professional APCs. Cancer Immunol Immunother 2012;61:1279-88.

BDCA3 expression is associated with high IFN-λ production by CD34^{+}-derived dendritic cells generated in the presence of GM-CSF, IL-4 and/or TGF-β

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Chapter 4

Abstract

High BDCA3 expression is associated with a specific human IFN-λ-producing dendritic cell (DC) subset. However, BDCA3 has also been detected on other DC subsets. Thus far, development and function of BDCA3 expression on DCs remains poorly understood. Human Langerhans cells (LCs) and interstitial DCs (intDCs) can be generated in vitro by differentiation of $CD34^+$ hematopoietic progenitors via distinct precursor DCs (preDCs), $CD1a^+$ preDCs and $CD14^+$ preDCs, respectively. Here, we identified BDCA3 expression in this well-known GM-CSF/TNF-α-driven culture system and described the effect of IL-4 and/or TGF-β on induction of BDCA3 expression.

In control or TGF-β cultures, BDCA3 was only detected on $CD14^+$ preDC-derived intDCs. IL-4 induced BDCA3 expression in both $CD14^+$-derived and $CD1a^+$-derived cultures. TGF-β and IL-4 together further increased $CD14^+$-derived and $CD1a^+$-derived $BDCA3^+$ DC frequencies, which partly expressed CLEC9A, but were not identical to the $BDCA3^{high}CLEC9A^+$ DC subset in vivo. Importantly, $BDCA3^+$ cells, but not $BDCA3^-$ cells, in this system produced high IFNλ levels upon polyI:C stimulation. This culture system, in which BDCA3 expression is preferentially associated with the intDC lineage and IFNλ-producing capacity, will greatly contribute to further research on the function and regulation of BDCA3 expression and IFN-λ production by DCs.

Introduction

Dendritic cells (DCs) represent a heterogeneous population of professional antigen presenting cells that are found in almost all tissues [1]. Peripheral tissue DC that migrate to lymphoid organs are crucial to the initiation of immunity [2, 3]. These so-called migratory DCs include various subtypes with specialized (immune) functions [4]. In humans, the best described migratory DC subtypes are those localized in the skin, including epidermal Langerin$^+$ Langerhans cells (LCs) and two types of dermal interstitial DCs (intDCs) [5]. Additionally, BDCA3$^+$CLEC9A$^+$ DCs, specialized in antiviral functions including cross-presentation and IFNλ production [6-8], were recently identified in human dermis [9]. BDCA3 (also known as Thrombomodulin or CD141) is widely used to identify the IFNλ-producing BDCA3$^+$CLEC9A$^+$ DC subset. However, intermediate levels of BDCA3 expression were also detected on monocytes [10] and other DCs, including blood pDCs [11], pulmonary intDCs and LCs [12], and skin intDCs [9, 13, 14], indicating that BDCA3 is not a discriminating marker for the BDCA3$^+$CLEC9A$^+$ DC subset [9, 13, 14]. BDCA3 is a cell surface-expressed transmembrane glycoprotein. Besides its expression on DCs, BDCA3 is predominantly expressed on vascular endothelial cells, where it is well known for its anticoagulant activity [15]. However, the exact function of BDCA3 expression on immune cells remains to be elucidated. In addition, it remains poorly understood under which conditions BDCA3 expression is induced and whether this is restricted to specific cell subsets.

Due to the rarity and limited accessibility of human DCs and their precursors, studies on their functions and developmental pathways remain largely dependent on *in vitro* culture systems [6, 16, 17]. The possibility to simultaneously generate intDCs and LCs by culturing CD34$^+$ hematopoietic progenitor cells (HPC) with GM-CSF and TNF-α has been of great value to their characterization [17-21]. In this culture system, HPCs differentiate via two different precursor cells, CD1a$^+$CD14$^-$ precursor DCs (preDCs) and CD1a$^-$CD14$^+$ preDCs, into CD1a$^+$Langerin$^+$ LCs and CD1a$^+$DC-SIGN$^+$ intDCs, respectively [17, 19, 20]. It has been described that the differentiation of these cells can be manipulated by addition of TGF-β, polarizing towards LCs, and IL-4, polarizing towards intDCs [22]. In the present study, we investigated whether human DCs generated from CD34$^+$ HPCs by this accepted culture method expressed BDCA3 and examined the effect of TGF-β and IL-4 on the development of these BDCA3-expressing DC and their function.

Upon differentiation of CD34$^+$ HPCs in this GM-CSF/TNF-α-driven culture system we identified BDCA3 expression on DCs in this system. Without additional cytokine stimulation BDCA3 expression was specifically found on cells of the CD14$^+$-derived intDC lineage. Addition of TGF-β enhanced BDCA3 expression in CD14$^+$-derived cultures, whereas IL-4 enhanced BDCA3 expression in both CD14$^+$-derived and CD1a$^+$-derived cultures. Addition of both TGF-β and IL-4 further increased the frequencies of BDCA3$^+$ DCs. Importantly, in contrast to CD34$^+$-derived BDCA3$^-$ cells, BDCA3$^+$ cells, either CD14$^+$- or CD1a$^+$-derived and generated in the presence or absence of IL-4 and/or TGF-β, produced high levels of IFN-λ upon polyinosinic:polycytidylic acid (polyI:C) stimulation, irrespective of CLEC9A co-expression. This indicates that IFN-λ production is a specific characteristic shared by the BDCA3$^+$ DCs generated in this system. These findings demonstrate that this CD34$^+$-derived culture system is valuable for future research into DC biology aiming at the identification of the cellular and molecular mechanisms underlying development of BDCA3-expressing DCs and their functional regulation.

Materials and methods

Isolation of CD34$^+$ hematopoietic progenitor cells and peripheral-blood DCs

CD34$^+$ HPCs were isolated from umbilical cord blood samples, obtained from healthy donors according to legal guidelines, as described previously [17, 19, 20]. In brief, CD34$^+$ HPCs were isolated from mononuclear cell fractions by positive immunomagnetic selection using anti-CD34-coated microbeads and MS separation columns according to the manufacturer's protocol (Miltenyi Biotec, Bergish Gladbach, Germany). Isolated cells were cryopreserved in 10% DMSO (Sigma-Aldrich, St. Louis, MO) diluted in RPMI 1640 medium (Invitrogen, Breda, The Netherlands) supplemented with 9% heat inactivated fetal bovine serum (FBS; Sigma-Aldrich) and penicillin/streptomycin (Invitrogen).

Peripheral blood DCs were isolated from leukocyte-enriched buffy coats obtained from healthy donors. First, mononuclear cells were isolated by Ficoll-Paque density gradient centrifugation. Subsequently, BDCA3$^+$ DCs were isolated either by enrichment for DCs using a Dynabeads Human DC Enrichment kit (Invitrogen) followed by sorting of BDCA3$^+$CLEC9A$^+$ DCs using a FACSAria (BD Biosciences), or by immunomagnetic enrichment using PE-labeled BDCA3 antibody (Ab), anti-PE microbeads and MS separation columns (Miltenyi).

***In vitro* generation of CD34$^+$ HPC-derived myeloid DCs**

After cryopreservation, CD34$^+$ HPCs were cultured as described previously [17]. Cells were seeded in RPMI 1640 containing 9% heat inactivated FBS, penicillin/streptomycin, 10 mM HEPES (Lonza, Walkersville, MD), 2 mM L-glutamine (Invitrogen) and 50 μM β-mercaptoethanol (Merck, Darmstadt, Germany; referred to as complete medium), supplemented from day 0-6 with 100 ng/ml Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF, PeproTech, London, U.K.), 25 ng/ml Stem Cell Factor (SCF, PeproTech), 2.5 ng/ml Tumor Necrosis Factor α (TNF-α, PeproTech) and 5% heat-inactivated AB$^+$ pooled human serum (NHS-AB, Lonza). From day 6, cells were cultured in 100 ng/ml GM-CSF. 1 ng/ml Transforming Growth Factor β (TGF-β, PeproTech) and/or 20 ng/ml Interleukin 4 (IL-4, eBioscience, San Diego, CA) were added where indicated.

Flow cytometry

For phenotypic analysis, cells were washed with PBS containing 1% bovine serum albumin (BSA; Sigma-Aldrich), 1% heat inactivated human serum and 0.02% NaN_3 (Merck, Darmstadt, Germany; referred to as FACS buffer) and subsequently labeled with fluorochrome-conjugated Abs. Labeling was performed during 30 minutes of incubation on ice with Abs recognizing BDCA3/CD141 (AD5-14H12, Miltenyi); BDCA1/CD1c (AD5-8E7, Miltenyi); CD1a (HI149, eBioscience); CD11c (3.9, eBioscience); CD14 (61D3, eBioscience); HLA-DR (LN3, eBioscience); CD11b (ICRF44, BD Biosciences, Breda, the Netherlands); CD86 (Fun-1, BD Biosciences); DC-SIGN/CD209 (DCN47, BD Biosciences); CLEC9A (8F9, BioLegend, San Diego, CA) and Langerin/CD207 (DCFM4, Beckman Coulter, Woerden, the Netherlands). Fluorescence was measured using a FACS Canto II (BD Biosciences) and resulting data were analyzed using FlowJo software (Tree Star, Inc.).

For sorting experiments, day 6 cultures were sorted based on CD1a and CD14 expression using a FACSAria (BD Biosciences). For some experiments, CD14$^+$CD1a$^-$-, CD14$^-$CD1a$^+$- or CD1a$^+$CD14$^+$-derived cultures were sorted again at day 12 based on BDCA3 and Langerin expression or at day 14 based on BDCA3/CD141 and CLEC9A expression. Sorted cells were seeded in complete medium supplemented with GM-CSF, either with or without TGF-β and/

or IL-4, or stimulated as described below.

RNA isolation and gene expression analysis
RNA was extracted according to the manufacturer's instructions using an RNAeasy Micro kit or a Nucleospin RNA II kit for samples containing <5x10^5 or >5x10^5 cells, respectively. For qPCR, OD260/280 ratios were measured to determine RNA concentration and purity prior to cDNA synthesis using a PrimeScript cDNA synthesis kit (Takara Bio, Tokyo, Japan). qPCR was performed using a MyiQ Real-Time PCR detection system and primers as shown in table 1. For gene expression profiling using Nanostring nCounter system, RNA concentration and integrity was assessed using a Bioanalyzer system (Agilent Technologies). Human immunology-related genes were profiled using the nCounter GX Human Immunology v2 kit Gene Expression CodeSet (Nanostring Technologies). Gene expression levels were normalized according to 15 housekeeping genes. Principal component analysis (PCA) was performed using MeV software.

Cytokine secretion
DC were stimulated for 24 or 48 hours with PolyI:C (20 µg/ml, Invivogen, Toulouse, France). The levels of secreted human IFN-λ1 (IL-29, eBioscience), IFN-λ2 (IL-28A), IFN-λ3 (IL-28B), CXCL10 (R&D systems) and IL-12p70 (eBioscience) were measured using commercially available ELISA kits according to the manufacturer's protocol. Detection limits were 8 pg/ml (IFN-λ1), 30 pg/ml (IFN-λ2/ IFN-λ3), 1.67 pg/ml (CXCL10) and 4 pg/ml (IL-12p70).

Statistical analysis
Statistical analysis was performed using Graphpad Prism version 5.01 for Windows (GraphPad Software, San Diego, California USA). A paired Student's t-test was used to determine the p-values.

Table 1. RT-PCR primers

CLEC9A	Forward 5'-ATCCAACAAGAGAGGGCAC-3' Reverse 5'-TTGTTTGGACAAGGACTGC-3'
XCR1	Forward 5'- CAAGACGCATGTAAAGAGG-3' Reverse 5'- CTGGCTCTGAAGGTCATAG-3'
TLR3	Forward 5'-AGTTGTCATCGAATCAAATTAAAGAG-3' Reverse 5'-AATCTTCCAATTGCGTGAAAA-3'
CADM1	Forward 5'- TGCCAGCTCTATACCGATC-3' Reverse 5'- AATCTCCTCACCTTCCACC-3'
BATF3	Forward 5'-ATGATGACAGGAAGGTCCG-3' Reverse 5'-TTTCTTGCTCCAGGCTCTC-3'
IRF8	Forward 5'- TCCCAACTGGACATTTCCG-3' Reverse 5'- ACTTCATTCACGCAGCCAG-3'
GAPDH	Forward 5'-TGCACCACCAACTGCTTAGC-3' Reverse 5'-GGCATGGACTGTGGTCATGAG-3'

Results

CD34$^+$-derived intDCs express BDCA3

We first aimed to fully elucidate the known culture system that generates DCs from CD34$^+$ HPCs under the influence of GM-CSF and TNF-α [17]. As previously described, this system generated a BDCA1$^+$CD1a$^+$ DC population comprising Langerin$^+$ LCs and DC-SIGN$^+$ intDCs (Figure 1A), which are known to differentiate through independent pathways via CD1a$^+$CD14$^-$ preDCs and CD14$^+$CD1a$^-$ preDCs, respectively [17, 19]. In addition, we observed that DC-SIGN$^+$ intDCs, but not Langerin$^+$ LCs, partially co-expressed BDCA3 (Figure 1A).

To determine the route of differentiation of these BDCA3$^+$ DCs, day 6 CD14$^+$ preDCs and CD1a$^+$ preDCs shown in Figure 1A were sorted and cultured separately. Indeed, BDCA3 expression was particularly observed within the CD14$^+$-derived cultures (Figure 1B,C). By day 6, CD14$^+$ preDCs already included a BDCA3$^+$ population, which increased in frequency (2±0.5-fold, $p<0.05$), absolute cell numbers and BDCA3 expression level upon further differentiation from day 10 until day 14 (Figure 1B, C). Comparison of BDCA3$^+$ and BDCA3$^-$ CD14$^+$-derived cells for several phenotypic markers revealed that both populations had a CD11c$^+$HLA-DR$^+$CD86$^{-/low}$ immature DC phenotype, expressed BDCA1 and CD1a, partially expressed CD11b and DC-SIGN, but lacked CLEC9A (Figure 1B,D). Although the BDCA3$^-$ population included Langerin$^+$ cells, hardly any co-expression of Langerin and BDCA3 was detected (Figure 1D).

Thus, BDCA3 expression was detected on CD34$^+$-derived DCs generated in the presence of GM-CSF and TNF-α and particularly associated with the CD14$^+$-derived intDC lineage. CD14$^+$-derived BDCA3$^+$ DCs had an immature phenotype and did not differ much in the expression of BDCA1, CD1a, DC-SIGN and CD11b from their BDCA3$^-$ CD14$^+$-derived counterparts.

IL-4 induces CD1a$^+$-derived BDCA3$^+$ DCs and, like TGFβ, increases CD14$^+$-derived BDCA3$^+$ DCs

TGF-β and IL-4 have been described to skew the differentiation of CD34$^+$-derived DCs towards LCs and intDCs, respectively [22]. Indeed, addition of TGF-β from day 6 strongly enhanced expression of the LC marker Langerin at day 14, whereas IL-4 decreased Langerin expression and increased expression of the intDC marker DC-SIGN (Figure 2A). Additionally, IL-4 enhanced differentiation towards CD1a$^+$BDCA1$^+$ DCs and expression of CD11b (Figure 2A). Although addition of TGF-β and IL-4 together decreased the upregulation of DC-SIGN compared with IL-4 alone, the combination of TGF-β and IL-4 mostly resembled the effect of IL-4 alone with respect to Langerin, CD1a, BDCA1, and CD11b expression (Figure 2A).

In addition, we investigated the effect of TGF-β and/or IL-4 on the induction of BDCA3 expresion on DCs in this system. As shown in Figure 1, in the absence of TGF-β and/or IL-4, BDCA3 was particularly expressed by CD14$^+$-derived intDCs. To investigate whether addition of TGF-β and/or IL-4 affected this preferential expression by intDCs, we sorted CD14$^+$ and CD1a$^+$ preDCs at day 6 and cultured the two populations separately with or without TGF-β and/or IL-4. TGF-β enhanced the frequencies of BDCA3-expressing CD14$^+$-derived DCs, but was not able to induce BDCA3 expression on CD1a$^+$-derived DCs (Figure 2B, C, D, E). Since we already observed that BDCA3 expression specifically developed within the CD14$^+$ preDC population in control cultures without additional cytokines (Figure 1), it was not unexpected that IL-4, a cytokine known to accelerate intDC development from CD14$^+$ preDCs, enhanced the frequency and absolute numbers of BDCA3$^+$ DCs in CD14$^+$-derived cultures (Figure 2B, C, D, E). Interestingly, culture of CD1a$^+$ preDCs in the presence of IL-4 also resulted in highly increased frequencies and absolute numbers of BDCA3$^+$ DCs (Figure 2B, C, D, E). The

combination of TGF-β plus IL-4 induced the highest expression of BDCA3 and the highest frequency of BDCA3⁺ cells in both CD14⁺ and CD1a⁺ sorted cultures (Figure 2B, C, D, E).
Furthermore, in depth analysis of BDCA3 expression by cells in the different cytokine cultures revealed that in CD14⁺-derived control cultures without additional cytokines, BDCA3 was mainly expressed by DC-SIGN⁺ intDCs (Figure 2F). CD14⁺-derived cultures to which also TGF-β was added contained a Langerin^{hi}BDCA3⁻ DC population that most likely represents LCs.
Very remarkable, however, is the finding that these TGF-β-induced cultures also included a

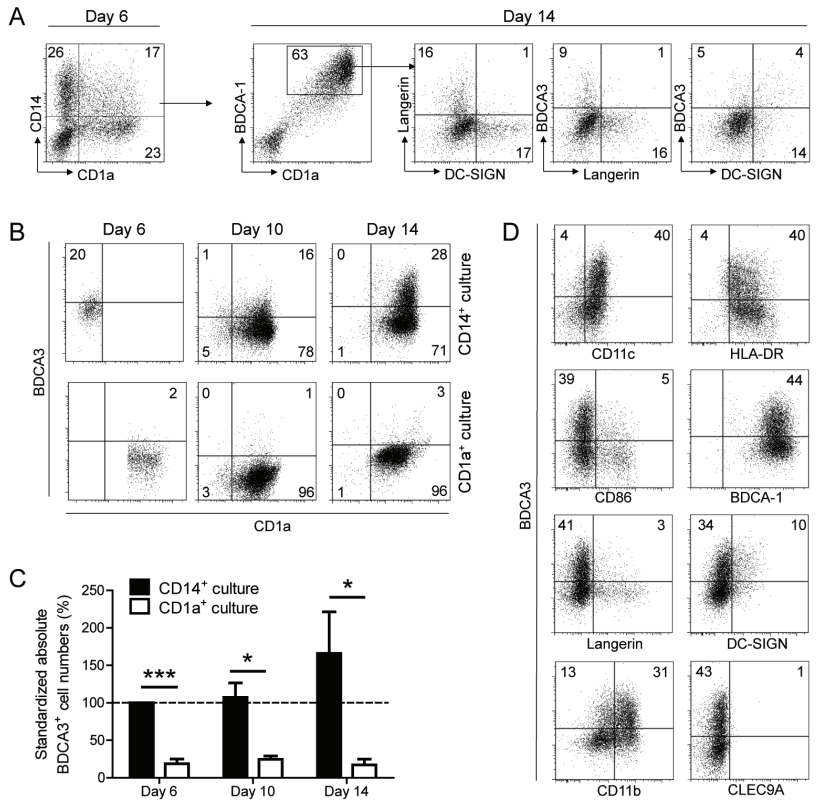

Figure 1. CD34⁺-derived DC cultures contain a BDCA3⁺ DC subset in addition to intDCs and LCs.
(A) CD34⁺ cells were plated and cultured for 6 days in the presence of GM-CSF, TNF-α and SCF, followed by an 8-day culture in the presence of GM-CSF alone. CD34⁺-derived cells were analyzed at day 6 for expression of CD1a and CD14, and at day 14 for BDCA1, CD1a, Langerin, DC-SIGN and BDCA3 by flow cytometry. **(B and C)** Day 6 cells were FACSorted into CD1a⁺CD14⁻ and CD1a⁻CD14⁺ fractions, and cultured separately. At day 6, 10 and 14, cells were counted with trypan blue exclusion and BDCA3 expression of FSC/SSC-gated viable cells was analyzed by flow cytometry. Isotype controls were used to determine the positions of the gates at each timepoint. Absolute numbers of BDCA3⁺ DC were calculated and standardized to day 6 CD1a⁻CD14⁺ fractions. (B) Representative FACS plots and (C) mean ±SEM standardized absolute BDCA3⁺ cell numbers are shown (*n*=5). (C) Data are pooled from 5 independent experiments, each performed with a different donor. **p*<0.05, ****p*<0.001, paired Student's t-test. **(D)** Day 14 CD14⁺-derived DCs were analyzed for BDCA3, CD11c, HLA-DR, CD86, BDCA1, CD11b, DC-SIGN, Langerin and CLEC9A expression. (A-D) FACS plots are representative of five independent experiments, each performed with a different donor.

BDCA3$^+$ DC population with intermediate Langerin expression. In addition, BDCA3$^+$Langerin$^-$ DCs were observed (Figure 2F).
In IL-4-induced CD14$^+$-derived cultures BDCA3 was expressed by DC-SIGN$^+$ intDCs, whereas in CD1a$^+$-derived cultures BDCA3 was expressed by DC-SIGN$^+$ DCs as well as DC-SIGN$^-$ DCs (Figure 2F). CD14$^+$-derived DCs generated in the presence of IL-4 and TGF-β almost all expressed BDCA3. 70% of these cells co-expressed DC-SIGN and a small Langerin$^+$ cell population was observed within the DC-SIGN$^-$ population. Development of BDCA3$^+$ DCs was boosted in CD1a$^+$-derived cultures by IL-4 and TGF-β compared with IL-4 alone. Both Langerin-expressing BDCA3$^+$ DCs and more intDC-related DC-SIGN$^+$ BDCA3$^+$ DCs were observed and a Langerin$^+$BDCA3$^-$ DC population remained present, which is likely to represent LCs.
Together, these data show that IL-4 as well as TGF-β induce BDCA3$^+$ DCs within the cell population derived from CD14$^+$ preDCs, known as precursors of intDCs (pre-intDCs). Only IL-4, a cytokine known to stimulate intDC development, induces BDCA3$^+$ DC development in cultures of CD1a$^+$ preDCs, known as precursors of Langerhans cells (pre-LCs). In the presence of both TGF-β and IL-4, almost all CD14$^+$-derived DCs express BDCA3 and most CD1a$^+$-derived DCs express BDCA3, but within this latter condition also a BDCA3$^-$Langerin$^+$ DC population is present which does not respond to skewing towards a more intDC related cell type.

Phenotypic and functional characteristics of TGFβ/IL-4-induced BDCA3$^+$CLEC9A$^+$ DCs
Since high BDCA3 expression is known to be specific for a distinct human DC subset *in vivo* that can be further characterized by CLEC9A expression and IFN-λ production, we first investigated whether the BDCA3$^+$ DCs induced in this *in vitro* culture system also expressed CLEC9A and exhibited other phenotypical and functional characteristics associated with this human DC subset. In contrast to other conditions, cultures containing both TGF-β and IL-4 included BDCA3$^+$CLEC9A$^+$ cells (Figure 3A). Highest frequencies and absolute numbers of BDCA3$^+$CLEC9A$^+$ DCs developed from CD1a$^+$-preDCs (Figure 3A, B, C, D). Whereas the percentage and absolute numbers of CLEC9A$^+$ cells within CD1a$^+$ cultures did not further increase from day 10, the CLEC9A expression level significantly increased from day 10 till day 14 (Figure 3B).
Next, day 6 CD14$^+$ preDCs and CD1a$^+$ preDCs were cultured together in the presence of TGF-β and IL-4, and the total population of BDCA3$^+$CLEC9A$^+$ DCs was FACSorted at day 14. We investigated whether these *in vitro*-generated BDCA3$^+$CLEC9A$^+$ DCs resembled peripheral blood-derived BDCA3$^+$CLEC9A$^+$ DCs. Both DC types were profiled for expression of 579 immunology-related human genes (Supplemental Table 1). Principal component analysis (PCA) indicated that along the PC1 axis, the variance between the two DC types is larger than the variance between the replicates. However, the adjacent position of the two DC types along the PC2 and PC3 axis indicates partial analogy in gene expression profiles of *in vitro*-generated DCs and blood-derived DCs (Figure 4A). In addition, there is a significant correlation in gene expression between the two DC types ($r=0.84$, $p<0.0001$, Figure 4B). 43% of genes showed a ≤3-fold change between *in vitro*-generated DCs and blood-derived DCs (Supplemental Table 1). Several of these genes encode proteins that are involved in antigen processing and presentation, receptors, and IFN-λ-regulatory elements (Figure 4C). Genes showing a >3-fold change in expression levels between *in vitro*-generated DCs and blood-derived DCs (57%) included genes that were previously identified as being highly expressed in *in vivo* BDCA3$^+$CLEC9A$^+$ DCs, i.e. IRF8, XCR1, IDO1, NFIL3 and TLR3 (Figure 4C, Supplemental Table 1) [6, 8, 23, 24]. The expression levels of these genes were higher in blood-derived

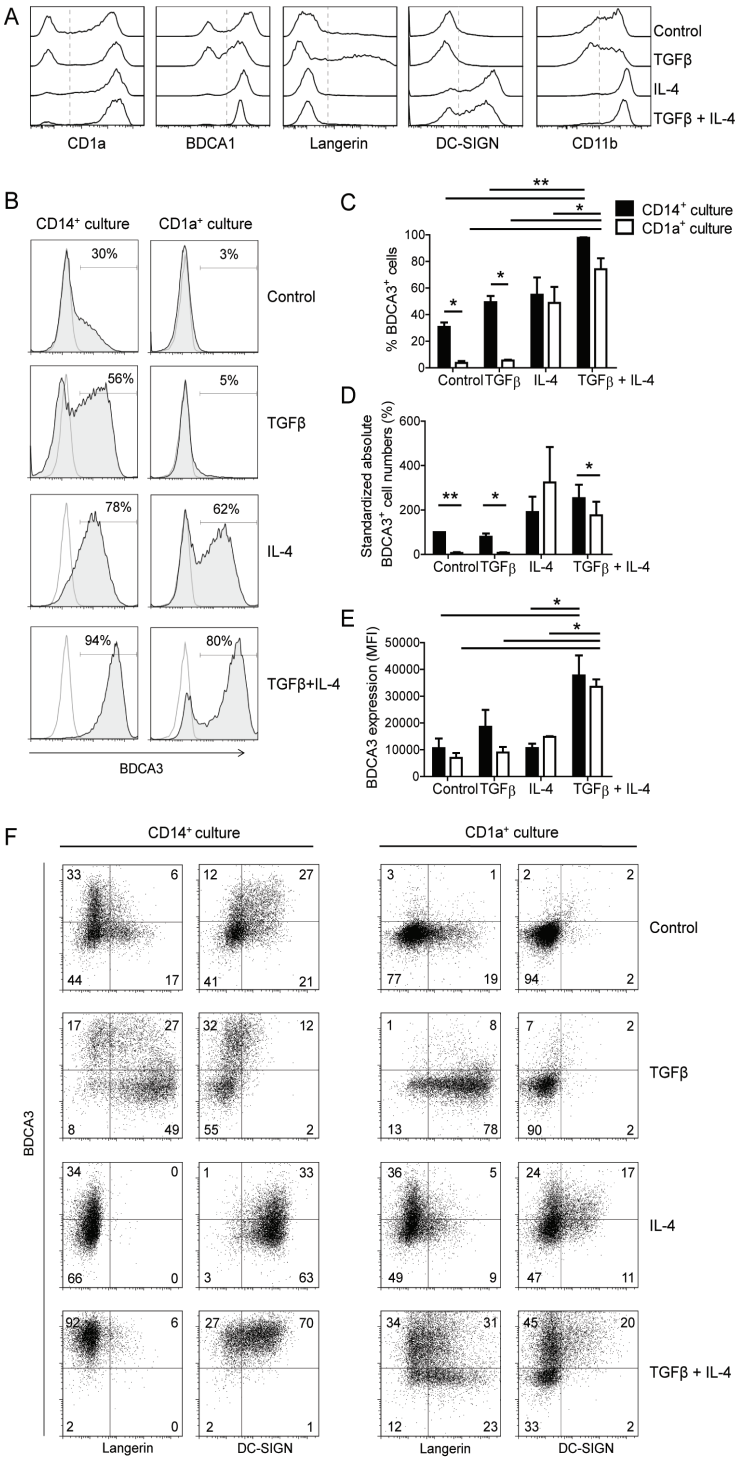

Figure 2. In contrast to TGF-β, IL-4 not only induces BDCA3⁺ DCs within CD14⁺-derived cultures, but also within CD1a⁺-derived cultures.

CD34⁺ cells were plated and cultured for 6 days in the presence of GM-CSF, TNF-α and SCF, followed by a 6-day culture in the presence of GM-CSF, either with or without TGF-β, IL-4 or TGF-β and IL-4. **(A)** CD1a, BDCA1, Langerin, DC-SIGN and CD11b expression was determined at day 14. FACS plots of FSC/SSC gated viable cells are representative of 3 independent experiments, each performed with a different donor. The maximum level of isotype staining is indicated by the dashed lines. **(B-E)** Day 6 cells were FACSorted into $CD1a^+CD14^-$ and $CD1a^-CD14^+$ fractions and cultured separately in the presence of GM-CSF with or without TGF-β, IL-4 or TGF-β and IL-4. At day 14, cells were counted with trypan blue exclusion and BDCA3 expression of FSC/SSC-gated viable cells was analyzed by flow cytometry (n=4). (B) Representative FACS plots are depicted in which the dotted line represents the isotype control staining. (C) Percentage of $BDCA3^+$ cells within the different cultures. (D) Absolute numbers of $BDCA3^+$ DCs were calculated

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Figure 2. (continued)
and standardized to day 14 $CD14^+$derived control fractions. These relative values correspond with an average $CD14^+$-derived $BDCA3^+$ cell yield of $1.7*10^4$ cells after 14 days of culture under standard culture conditions starting from $1.0*10^6$ $CD34^+$ HPCs. (E) Mean fluorescence intensity (MFI) of BDCA3 staining on the $BDCA3^+$ population. (C-E) Data are shown as mean ±SEM (n=4). *p<0.05, **p<0.01, paired Student's t-test. (F) Day 6 cells were FACSorted into $CD1a^+CD14^-$ and $CD1a^-CD14^+$ fractions and cultured separately in the presence of GM-CSF with or without TGF-β, IL-4 or TGF-β and IL-4. At day 14, BDCA3, Langerin and DC-SIGN expression were determined by flow cytometry. Representative FACS plots of FSC/SSC gated viable cells are shown. (B-F) Data are pooled from four independent experiments, each performed with a different donor.

DCs than in *in vitro*-generated DCs. Additionally, many cytokine-receptor and chemokine-receptor genes varied >3-fold between the two DC types (Figure 4C). These results indicate that *in vitro*-generated $BDCA3^+CLEC9A^+$ DCs are not identical to peripheral blood-derived $BDCA3^+CLEC9A^+$ DCs, which may be partially explained by differences between *in vitro*-generated and *in vivo*-derived DC in general.
To further investigate phenotypic and functional differences and similarities between *in vitro*-generated $BDCA3^+CLEC9A^+$ DCs and their $BDCA3^+CLEC9A^-$ and $BDCA3^-CLEC9A^-$ counterparts on one hand and to confirm differential gene expression between *in vitro*-generated and blood-derived $BDCA3^+CLEC9A^+$ DCs on the other hand, DC populations were analyzed for CLEC9A, XCR1, BATF3, CADM1 and IRF8 by qPCR (Supplemental Figure 1). CADM1, TLR3 and BATF3 mRNA expression was observed for all three FACSorted *in vitro*-generated cell populations, although $BDCA3^+CLEC9A^+$ DCs expressed significantly higher levels of CADM1 and BATF3 than the other populations. Neither XCR1 nor IRF8 mRNA expression could be detected for any of the *in vitro*-generated DC populations (Supplemental Figure 1). Functional analysis revealed that all three *in vitro*-generated DC populations produced CXCL10 (Figure 4D). Interestingly, despite the similar TLR3 expression by $BDCA3^-$ and $BDCA3^+$ cells, only $BDCA3^+$ DCs produced high levels of IFN-λ1 upon polyI:C stimulation, irrespective of the expression of CLEC9A (Figure 4D, E). Although IFN-λ1 levels produced by *in vitro*-generated DCs were lower than those produced by blood-derived $BDCA3^+CLEC9A^+$ DCs, they were at least 18-fold higher than levels produced by polyI:C-stimulated blood-derived $BDCA1^+$ DCs and 80-fold higher than CpG-A-stimulated blood-derived pDCs (Figure 4D). IL-12p70 production in response to polyI:C stimulation could not be detected (data not shown).
Together, these data show that the *in vitro*-generated $BDCA3^+CLEC9A^+$ DCs are, based on the above-mentioned markers, not distinct from $CLEC9A^-$ DCs generated in this system. However, like *in vivo* $BDCA3^+CLEC9A^+$ DCs, *in vitro*-generated $BDCA3^+$ DCs specifically produce high levels of IFN-λ upon polyI:C stimulation, irrespective of CLEC9A expression.

IFN-λ1 production levels correlate with $BDCA3^+$ DC development
The observation that high IFN-λ production was restricted to $BDCA3^+$ DCs, prompted us to also investigate the IFN-λ-producing capacity of cells derived from control, TGF-β, IL-4 and TGF-β/IL-4-induced cultures, each containing different frequencies of $BDCA3^+$ DCs. Functional analysis of polyI:C-stimulated cultures revealed that IFN-λ1 was not only produced by TGF-β/IL-4-induced DCs, but specifically by all cultures containing $BDCA3^+$ DCs, i.e. all $CD14^+$-derived cultures and $CD1a^+$ preDC cultures containing IL-4 either with or without TGF-β, but not in control or TGF-β-treated $CD1a^+$ preDC cultures (Figure 5A). Moreover, statistical analysis of the relation between the percentage of $BDCA3^+$ DCs present in the cultures exposed to polyI:C and the level of IFN-λ produced by these cell cultures revealed a positive correlation between the percentage $BDCA3^+$ cells and the level of IFN-λ1

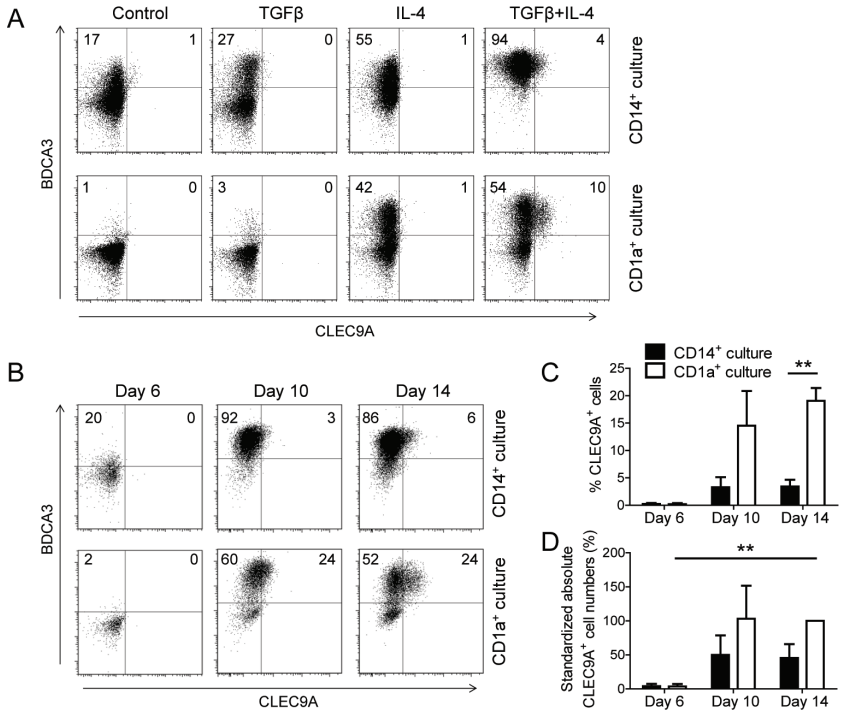

Figure 3. TGF-β/IL-4-induced cultures contain CLEC9A$^+$ cells.
CD34$^+$ cells were plated and cultured for 6 days in the presence of GM-CSF, TNF-α and SCF. Day 6 cells were FACSorted into CD1a$^+$CD14$^-$ and CD1a$^-$CD14$^+$ fractions and cultured separately in the presence of GM-CSF with TGF-β and IL-4. **(A)** At day 14, CLEC9A and BDCA3 expression were analysed by flow cytometry. Representative FACS plots of FSC/SSC gated viable cells show CLEC9A and BDCA3 expression (*n*=3). **(B-D)** Cells cultured in the presence of GM-CSF, TGF-β and IL-4 were counted with trypan blue exclusion and analyzed for CLEC9A and BDCA3 expression by flow cytometry at day 6, 10 and 14. (B) Representative FACS plots of FSC/SSC gated viable cells. (C) Data are shown as mean ±SEM percentages and (D) standardized absolute CLEC9A$^+$ cell numbers (*n*=3). ***p*<0.01, paired Student's t-test. (A-D) Data are pooled from three independent experiments, each performed with a different donor.

(Figure 5B), suggesting that IFN-λ1 is particularly produced by BDCA3$^+$ DCs. In addition, assessment of IFN-λ2 and IFN-λ3 production resulted in a similar pattern. Both IFN-λ2 and IFN-λ3 were also specifically detected in supernatants derived from cell populations which included BDCA3$^+$ DCs, although levels produced by the IL-4-induced CD14$^+$-derived cell population were very low (Figure 5C, D). Interestingly, IFN-λ2 and IFN-λ3 levels produced by TGF-β-differentiated CD14$^+$-derived cells were remarkably higher compared with those of the other conditions.

To investigate whether indeed the BDCA3$^+$, but not the BDCA3$^-$ cells, were the major IFN-λ producers in these cultures and to investigate the high IFN-λ production by TGF-β-induced CD14$^+$-derived DCs in more detail, TGF-β-induced CD14$^+$-derived BDCA3$^+$ and BDCA3$^-$ cells were FACSorted and analyzed separately for IFN-λ production upon polyI:C stimulation. Furthermore, since TGF-β is known as a cytokine promoting development of CD14$^+$-derived Langerin$^+$ cells, previously considered as Langerhans-like cells [22] (Figure 2F), and BDCA3 expression was observed on Langerin$^+$ as well as Langerin$^-$ cells, cells were also FACSorted based on Langerin expression. Indeed, BDCA3$^+$ DCs, but not BDCA3$^-$ DCs,

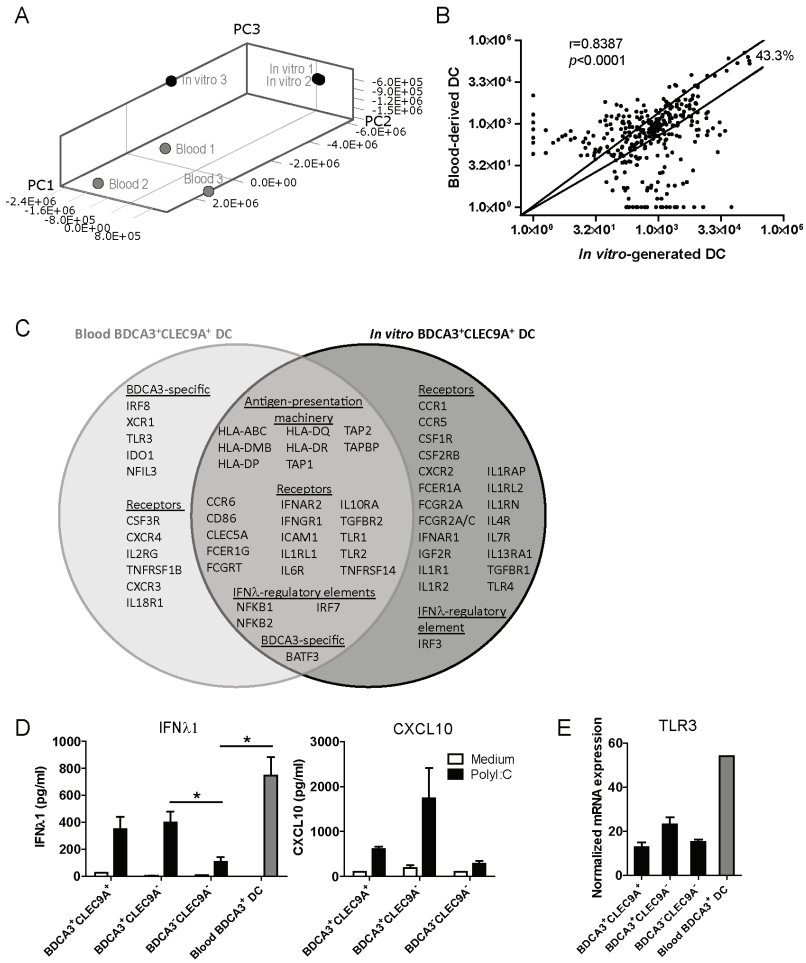

Figure 4. TGF-β and IL-4-induced CLEC9A$^+$BDCA3$^+$ DCs do not resemble blood-derived BDCA3$^+$CLEC9A$^+$ DCs, but produce CXCL10 and IFN-λ upon polyI:C stimulation. CD34$^+$ cells were plated and cultured for 6 days in the presence of GM-CSF, TNF-α and SCF. At day 6, CD14$^+$ and CD1a$^+$ preDCs were sorted and cultured together in the presence of GM-CSF with TGF-β and IL-4. At day 14, cells were FACSorted based on BDCA3 and CLEC9A expression. **(A-C)** mRNA was isolated from *in vitro*-generated BDCA3$^+$CLEC9A$^+$ DCs (*n*=3) and FACSorted peripheral blood-derived BDCA3$^+$CLEC9A$^+$ DC (*n*=3). Gene expression levels were measured using the NanoString nCounter system. (A) PCA was performed using the MeV software, based on genes expressed in at least one of the two DC types (expression level >50). PC1 accounts for 80.1%, PC2 for 8.8%, and PC3 for 6.6% of the total variance. Each point represents a sample, classified by DC type. (B) The correlation between gene expression profiles of the two DC types with 3-fold change lines. (C) Genes >3-fold upregulated in blood-derived DCs (left circle), >3-fold upregulated in *in vitro*-generated DCs (right circle) and ≤3-fold changed between the two DC types (overlapping area) are shown. **(D)** *In vitro*-generated BDCA3$^+$CLEC9A$^+$, BDCA3$^+$CLEC9A$^-$ and BDCA3$^-$CLEC9A$^-$ fractions and FACSorted blood-derived BDCA3$^+$CLEC9A$^+$ DCs were cultured in the presence or absence of polyI:C for 24 hours. IFN-λ1 and CXCL10 concentrations were determined by ELISA. **(E)** mRNA was isolated from *in vitro*-generated BDCA3$^+$CLEC9A$^+$, BDCA3$^+$CLEC9A$^-$ and BDCA3$^-$CLEC9A$^-$ DC fractions and a MACS-enriched blood-derived BDCA3$^+$ DC fraction. TLR3 mRNA levels were measured by qPCR and normalized to GAPDH expression levels. (D and E) Data are shown as mean ±SEM of (D) IFNλ1 and CXCL10 production or (E) normalized expression levels (*n*=3). (A-E) Data are pooled from 3 independent experiments, each performed with a different donor. ND, not detected. *p<0.05, paired Student's t-test.

produced high levels of IFN-λ1, IFN-λ2 and IFN-λ3 upon polyI:C stimulation (Figure 5E, F, G). The high IFN-λ1 production by BDCA3$^+$ DCs was irrespective of Langerin expression (Figure 5E, F, G). By contrast, neither polyI:C nor LPS, R848 or CD40L induced IFN-λ production by TGF-β-differentiated BDCA3$^-$ cells (data not shown). Thus, BDCA3$^+$ DCs, either Langerin$^+$ or Langerin$^-$ that are generated in this *in vitro* culture system are potent producers of IFN-λ.

Discussion

The GM-CSF/TNF-α-driven culture system firstly described by Caux *et al.,* is known to generate LCs and intDCs [17]. In the present study we report for the first time the development of BDCA3-expressing DCs in this system that specifically produced IFN-λ. By using IL-4 and TGF-β, cytokines which were previously described to skew the differentiation towards interstitial DCs and LCs respectively, we observed that BDCA3 expression was strongly associated with the intDC lineage. Furthermore, BDCA3$^+$ DCs, but not BDCA3$^-$ DCs, from different conditions shared the capacity to produce high levels of IFN-λ upon polyI:C stimulation.

BDCA3 has proven a useful marker in the classification of human DCs, but phenotypic and functional heterogeneity between BDCA3$^+$ DCs has been described. High BDCA3 expression identifies DCs belonging to the CLEC9A$^+$ DC subset, but BDCA3 is also expressed by CD14$^+$ dermal DCs which do not belong to this CLEC9A$^+$ DC subset [9, 13]. Here, BDCA3 expression was particularly observed in CD14$^+$ pre-intDC cultures, which could be further promoted by IL-4, TGF-β, or both. In contrast, only when the intDC-skewing cytokine IL-4 was added to CD1a$^+$ preDC cultures, significant BDCA3 expression could be detected on DCs derived from these precursors, suggesting that BDCA3 expression is specifically associated with the intDC lineage. The link between BDCA3 expression and the intDC lineage corresponds to recent studies of dermal DCs, which described that co-expression of BDCA3 was only detected in the CD14$^+$ intDC population [13, 14]. It is currently unknown what the function of BDCA3 is on this DC subset and whether BDCA3$^+$ and BDCA3$^-$ intDCs are functionally different. BDCA3 has been described to have an anti-inflammatory function through several direct and indirect mechanisms, reviewed by Li *et al.* [15]. Indeed, several studies suggested that BDCA3 expression on DCs is associated with a tolerogenic function of these cells [14, 25, 26]. On the other hand, BDCA3$^+$CLEC9A$^+$ DCs in peripheral tissues and blood, are often associated with cross-presentation and anti-viral immunity and described as potent immunogenic DCs [6-9, 27]. In the present study we found no clear differences between CD34$^+$-derived BDCA3$^+$ DCs and BDCA3$^-$ DCs in CXCL10 and IL-12p70 production. Furthermore, it does not seem that BDCA3 is expressed by more mature DCs since the percentage of CD86$^+$ cells was even lower in the BDCA3$^+$ population than in the BDCA3$^-$ population in control cultures. However, a very interesting finding is that BDCA3 expression in this culture system is associated with polyI:C-induced IFN-λ production. This suggests an anti-viral function of BDCA3-expressing cells. However, the mechanisms underlying the relation between BDCA3 expression and IFN-λ production remain to be elucidated.

The superior IFN-λ2 and IFN-λ3 production as specifically observed for TGF-β-induced BDCA3$^+$ DCs may indicate that these cells are functionally different or further differentiated. On the other hand, TGF-β might positively influence the signalling leading to IFN-λ production, whereas IL-4 might have a negative effect on this process. Further research is required to obtain insight into the exact cause of these differences in IFN-λ levels between the distinct

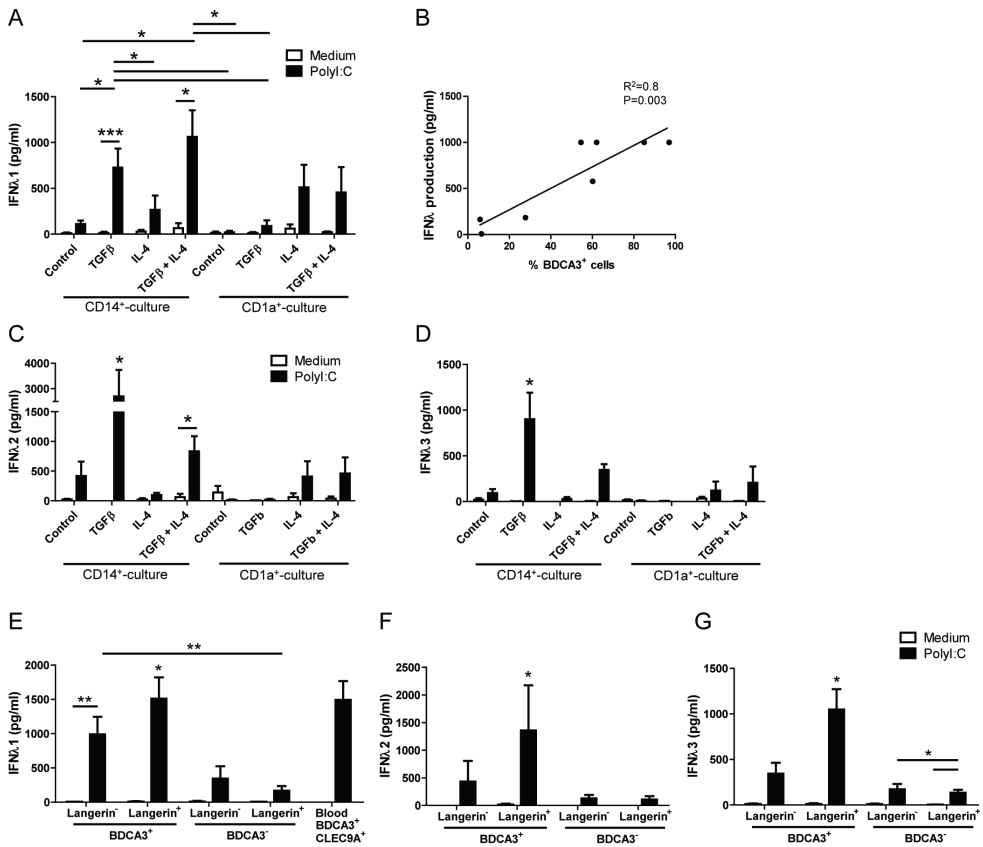

Figure 5. BDCA3$^+$ DCs are potent producers of IFN-λ in response to polyI:C stimulation. CD34$^+$ cells were plated and cultured for 6 days in the presence of GM-CSF, TNF-α and SCF. **(A-D)** Day 6 cells were FACSorted into CD1a$^+$CD14$^-$ and CD1a$^-$CD14$^+$ fractions and cultured separately in the presence of GM-CSF with TGF-β and/or IL-4. At day 14, cells were cultured in the presence or absence of polyI:C without additional cytokines for 24 hours. IFN-λ1, IFN-λ2 and IFN-λ3 concentrations were determined by ELISA. (A) Data are shown as mean ±SEM IFN-λ1 production (n=3) and are pooled from 3 independent experiments, each performed with a different donor. The correlation between the percentage of BDCA3$^+$ cells and IFN-λ1 production was determined. (B) Data from one representative experiment of three independent experiments with similar results is depicted. (C and D) Data are shown as mean ±SEM of (C) IFN-λ2 and (D) IFN-λ3 production (n=3) and are pooled from 3 independent experiments, each performed with a different donor. Statistical analysis was performed on IFN-λ1, IFN-λ2 and IFN-λ3 levels standardized to levels produced by TGF-β-induced CD14$^+$-derived cells. **(E-G)** CD14$^+$CD1a$^-$ cells were FACSorted at day 6 and cultured in the presence of GM-SCF and TGF-β. At day 12, BDCA3$^+$Langerin$^+$, BDCA3$^+$Langerin$^-$ and BDCA3$^-$Langerin$^+$ fractions were FACSorted and further cultured in the presence or absence of polyI:C without additional cytokines. After 2 days, supernatants of BDCA3$^+$Langerin$^+$, BDCA3$^+$Langerin$^-$ and BDCA3$^-$Langerin$^+$ cells were harvested and IFN-λ1, IFN-λ2 and IFN-λ3 concentrations were determined by ELISA. Data are shown as mean ±SEM production of IFN-λ1 (n=5, E), IFN-λ2 (n=3, F) and IFN-λ3 (n=3, G) and are pooled from 5 independent experiments, each performed with a different donor. Statistical analysis was performed on IFN-λ1, IFN-λ2 and IFN-λ3 levels standardized to levels produced by TGF-β-induced CD14$^+$-derived cells. polyI:C-stimulated BDCA3$^+$Langerin$^+$ cells. *p<0.05, **p<0.01, ***p<0.001, paired Student's t-test.

cytokine conditions.

So far, the IFN-λ-producing BDCA3$^+$ DCs *in vivo* have been identified by co-expression of CLEC9A, which is involved in the sensing and presentation of necrotic cell-derived antigens [6, 8, 27-30]. Here, CLEC9A was partially expressed on BDCA3$^+$ DCs induced by TGF-β and IL-4, which suggests that these *in vitro*-generated DC belong to the CLEC9A$^+$ lineage described *in vivo* by Poulin *et al.* [29].

However, transcriptional profiling showed that gene expression patterns of *in vitro*-generated BDCA3$^+$CLEC9A$^+$ DCs showed only partial analogy with blood-derived BDCA3$^+$CLEC9A$^+$ DCs. This is in line with the findings of Lundberg *et al.*, who show that many of immune-related genes differ across *in vitro* model DCs and *ex vivo* DCs and that *in vitro* model DCs are more similar to each other than to *ex vivo* DCs [31]. Nevertheless, BDCA3$^+$CLEC9A$^+$ DC-characteristic genes, i.e. IRF8, XCR1, IDO1, TLR3 and NFIL3, were absent or lower expressed by our *in vitro*-generated BDCA3$^+$CLEC9A$^+$ DCs. Thus, although *in vitro*-generated and *in vivo*-derived BDCA3$^+$CLEC9A$^+$ DC share several functional and phenotypic features, overall the immunology-related gene expression profiles indicate that *in vitro*-generated BDCA3$^+$CLEC9A$^+$ DCs are not identical to *bona fide* BDCA3$^+$CLEC9A$^+$ DCs present *in vivo*. This also suggests that not only BDCA3, but also CLEC9A, a marker commonly used for identification of DCs of the BDCA3$^+$CLEC9A$^+$XCR1$^+$ lineage in human and mice, can be expressed by cells that do not belong to this DC lineage. Moreover, BDCA3 and CLEC9A may be induced on each precursor cell when stimulated appropriately, and the induction of BDCA3 may require different stimulatory signals than induction of CLEC9A. Low CLEC9A expression was previously also detected on human B cells and murine pDC [32]. By what exact mechanism CLEC9A expression is induced remains to be further investigated. Given the expression of CLEC9A in this culture system under certain conditions, this system may contribute to assessment of pathways regulating CLEC9A expression. Of note, the combination of Flt3L and IL-4, which was used in a previously described *in vitro* culture system to generate BDCA3$^+$CLEC9A$^+$ DC [6], did not induce any CLEC9A$^+$ cells in this system (data not shown).

The increased expression of Langerin upon addition of TGF-β to the culture system confirms previous findings showing that exogenous TGF-β can induce Langerin expression and polarize differentiation of CD14$^+$ preDCs towards Langerin$^+$ LC-like cells [19, 22, 33].

However, previous studies reported that Birbeck granules were detected in only 30% of TGF-β-induced CD14$^+$-derived Langerin$^+$ cells [22], which may indicate that only part of these Langerin$^+$ cells could be considered as real LCs. Here, TGF-β was shown to induce Langerin^{int}BDCA3$^+$ cells besides Langerin^{hi}BDCA3$^-$ cells, of which the latter population showed a Langerin expression level comparable to that of CD1a$^+$-derived LCs (Figure 2F). Functional analysis of these two DCs subtypes showed that in contrast to Langerin^{hi}BDCA3$^-$ DCs, Langerin^{int}BDCA3$^+$ DCs produced high levels of IFN-λ upon polyI:C stimulation. These findings indicate that not all TGF-β-differentiated CD14$^+$-derived Langerin$^+$ cells represent LCs, but suggest the development of two distinct DC subsets, i.e. Langerin^{hi}BDCA3$^-$ LC and IFN-λ-producing Langerin^{int}BDCA3$^+$ DCs.

In conclusion, we identified BDCA3 expression in a culture system known to generate intDCs and LCs and provided insight into the influence of TGF-β and IL-4 on the induction of BDCA3 expression on DCs in this system. Importantly, we found that BDCA3 expression is preferentially associated with the intDC lineage and that BDCA3$^+$ DCs, but not BDCA3$^-$ DCs, have the capacity to produce high levels of IFN-λ upon polyI:C stimulation. This system may be of great use to obtain insight into the function and regulation of BDCA3 expression on DCs. In addition, this system will enable investigation of the mechanisms that regulate IFN-λ

production and how this is linked in particular to BDCA3-expressing DCs.

Acknowledgements

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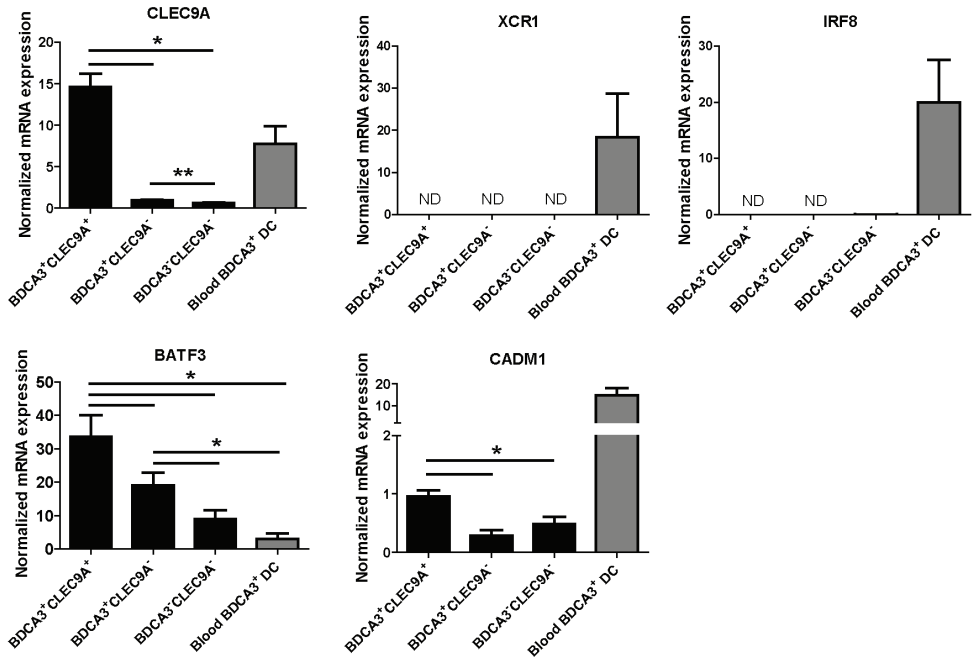
References

1. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature 1998;392:245-52.
2. Allenspach EJ, Lemos MP, Porrett PM, Turka LA, Laufer TM. Migratory and lymphoid-resident dendritic cells cooperate to efficiently prime naive CD4 T cells. Immunity 2008;29:795-806.
3. Allan RS, Waithman J, Bedoui S, Jones CM, Villadangos JA, Zhan Y, et al. Migratory dendritic cells transfer antigen to a lymph node-resident dendritic cell population for efficient CTL priming. Immunity 2006;25:153-62.
4. Collin M, McGovern N, Haniffa M. Human dendritic cell subsets. Immunology 2013;140:22-30.
5. Banchereau J, Briere F, Caux C, Davoust J, Lebecque S, Liu YJ, et al. Immunobiology of dendritic cells. Annu Rev Immunol 2000;18:767-811.
6. Poulin LF, Salio M, Griessinger E, Anjos-Afonso F, Craciun L, Chen JL, et al. Characterization of human DNGR-1+ BDCA3+ leukocytes as putative equivalents of mouse CD8alpha+ dendritic cells. J Exp Med 2010;207:1261-71.
7. Lauterbach H, Bathke B, Gilles S, Traidl-Hoffmann C, Luber CA, Fejer G, et al. Mouse CD8alpha+ DCs and human BDCA3+ DCs are major producers of IFN-lambda in response to poly IC. J Exp Med 2010;207:2703-17.
8. Jongbloed SL, Kassianos AJ, McDonald KJ, Clark GJ, Ju X, Angel CE, et al. Human CD141+ (BDCA-3)+ dendritic cells (DCs) represent a unique myeloid DC subset that cross-presents necrotic cell antigens. J Exp Med 2010;207:1247-60.
9. Haniffa M, Shin A, Bigley V, McGovern N, Teo P, See P, et al. Human Tissues Contain CD141(hi) Cross-Presenting Dendritic Cells with Functional Homology to Mouse CD103(+) Nonlymphoid Dendritic Cells. Immunity 2012.
10. Demedts IK, Brusselle GG, Vermaelen KY, Pauwels RA. Identification and characterization of human pulmonary dendritic cells. Am J Respir Cell Mol Biol 2005;32:177-84.
11. Dzionek A, Inagaki Y, Okawa K, Nagafune J, Rock J, Sohma Y, et al. Plasmacytoid dendritic cells: from specific surface markers to specific cellular functions. Hum Immunol 2002;63:1133-48.
12. Van Pottelberge GR, Bracke KR, Demedts IK, De Rijck K, Reinartz SM, van Drunen CM, et al. Selective accumulation of langerhans-type dendritic cells in small airways of patients with COPD. Respir Res 2010;11:35.
13. Segura E, Valladeau-Guilemond J, Donnadieu MH, Sastre-Garau X, Soumelis V, Amigorena S. Characterization of resident and migratory dendritic cells in human lymph nodes. J Exp Med 2012;209:653-60.
14. Chu CC, Ali N, Karagiannis P, Di Meglio P, Skowera A, Napolitano L, et al. Resident CD141 (BDCA3)+ dendritic cells in human skin produce IL-10 and induce regulatory T cells that suppress skin inflammation. J Exp Med 2012;209:935-45.
15. Li YH, Kuo CH, Shi GY, Wu HL. The role of thrombomodulin lectin-like domain in inflammation. J Biomed Sci 2012;19:34.
16. Sallusto F, Lanzavecchia A. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. J Exp Med 1994;179:1109-18.
17. Caux C, Vanbervliet B, Massacrier C, Dezutter-Dambuyant C, de Saint-Vis B, Jacquet C, et al. CD34+ hematopoietic progenitors from human cord blood differentiate along two independent dendritic cell pathways in response to GM-CSF+TNF alpha. J Exp Med 1996;184:695-706.
18. Klechevsky E, Morita R, Liu M, Cao Y, Coquery S, Thompson-Snipes L, et al. Functional specializations of human epidermal Langerhans cells and CD14+ dermal dendritic cells. Immunity 2008;29:497-510.

19. van de Laar L, Buitenhuis M, Wensveen FM, Janssen HL, Coffer PJ, Woltman AM. Human CD34-derived myeloid dendritic cell development requires intact phosphatidylinositol 3-kinase-protein kinase B-mammalian target of rapamycin signaling. J Immunol 2010;184:6600-11.
20. Woltman AM, Massacrier C, de Fijter JW, Caux C, van Kooten C. Corticosteroids prevent generation of CD34+-derived dermal dendritic cells but do not inhibit Langerhans cell development. J Immunol 2002;168:6181-8.
21. Yasmin N, Bauer T, Modak M, Wagner K, Schuster C, Koffel R, et al. Identification of bone morphogenetic protein 7 (BMP7) as an instructive factor for human epidermal Langerhans cell differentiation. J Exp Med 2013;210:2597-610.
22. Caux C, Massacrier C, Dubois B, Valladeau J, Dezutter-Dambuyant C, Durand I, et al. Respective involvement of TGF-beta and IL-4 in the development of Langerhans cells and non-Langerhans dendritic cells from CD34+ progenitors. J Leukoc Biol 1999;66:781-91.
23. Bachem A, Guttler S, Hartung E, Ebstein F, Schaefer M, Tannert A, et al. Superior antigen cross-presentation and XCR1 expression define human CD11c+CD141+ cells as homologues of mouse CD8+ dendritic cells. J Exp Med 2010;207:1273-81.
24. Crozat K, Guiton R, Guilliams M, Henri S, Baranek T, Schwartz-Cornil I, et al. Comparative genomics as a tool to reveal functional equivalences between human and mouse dendritic cell subsets. Immunol Rev 2010;234:177-98.
25. Takagi T, Taguchi O, Toda M, Ruiz DB, Bernabe PG, D'Alessandro-Gabazza CN, et al. Inhibition of allergic bronchial asthma by thrombomodulin is mediated by dendritic cells. Am J Respir Crit Care Med 2011;183:31-42.
26. Lindenberg JJ, Oosterhoff D, Sombroek CC, Lougheed SM, Hooijberg E, Stam AG, et al. IL-10 conditioning of human skin affects the distribution of migratory dendritic cell subsets and functional T cell differentiation. PLoS One 2013;8:e70237.
27. Nizzoli G, Krietsch J, Weick A, Steinfelder S, Facciotti F, Gruarin P, et al. Human CD1c+ dendritic cells secrete high levels of IL-12 and potently prime cytotoxic T-cell responses. Blood 2013;122:932-42.
28. Sancho D, Joffre OP, Keller AM, Rogers NC, Martinez D, Hernanz-Falcon P, et al. Identification of a dendritic cell receptor that couples sensing of necrosis to immunity. Nature 2009;458:899-903.
29. Poulin LF, Reyal Y, Uronen-Hansson H, Schraml BU, Sancho D, Murphy KM, et al. DNGR-1 is a specific and universal marker of mouse and human Batf3-dependent dendritic cells in lymphoid and nonlymphoid tissues. Blood 2012;119:6052-62.
30. Huysamen C, Willment JA, Dennehy KM, Brown GD. CLEC9A is a novel activation C-type lectin-like receptor expressed on BDCA3+ dendritic cells and a subset of monocytes. J Biol Chem 2008;283:16693-701.
31. Lundberg K, Albrekt AS, Nelissen I, Santegoets S, de Gruijl TD, Gibbs S, et al. Transcriptional profiling of human dendritic cell populations and models--unique profiles of in vitro dendritic cells and implications on functionality and applicability. PLoS One 2013;8:e52875.
32. Caminschi I, Proietto AI, Ahmet F, Kitsoulis S, Shin Teh J, Lo JC, et al. The dendritic cell subtype-restricted C-type lectin Clec9A is a target for vaccine enhancement. Blood 2008;112:3264-73.
33. Ju XS, Ruau D, Jantti P, Sere K, Becker C, Wiercinska E, et al. Transforming growth factor beta1 up-regulates interferon regulatory factor 8 during dendritic cell development. Eur J Immunol 2007;37:1174-83.

Supplementary information



Supplemental figure 1. TGFβ and IL-4-induced CLEC9A⁺BDCA3⁺ DC are not distinct from CLEC9A⁻ DC.
CD34⁺ cells were plated and cultured for 6 days in the presence of GM-CSF, TNFα and SCF. At day 6, CD14⁺ and CD1a⁺ preDC were sorted and cultured together in the presence of GM-CSF with TGFβ and IL-4. At day 14, cells were FACSorted based on BDCA3 and CLEC9A expression. mRNA was isolated from in vitro-generated BDCA3⁺CLEC9A⁺, BDCA3⁺CLEC9A⁻ and BDCA3⁻CLEC9A⁻ DC fractions and a MACS-enriched blood-derived BDCA3⁺CLEC9A⁺ DC fraction. CLEC9A, XCR1, CADM1, BATF3 and IRF8 mRNA levels were measured by qPCR and normalized to GAPDH expression levels. Mean±SEM normalized expression levels of 3 independent experiments with different donors are shown. ND, not detected. *p<0.05, **p<0.01, paired Student's t-test.

Supplementary table 1. Gene expression profiles of *in vitro*-generated and blood-derived BDCA3$^+$CLEC9A$^+$ DC.

Gene expression analysis was performed as described in Figure 4. Transcripts identified by expression level >50 in at least one of the two DC types are shown.

Genes with >3 fold-change in expression between blood and in vitro DC

Name	In vitro BDCA3+CLEC9A+ DC	Blood BDCA3+CLEC9A+ DC
IRF8	1	10,832
SELL	1	2,197
BTLA	1	1,136
CD45RA	1	649
XCR1	2	602
CTLA4	1	319
LILRA4	1	212
CD48	54	6,294
CAMP	1	84
TNFSF8	1	82
CD22	3	209
CSF3R	5	355
SMAD3	4	273
ADA	6	401
KIT	8	561
HLA-DOB	101	5,008
ICAM2	2	91
DUSP4	129	4,496
TLR3	58	1,937
LILRA5	8	273
CD5	10	312
DPP4	41	1,242
NFKBIZ	54	1,374
FYN	77	1,884
CD97	21	506
IDO1	324	7,536
TNFAIP3	175	3,229
MAP4K1	114	2,058
JAK3	18	299
NLRP3	58	905
PTK2	103	1,589
NOD2	16	224
CD45RB	72	990
IRAK2	28	380
ITGAL	149	1,877
CXCR4	1,891	22,089
MYD88	458	4,709
NFKBIA	2,619	25,981
SLAMF7	205	1,931
IL2RG	303	2,803
TNFRSF1B	22	199
MCL1	6,425	59,024
CD83	2,750	24,329
NOTCH2	143	1,238
S100A9	77	661
CFP	139	1,126
BCL2	18	139
POU2F2	60	448
TRAF4	150	1,051
NFIL3	402	2,631
LILRA2	51	307
IRF1	198	1,187
ICAM3	1,363	8,064
IL16	506	2,967
MAPKAPK2	313	1,760
C5	14	74
CXCR3	130	652
MX1	210	1,052
HLA-DPB1	49,850	234,676
TGFB1	409	1,760
IL18R1	167	713
GPR183	1,578	6,516
ABL1	366	1,510

Genes with ≤3 fold-change in expression between blood and in vitro DC

Name	In vitro BDCA3+CLEC9A+ DC	Blood BDCA3+CLEC9A+ DC
ILF3	1,533	4,555
HLA-A	9,152	25,481
TAPBP	1,172	3,255
LTB4R	38	102
TAP2	443	1,183
FCGRT	2,821	7,320
HLA-C	1,970	5,073
NCF4	142	363
ATG12	29	74
IKZF1	60	150
IRAK1	202	505
TAP1	314	773
MIF	1,026	2,510
BCL3	321	777
HLA-B	9,117	21,620
HLA-DQA1	23,609	55,140
PSMB10	601	1,381
ARHGDIB	3,167	7,016
IFNGR1	2,016	4,175
CASP2	316	643
TRAF6	363	710
HLA-DRA	81,673	156,657
PSMB7	2,185	4,142
HLA-DRB3	45,058	83,971
ICAM1	1,992	3,622
HLA-DPA1	145,869	263,906
BCL2L11	212	382
C14orf166	2,846	5,131
IL1RL1	31	54
PECAM1	1,529	2,682
CD86	617	1,075
PTPN22	699	1,186
LGALS3	1,297	2,174
PTPRC_all	4,589	7,672
JAK1	918	1,499
TNFAIP6	63	102
STAT2	944	1,515
RELB	384	607
CTSS	6,929	10,802
IFIH1	184	279
SYK	2,823	4,275
ITGA4	1,019	1,536
CLEC5A	65	95
IFNAR2	1,280	1,870
NFATC2	1,096	1,552
BCL10	1,787	2,524
PSMB8	3,709	5,227
TNFSF13B	446	614
IRF7	171	226
XBP1	762	979
MALT1	883	1,131
RELA	403	516
TYK2	753	958
IL18	282	359
TP53	1,022	1,296
TGFBI	6,598	8,017
CD74	157,532	190,388
ITGB2	5,936	7,090
CXCL2	294	339
NFKB2	362	416
CIITA	1,850	2,115
RUNX1	715	814
MAP4K4	788	894

Genes with >3 fold-change in expression between blood and in vitro DC		
Name	In vitro BDCA3+CLEC9A+ DC	Blood BDCA3+CLEC9A+ DC
NT5E	31	120
SIGIRR	20	75
PTGER4	210	784
HLA-DMA	4,195	15,502
HLA-DRB1	15,369	55,543
CASP8	561	1,975
BCL6	907	3,012
sCTLA4	81	262
ATG16L1	244	788
LILRB2	42	134
C1QBP	1,750	5,544
IL32	121,206	381,165
PSMB9	1,647	5,142
HLA-DQB1	7,806	24,339
LTB4R2	30	93
ITGAE	161	495
TRAF5	40	121
ATG10	226	74
IKBKG	493	154
CD58	3,003	927
IRF5	2,953	890
MAPK14	1,440	433
CASP1	679	199
ITGAX	9,088	2,632
CD45R0	6,370	1,829
IRF3	140	40
GFI1	160	44
IKBKB	486	134
TGFBR1	1,133	290
CCR5	170	40
LY96	1,862	429
NFATC1	295	66
ETS1	202	45
TOLLIP	1,023	228
TLR4	91	19
IGF2R	2,165	429
NOD1	220	43
CLEC7A	6,608	1,270
NOTCH1	313	57
IL4R	1,592	276
ITGA5	1,518	259
CD59	15,947	2,713
PTGS2	335	56
CD81	14,546	2,396
TRAF2	368	60
IFI16	6,377	909
PSMB5	2,823	384
TIRAP	58	8
SKI	1,423	189
APP	8,951	1,166
CCRL2	101	12
IFNAR1	159	18
ATG7	535	60
CD53	14,726	1,614
CRADD	99	11
MR1	311	33
TNF	3,320	349
CASP3	1,513	136
IL1R2	982	73
CSF2RB	8,595	617
IRF4	9,572	632
FADD	81	4
PPBP	1,688	72
CD9	2,226	94
CD40	9,152	382
IL8	30,722	1,157
CXCR2	150	5
CTSC	15,965	551
CDKN1A	21,125	699
CXCL1	7,859	231

Genes with ≤3 fold-change in expression between blood and in vitro DC		
Name	In vitro BDCA3+CLEC9A+ DC	Blood BDCA3+CLEC9A+ DC
PTPN6	1,949	2,148
CFB	139	152
HLA-DMB	9,182	9,886
GPI	1,047	1,123
PSMD7	3,238	3,466
IL6R	634	668
SOCS3	616	648
CUL9	109	113
CEBPB	136	140
PYCARD	463	470
NFKB1	695	699
TIGIT	77	76
TFRC	1,385	1,358
UBE2L3	361	354
NFATC3	740	714
B2M	161,096	153,262
STAT5B	830	770
MBP	655	606
IL10RA	4,856	4,444
ENTPD1	88	79
BST2	733	657
LCP2	980	828
TICAM1	588	496
LTBR	1,000	841
CD164	5,779	4,847
RAF1	1,146	945
STAT6	1,950	1,607
TNFSF12	85	69
TAGAP	234	186
PLAUR	1,469	1,153
BTK	661	510
PSMC2	2,159	1,653
CD46	2,664	2,033
CCND3	698	522
PTPN2	1,362	1,000
FCER1G	1,171	851
AHR	8,004	5,721
ICOSLG	939	658
MAP4K2	517	362
ATG5	684	475
CD4	2,701	1,863
STAT1	722	490
CHUK	951	646
SLC2A1	847	571
CD44	4,228	2,847
PML	319	215
IKBKAP	128	85
SMAD5	303	200
CTNNB1	8,378	5,387
HAVCR2	1,912	1,214
CCR6	215	136
TRAF3	281	175
CD99	5,823	3,631
PRKCD	509	309
IRAK4	407	244
STAT3	1,601	948
TNFRSF14	1,112	652
LILRB4	270	156
LITAF	3,584	2,072
TRAF1	215	124
IL6ST	979	563
IFI35	264	149
TLR1	633	353
TMEM173	125	69
ZEB1	395	212
BAX	4,040	2,153
TBK1	825	439
HRAS	96	49
TCF4	779	391
TLR2	227	113

Genes with >3 fold-change in expression between blood and in vitro DC

Name	In vitro BDCA3+CLEC9A+ DC	Blood BDCA3+CLEC9A+ DC
IL1RL2	185	5
IL13RA1	15,202	398
CCL4	163	4
CARD9	1,441	28
CD8A	422	8
LAMP3	1,119	17
PRDM1	3,543	53
CFI	1,305	18
CD36	41,474	562
CLEC4A	30,981	404
MAF	568	5
KCNJ2	697	6
IL1R1	675	5
CCR1	995	8
SPP1	198	2
LTA	554	4
IL1RN	174	1
GBP5	183	1
IL7R	190	1
CD80	907	4
IKZF2	204	1
PDGFB	247	1
FCER1A	27,731	97
SOCS1	1,786	6
SLAMF1	299	1
CXCL13	321	1
IL1RAP	6,977	21
C1QA	967	2
CD24	397	1
FCGR2A/C	2,552	6
CFH	477	1
CSF1	1,643	3
PTAFR	526	1
TAL1	3,998	7
CSF1R	553	1
BLNK	1,220	2
PLAU	4,983	8
CD274	895	1
FCGR2A	1,168	1
CCL22	1,285	1
CISH	1,295	1
PDCD1LG2	1,611	1
ITGAM	1,950	1
EGR2	2,545	1
MRC1	2,628	1
CD276	2,858	1
CD209	4,172	1
CCL13	7,478	1
C1QB	12,337	1
CD1A	58,820	1

Genes with ≤3 fold-change in expression between blood and in vitro DC

Name	In vitro BDCA3+CLEC9A+ DC	Blood BDCA3+CLEC9A+ DC
SELPLG	149	71
BATF3	7,262	3,367
MAPK1	4,005	1,823
TGFBR2	1,339	598
PDCD2	278	121
SRC	898	374
FKBP5	2,270	898
TNFRSF11A	763	292
JAK2	3,039	1,114
STAT5A	1,030	372
BCAP31	2,474	863
ITGB1	5,641	1,913

Transcriptional patterns associated with BDCA3 expression on BDCA1$^+$ myeloid dendritic cells

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In preparation

Chapter 5

Abstract

Myeloid dendritic cells, which include BDCA1^{+} DCs and BDCA3^{hi} DCs, play a pivotal role in the induction and regulation of immune responses. Interestingly, BDCA1^{+} DCs also express low to intermediate levels of BDCA3. We have previously shown that BDCA3 expression on CD34^{+} progenitor cell-derived DCs is associated with an increased capacity to secrete IFN-λ, a hallmark of BDCA3^{hi} DCs. This raised the question whether BDCA3^{+}BDCA1^{+} DCs share also other traits with BDCA3^{hi} DCs that are absent in BDCA3^{-}BDCA1^{+} DCs or whether their higher capacity to secrete IFN-λ is a stand-alone observation.

Here, we used immunology-related gene expression analysis to determine divergence between BDCA3-expressing and BDCA3-negative BDCA1^{+} DCs and their relation to *bona fide* BDCA3^{hi} DCs. Results showed that transcriptomic fingerprints of BDCA3^{+}BDCA1^{+} DCs and BDCA3^{-}BDCA1^{+} DCs are very similar, and clearly distinct from that of BDCA3^{hi} DCs. Differences in mRNA expression, however, were observed between BDCA3^{+}BDCA1^{+} DCs and BDCA3^{-}BDCA1^{+} DCs that pointed towards a more activated status of BDCA3^{+}BDCA1^{+} DCs. In line with this, higher maturation marker expression and inflammatory cytokine production by BDCA3^{+}BDCA1^{+} DCs was observed. This dataset provides better insight into differences between myeloid DC populations and contributes to further study of DC immunobiology.

Introduction

Dendritic cells (DCs) represent a family of professional antigen presenting cells (APC) that play a crucial role in the immune defense against infections and cancer. DCs are located in peripheral blood, lymphoid and non-lymphoid tissues, where they continuously sample the environment for invading pathogens. Recognition of foreign antigens by pathogen recognition receptors (PRR) induces activation of DCs and migration to secondary lymphoid organs where they activate naïve T cells. Activation of DCs induces upregulation of several surface receptors and production of cytokines that enable DCs to migrate and to interact with and instruct T cells and other immune cells [1].

The human DC family comprises several subsets diverging in ontogeny, localization and phenotype. Although all DC subsets share the functional capacity to induce T cell responses, they vary in their specialized immune functions. These DC subsets can be divided into BDCA2$^+$BDCA4$^+$ plasmacytoid DCs and myeloid DCs, which includes BDCA1$^+$ DCs and BDCA3^{hi}CLEC9A$^+$XCR1$^+$ DCs [2]. These markers, BDCA1, BDCA2, BDCA3 and BDCA4, are generally used to classify human DC. However, BDCA3 is not exclusively expressed by BDCA3^{hi}CLEC9A$^+$XCR1$^+$ DCs (further referred to as BDCA3^{hi} DCs), but also at intermediate levels by other DCs, such as BDCA1$^+$ DCs in blood [3] (chapter 6), and even more so by BDCA1$^+$ DCs in lymphoid tissues [4] and non-lymphoid tissues [5].

BDCA3, also known as CD141 or thrombomodulin, is a cell surface-expressed transmembrane glycoprotein. On vascular endothelial cells, BDCA3 is known for its anticoagulant activity, and has been described to have an anti-inflammatory function as it blocks pro-inflammatory proteins and activates anti-inflammatory proteins and inhibitors of the complement system [6, 7]. However, the exact function of BDCA3 expression on immune cells is not completely understood. Although BDCA1$^+$ DCs have been shown to represent a subset that is distinct from BDCA3^{hi} DCs [5, 8], we find that BDCA3$^+$BDCA1$^+$ DCs share a higher IFN-λ-producing capacity with BDCA3^{hi} DCs, which is almost absent in BDCA3$^-$BDCA1$^+$ DCs. Here, we compared the immunology-related gene signature of BDCA3$^+$BDCA1$^+$ DCs, BDCA3$^-$BDCA1$^+$ DCs, and BDCA3^{hi} DCs, and analyzed differences between BDCA3$^+$ and BDCA3$^-$ DCs within the BDCA1$^+$ DC population. Results showed that although BDCA3$^+$BDCA1$^+$ DCs and BDCA3$^-$BDCA1$^+$ DCs are highly comparable and distinct from *bona fide* BDCA3^{hi} DCs, BDCA3-expressing BDCA1$^+$ DCs have a more activated status than BDCA1$^+$ DCs that do not express BDCA3, and as a result produce higher levels of cytokines.

Materials and methods

Cell isolation

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized peripheral blood samples or from buffy coats from healthy blood donors using Ficoll density gradient centrifugation. All healthy controls gave written informed consent before blood donation. Cells were enriched for DCs using Dynabeads (Thermo Fisher) and DC subsets were isolated based on BDCA1, BDCA3 and BDCA4 expression by FACSorting using a FACSAria (BD Biosciences).

Flow cytometric analysis

For phenotypic analysis, cells were washed with PBS containing 1% bovine serum albumin

(BSA; Sigma-Aldrich, St. Louis, MO), 1% heat inactivated human serum and 0.02% NaN_3 (Merck, Darmstadt, Germany; referred to as FACS buffer) and subsequently labeled with fluorochrome-conjugated antibodies (Abs). Labeling was performed during 30 minutes of incubation on ice with Abs recognizing BDCA3/CD141 (AD5-14H12, Miltenyi Biotec, Bergish Gladbach, Germany); BDCA1/CD1c (AD5-8E7, Miltenyi and L161, BioLegend) and BDCA4/CD304 (12C2, BioLegend). Fluorescence was measured using a FACS Canto II (BD Biosciences).

Gene expression analysis using Nanostring nCounter system

RNA was extracted according to the manufacturer's instructions using an RNAeasy Micro kit or a Nucleospin RNA II kit for samples containing $<5x10^5$ or $>5x10^5$ cells, respectively. RNA concentration and integrity was assessed using a Bioanalyzer system (Agilent Technologies). Human immunology-related genes were profiled using the nCounter GX Human Immunology v2 kit Gene Expression CodeSet (Nanostring Technologies). Gene expression levels were normalized according to 15 housekeeping genes. Gene clustering analysis was performed using GENE-E software (http://www.broadinstitute.org/cancer/software/GENE-E/). Principal component analysis (PCA) was performed using MeV software. Geneset enrichment analysis was performed using GSEA software [9].

DC activation and cytokine secretion

FACSorted DCs were stimulated with or without polyI:C (20 μg/ml, Invivogen, Toulouse, France) for 24 hours at 37°C in the presence of 10 ng/ml GM-CSF. The levels of secreted human IL-1β, IL-6, IL8, and TNF-α were measured in the supernatant using a BD cytometric bead array human inflammatory cytokines kit (CBA, BD Biosciences) according to the manufacturer's protocol. Detection limits were 7.2 pg/ml (IL-1β), 2.5 pg/ml (IL-6), 3.6 pg/ml (IL-8) and 3.7 pg/ml (TNFα).

Results

Immunology-related gene expression patterns of $BDCA3^+BDCA1^+$ DCs are similar to those of $BDCA3^-BDCA1^+$ DCs

To assess how $BDCA3^+BDCA1^+$ DCs relate to their BDCA3-negative counterpart, DC populations shown in Figure 1 were sorted and expression of immunology-related genes was analyzed (for complete list of gene expression values, see supplementary table 1). BDCA3 expression on $BDCA1^+$ DCs was highly variable between donors and ranged from 41-85 % (Figure 1). Sample clustering and principal component analysis (PCA) revealed a high level of overlap between the immunology-related gene expression profile of $BDCA3^{hi}$, $BDCA3^-BDCA1^+$ and $BDCA3^+BDCA1^+$ DC subtypes (Figure 2AB). $BDCA1^+BDCA3^+$ DCs and $BDCA3^-BDCA1^+$ DCs, however, appeared far more comparable to each other than to $BDCA3^{hi}$ DCs (Figure 2AB). Although $BDCA1^+$ DCs and $BDCA3^+BDCA1^+$ DCs were found to be most similar, some minor differences in immunology-related gene expression levels were observed (Figure 2A).

Comparison of $BDCA3^{hi}$ DCs and $BDCA3^+BDCA1^+$ DCs

In total, 10 genes showed a trend towards differential expression between $BDCA3^+BDCA1^+$ DCs and $BDCA3^-BDCA1^+$ DCs by t-testing, but none survived multiple testing correction

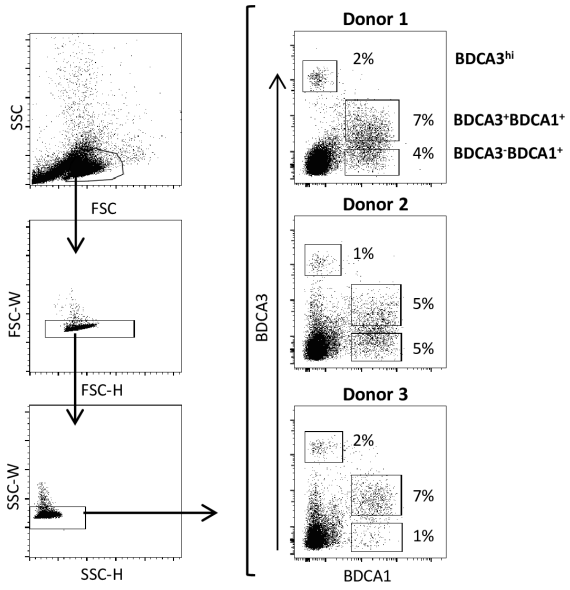

Figure 1. Gating strategy of DC populations
(A) PBMC were isolated from healthy controls and enriched for DCs using a Dynabeads Human DC Enrichment kit. Duplicates were excluded based on size, and DC populations were subsequently identified as BDCA3^{hi} cells, BDCA3^{+}BDCA1^{+} cells and BDCA3^{-} BDCA1^{+} cells. Representative results of 3 different donors in 3 different experiments are shown.

(Table 1). Yet, we were interested to see whether these 10 genes were also higher expressed in *bona fide* BDCA3^{hi} DCs. Interestingly, 9 genes were also differentially expressed between BDCA3^{-}BDCA1^{+} DCs and BDCA3^{hi} DCs (up in BDCA3^{hi} DCs: SLAMF7, CD83, SRC, CXCR3, and down: CD99, STAT5A, PRKCD, CD4, IRF4), indicating that BDCA3 expression on BDCA1^{+} DCs is accompanied by a trend for higher expression of also other genes highly expressed on BDCA3^{hi} DCs (Figure 3A).
To further asses the overlap between genes differentially expressed between BDCA3^{hi} DCs and BDCA3^{-}BDCA1^{+} DCs, and those between BDCA3-expressing and BDCA3-lacking BDCA1^{+} DCs, we used Gene Set Enrichment Analysis (GSEA). First, two gene sets containing the top 50 genes higher and lower expressed in BDCA3^{hi} DCs were generated. For visual insight into the composition of the resulting gene sets, STRING network analysis was used (Figure 3BC). As expected, the BDCA3^{hi}-overexpressed network contained the BDCA3^{hi} DC hallmark genes TLR3, BATF3 and XCR1 [10], but also genes related to antigen (cross)-presentation (HLA genes, proteasome- and transporter-associated with antigen presentation subunits (TAP). The BDCA1^{+} DC network in contrast was centered around the pattern recognition receptor (PRR) TLR4 for bacterial recognition and ITGAM, the alpha subunit of the MAC-1 integrin heterodimer that facilitates phagocytosis [11-13].
Using GSEA we next analyzed and visualized how the genes in these BDCA3^{hi} DC over-expressed and lower-expressed gene sets are behaving in BDCA3^{+}BDCA1^{+} DCs with respect

Table 1. Genes differentially expressed in BDCA3^{+}BDCA1^{+} DCs as compared to BDCA3^{-}BDCA1^{+} DCs.

Up	Down
SLAMF7	CD99
CD83	TOLLIP
SRC	STAT5A
IRF4	PRKCD
CXCR3	
CD4	

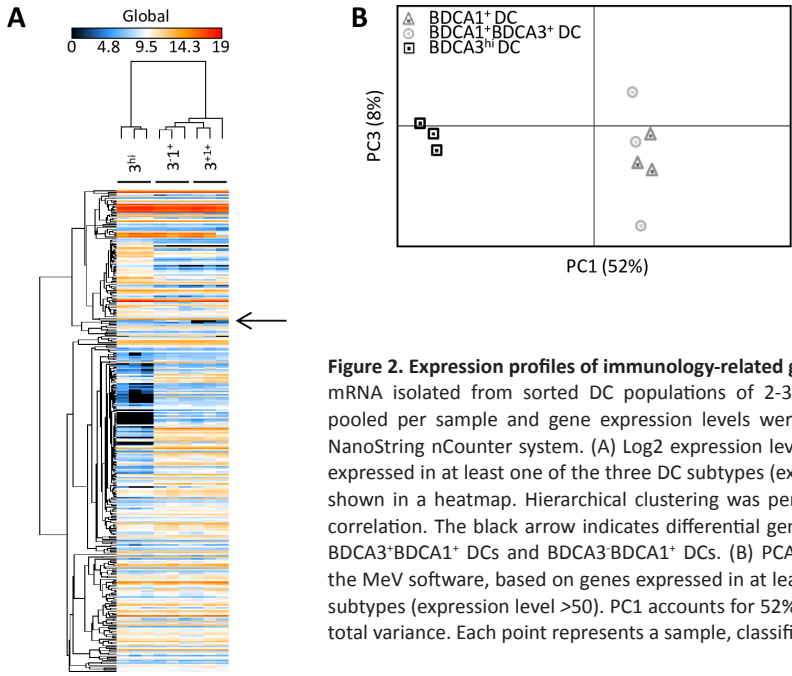

Figure 2. Expression profiles of immunology-related genes
mRNA isolated from sorted DC populations of 2-3 different donors was pooled per sample and gene expression levels were measured using the NanoString nCounter system. (A) Log2 expression levels of genes that were expressed in at least one of the three DC subtypes (expression level >50) are shown in a heatmap. Hierarchical clustering was performed using Pearson correlation. The black arrow indicates differential gene expression between BDCA3$^+$BDCA1$^+$ DCs and BDCA3$^-$BDCA1$^+$ DCs. (B) PCA was performed using the MeV software, based on genes expressed in at least one of the three DC subtypes (expression level >50). PC1 accounts for 52% and PC3 for 8% of the total variance. Each point represents a sample, classified by DC type.

to BDCA3$^-$BDCA1$^+$ DCs. Although GSEA showed that the majority of the BDCA3^{hi} DC over-expressed gene set was higher expressed in BDCA3$^+$BDCA1$^+$ DCs relative to BDCA1$^+$ DCs lacking BDCA3, enrichment of the gene set was not high (NES 1,6; FDR q-value 0.285, Figure 3D). Enrichment of the lower-expressed gene set in BDCA3-expressing BDCA1$^+$ DCs was also present but even less apparent (FDR q-value 0.925, Figure 3E).
Finally we assessed the expression of several established hallmark genes of either BDCA3^{hi} DCs or BDCA1$^+$ DCs. For these key genes, BDCA3-expressing BDCA1$^+$ DCs behaved almost identical to their BDCA3-lacking counterparts and higher expression of BDCA3^{hi} DC-associated genes was not observed (Figure 3FG). In contrast, the BDCA1$^+$ DC hallmark gene IRF4 was even higher expressed in BDCA3-expressing BDCA1$^+$ DCs. Thus, although BDCA3^{hi} DCs and BDCA3$^+$BDCA1$^+$ DCs are distinct populations, BDCA3$^+$BDCA1$^+$ DCs are slightly more similar to BDCA3^{hi} DCs than their BDCA3$^-$ counterparts.

BDCA3$^+$BDCA1$^+$ DCs have a higher cytokine-producing capacity than BDCA3$^-$BDCA1$^+$ DCs
Enhanced expression of SLAMF7 [14], CD83, SRC [15], IRF4 [16], and CXCR3, as well as reduced expression of TOLLIP [17] suggest a more activated status and differential cytokine production by BDCA3$^+$BDCA1$^+$ DCs compared to BDCA3$^-$BDCA1$^+$ DCs. To further investigate this, the surface expression of activation markers by freshly isolated cells, and the cytokine-producing capacity of the two cell types after *in vitro* TLR stimulation was assessed. Although CD83 and CD80 expression measured by flow cytometry was not significantly different between BDCA3$^-$BDCA1$^+$ DCs and BDCA3$^+$BDCA1$^+$ DCs, CD83 expression was consistently higher on BDCA3$^+$BDCA1$^+$ DCs (Figure 4A). However, CD40 and CD86 expression was significantly increased on BDCA3$^+$BDCA1$^+$ DCs, confirming a more activated status of these cells (Figure 4A). In addition, cytometric bead array analysis of cytokine production after 24 hour polyI:C stimulation revealed that IL-6, IL-8, IL-1β and TNFα production was consistently

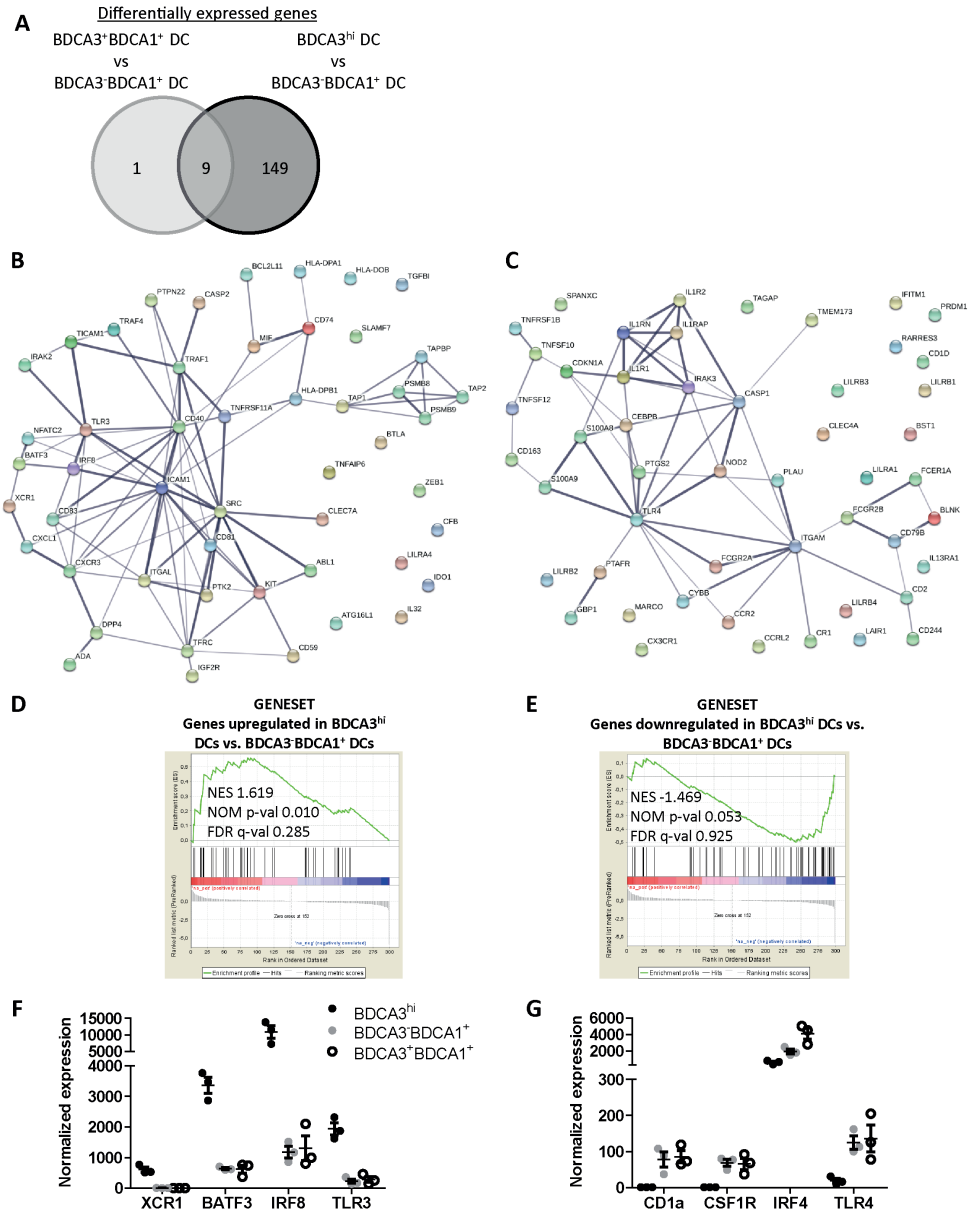

Figure 3. Analysis of DC population gene signatures by GSEA

mRNA isolated from FACSorted DC populations of 2-3 different donors was pooled per sample. Gene expression levels were measured using the NanoString nCounter system. (A) The Venn diagram represents the number of genes significantly differentially expressed between the indicated DC populations ($p<0.05$ by unpaired *t*-test). (B-C) String analysis of the top 50 genes that were significantly upregulated (B) or downregulated (C) in BDCA3^{hi} DCs compared to BDCA3^{-}BDCA1^{+} DCs ($p<0.05$ by unpaired *t*-test). (D-E) Enrichment plots of GSEA performed using the genesets shown in 3C and 3D, and a ranked list containing genes ranked by fold-change in expression values between BDCA3^{+}BDCA1^{+} DCs compared to BDCA3^{-}BDCA1^{+} DCs. The genesets were complemented by the curated C2 genesets published on software.broadinstitute.org to properly determine the normalized enrichment score (NES), nominal p-value (NOM p-val), and false discovery rate (FDR). (F-G) Normalized expression of hallmark genes of BDCA3^{hi} DCs (F) or BDCA1^{+} DCs (G) by the different DC populations.

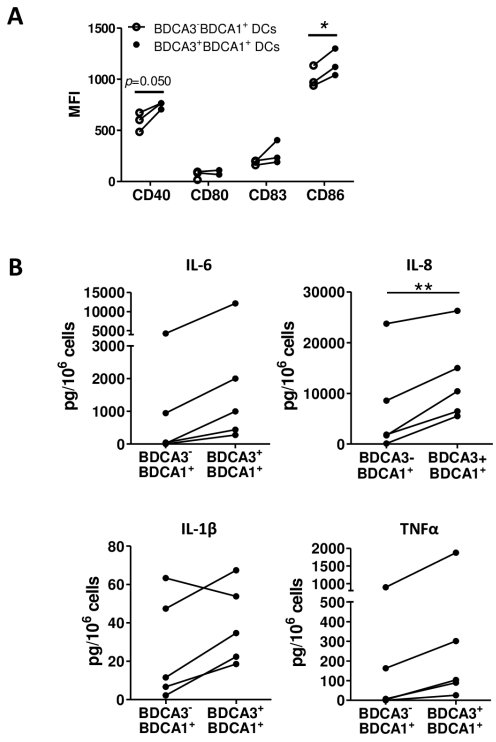

Figure 4. BDCA3$^+$BDCA1$^+$ DCs produce higher cytokine levels than BDCA3$^-$BDCA1$^+$ DCs.
(A) Expression of the indicated markers by freshly isolated DC as measured by flow cytometry. Data of 2 independent experiments, performed with 3 different donors are shown. *p<0.05, paired Student's t-test. (B) BDCA3$^+$BDCA1$^+$ DCs and BDCA3$^-$BDCA1$^+$ DCs were FACSorted from PBMCs enriched for DCs and stimulated for 24 hours with or without polyI:C. Mean±SEM cytokine levels as determined by CBA (n=5). Data are pooled from 5 independent experiments, each performed with a different donor. **p<0.01, paired Student's t-test.

higher for BDCA3$^+$BDCA1$^+$ DCs compared to BDCA3$^-$BDCA1$^+$ DCs (Figure 4B). TLR3 expression levels were not significantly different between BDCA3$^+$BDCA1$^+$ DCs and BDCA3$^-$BDCA1$^+$ DCs, indicating that a more abundant activation of TLR3 was likely not the underlying cause (Figure 3F). Together our data indicate that BDCA3$^+$BDCA1$^+$ DCs have an enhanced activation status, also reflected by a higher cytokine producing capacity as compared to their BDCA3$^-$ counterparts.

Discussion

Myeloid DCs include BDCA1$^+$ DCs and BDCA3^{hi} DCs. Since BDCA1$^+$ DCs can co-express intermediate levels of BDCA3 *in vivo*, this study aimed to provide insight into the relation between BDCA3 expression and other immunology-related genes in BDCA1$^+$ DCs, and whether this also endows these cells with a more BDCA3^{hi} DC-like phenotype. Results confirmed that BDCA3-expressing BDCA1$^+$ DCs are most similar to their BDCA3-lacking counterpart and are distinct from BDCA3^{hi} DCs [5, 8]. BDCA3$^+$BDCA1$^+$ DCs are highly comparable to BDCA3$^-$BDCA1$^+$ DCs, yet differences in gene expression and cytokine production that were observed suggested a more activated status of BDCA3$^+$BDCA1$^+$ DCs relative to BDCA3$^-$BDCA1$^+$ DCs. The more activated status of BDCA3-expressing BDCA1$^+$ DCs was confirmed for several activation markers on cells directly after isolation, and also for inflammatory cytokines after further *in vitro* TLR activation.
The association between BDCA3 expression *in vivo* and enhanced DC activation and

cytokine production is in line with an increase in BDCA3 expression on pDCs and BDCA1$^+$ DCs upon maturation of these cells *in vitro* [18]. In addition, BDCA3 expression is upregulated on monocyte-like cells upon TNF-α and IL-1β stimulation [19], and also associated with increased IFN-λ-producing capacity by CD34$^+$-derived DCs [20]. BDCA3 is also higher expressed on BDCA1$^+$ DCs in peripheral tissues compared to BDCA1$^+$ DCs in peripheral blood [5]. Previously, functional comparison of BDCA3$^+$ and BDCA3$^-$ monocyte-derived DC (moDC) showed that BDCA3$^+$ moDC induced a more strongly Th2-polarized response compared to BDCA3$^-$ moDC [21]. Since BDCA3 has been described to exhibit direct and indirect anti-inflammatory functions [6, 7, 22], it is tempting to speculate that although BDCA3 is expressed on more activated DCs, BDCA3 may play a role in dampening and/or balancing pro-inflammatory responses.

The gene expression dataset presented here is valuable for the study of DC immunobiology, as it provides useful information regarding phenotypic, functional and developmental characteristics of the different DC subpopulations and the relation of the marker BDCA3 to cell function. Nevertheless, further research is required to determine the exact function of BDCA3 expression on BDCA1$^+$ DCs or other DC subsets.

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The authors declare no conflict of interest.

References

1. Steinman, R. M. (2008) Dendritic cells in vivo: a key target for a new vaccine science. Immunity 29, 319-24.
2. Merad, M., Sathe, P., Helft, J., Miller, J., Mortha, A. (2013) The dendritic cell lineage: ontogeny and function of dendritic cells and their subsets in the steady state and the inflamed setting. Annu Rev Immunol 31, 563-604.
3. Crozat, K., Guiton, R., Contreras, V., Feuillet, V., Dutertre, C. A., Ventre, E., Vu Manh, T. P., Baranek, T., Storset, A. K., Marvel, J., Boudinot, P., Hosmalin, A., Schwartz-Cornil, I., Dalod, M. (2010) The XC chemokine receptor 1 is a conserved selective marker of mammalian cells homologous to mouse CD8alpha+ dendritic cells. J Exp Med 207, 1283-92.
4. Segura, E., Durand, M., Amigorena, S. (2013) Similar antigen cross-presentation capacity and phagocytic functions in all freshly isolated human lymphoid organ-resident dendritic cells. J Exp Med 210, 1035-47.
5. Haniffa, M., Shin, A., Bigley, V., McGovern, N., Teo, P., See, P., Wasan, P. S., Wang, X. N., Malinarich, F., Malleret, B., Larbi, A., Tan, P., Zhao, H., Poidinger, M., Pagan, S., Cookson, S., Dickinson, R., Dimmick, I., Jarrett, R. F., Renia, L., Tam, J., Song, C., Connolly, J., Chan, J. K., Gehring, A., Bertoletti, A., Collin, M., Ginhoux, F. (2012) Human tissues contain CD141hi cross-presenting dendritic cells with functional homology to mouse CD103+ nonlymphoid dendritic cells. Immunity 37, 60-73.
6. Conway, E. M. (2012) Thrombomodulin and its role in inflammation. Semin Immunopathol 34, 107-25.
7. Li, Y. H., Kuo, C. H., Shi, G. Y., Wu, H. L. (2012) The role of thrombomodulin lectin-like domain in inflammation. J Biomed Sci 19, 34.
8. Crozat, K., Guiton, R., Guilliams, M., Henri, S., Baranek, T., Schwartz-Cornil, I., Malissen, B., Dalod, M. (2010) Comparative genomics as a tool to reveal functional equivalences between human and mouse dendritic cell subsets. Immunol Rev 234, 177-98.
9. Subramanian, A., Tamayo, P., Mootha, V. K., Mukherjee, S., Ebert, B. L., Gillette, M. A., Paulovich, A., Pomeroy, S. L., Golub, T. R., Lander, E. S., Mesirov, J. P. (2005) Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci U S A 102, 15545-50.
10. van der Aa, E., van Montfoort, N., Woltman, A. M. (2014) BDCA3CLEC9A human dendritic cell function and development. Semin Cell Dev Biol.
11. Lindstedt, M., Lundberg, K., Borrebaeck, C. A. (2005) Gene family clustering identifies functionally associated subsets of human in vivo blood and tonsillar dendritic cells. J Immunol 175, 4839-46.
12. Chen, J., Namiki, S., Toma-Hirano, M., Miyatake, S., Ishida, K., Shibata, Y., Arai, N., Arai, K., Kamogawa-Schifter, Y. (2008) The role of CD11b in phagocytosis and dendritic cell development. Immunol Lett 120, 42-8.
13. Le Cabec, V., Carreno, S., Moisand, A., Bordier, C., Maridonneau-Parini, I. (2002) Complement receptor 3 (CD11b/CD18) mediates type I and type II phagocytosis during nonopsonic and opsonic phagocytosis, respectively. J Immunol 169, 2003-9.
14. Bouchon, A., Cella, M., Grierson, H. L., Cohen, J. I., Colonna, M. (2001) Activation of NK cell-mediated cytotoxicity by a SAP-independent receptor of the CD2 family. J Immunol 167, 5517-21.
15. Lowell, C. A. (2011) Src-family and Syk kinases in activating and inhibitory pathways in innate immune cells: signaling cross talk. Cold Spring Harb Perspect Biol 3.
16. Williams, J. W., Tjota, M. Y., Clay, B. S., Vander Lugt, B., Bandukwala, H. S., Hrusch, C. L., Decker, D. C., Blaine, K. M., Fixsen, B. R., Singh, H., Sciammas, R., Sperling, A. I. (2013) Transcription factor IRF4 drives dendritic cells to promote Th2 differentiation. Nat Commun 4, 2990.
17. Zhang, G. and Ghosh, S. (2002) Negative regulation of toll-like receptor-mediated signaling

by Tollip. J Biol Chem 277, 7059-65.

18. Dzionek, A., Fuchs, A., Schmidt, P., Cremer, S., Zysk, M., Miltenyi, S., Buck, D. W., Schmitz, J. (2000) BDCA-2, BDCA-3, and BDCA-4: three markers for distinct subsets of dendritic cells in human peripheral blood. J Immunol 165, 6037-46.
19. Grey, S. T., Csizmadia, V., Hancock, W. W. (1998) Differential effect of tumor necrosis factor-alpha on thrombomodulin gene expression by human monocytoid (THP-1) cell versus endothelial cells. Int J Hematol 67, 53-62.
20. van der Aa, E., van de Laar, L., Janssen, H. L., van Montfoort, N., Woltman, A. M. (2015) BDCA3 expression is associated with high IFN-lambda production by CD34(+)-derived dendritic cells generated in the presence of GM-CSF, IL-4, and/or TGF-beta. Eur J Immunol 45, 1471-81.
21. Yerkovich, S. T., Roponen, M., Smith, M. E., McKenna, K., Bosco, A., Subrata, L. S., Mamessier, E., Wikstrom, M. E., Le Souef, P., Sly, P. D., Holt, P. G., Upham, J. W. (2009) Allergen-enhanced thrombomodulin (blood dendritic cell antigen 3, CD141) expression on dendritic cells is associated with a TH2-skewed immune response. J Allergy Clin Immunol 123, 209-216 e4.
22. Van de Wouwer, M., Collen, D., Conway, E. M. (2004) Thrombomodulin-protein C-EPCR system: integrated to regulate coagulation and inflammation. Arterioscler Thromb Vasc Biol 24, 1374-83.

Supplementary information

Supplementary table 1. Expression levels of immunology-related genes of DC subpopulations.

Name	BDCA3^{hi} DCs	BDCA3^{hi} DCs	BDCA3^{hi} DCs	BDCA3^{+} BDCA1^{+} DCs	BDCA3^{+} BDCA1^{+} DCs	BDCA3^{+} BDCA1^{+} DCs	BDCA3^{-} BDCA1^{+} DCs	BDCA3^{-} BDCA1^{+} DCs	BDCA3^{-} BDCA1^{+} DCs
ABCB1	1	1	1	1	10	7	2	12	1
ABL1	1558	1484	1488	589	688	704	689	753	579
ADA	430	373	400	218	321	301	166	226	202
AHR	6451	6495	4216	2166	6662	4615	5716	7650	2256
AICDA	1	1	1	1	1	1	1	1	1
AIRE	27	28	21	30	24	1	16	14	17
APP	1031	1308	1158	1389	1389	1354	1225	1322	1515
ARG1	13	1	11	1	7	1	1	2	10
ARG2	1	1	1	1	1	1	1	1	1
ARHGDIB	6746	6822	7481	6681	6609	4972	6000	5630	7130
ATG10	81	63	79	71	61	108	81	65	103
ATG12	61	82	78	125	75	108	111	90	117
ATG16L1	784	878	702	417	405	682	473	523	450
ATG5	516	502	408	509	614	635	540	621	449
ATG7	96	27	57	112	116	259	147	215	196
ATM	18	1	1	1	1	1	3	8	1
B2M	149514	167143	143130	138462	142270	121460	158637	148017	127355
B3GAT1	3	1	1	1	3	1	1	4	1
BATF	8	1	1	3	7	15	28	31	34
BATF3	3470	2867	3763	738	758	369	704	619	601
BAX	1861	2322	2275	2816	2756	2072	2471	2512	2747
BCAP31	798	843	949	1106	947	811	947	872	1158
BCL10	2737	2489	2347	2077	2320	2289	2302	2698	1980
BCL2	121	137	161	199	328	215	222	280	207
BCL2L11	435	404	307	105	207	301	202	232	115
BCL3	869	749	713	645	1562	1180	1029	1493	870
BCL6	2617	2576	3842	1381	2613	1676	2213	2226	1113
BID	67	1	33	99	79	105	53	86	85
BLNK	4	1	1	40	53	49	50	61	69
BST1	51	9	28	282	249	454	487	425	715
BST2	652	604	713	588	584	494	539	530	601
BTK	529	426	575	752	814	718	615	637	820
BTLA	1394	949	1064	23	53	17	27	66	13
C14orf166	5415	5246	4731	4612	4475	4784	4422	5009	4045
C1QA	5	1	1	13	7	1	10	1	13
C1QB	1	1	1	11	19	17	26	25	5
C1QBP	5524	5369	5740	4754	3617	3289	4139	3700	4290
C1R	1	1	1	13	3	12	9	22	47
C1S	29	1	1	1	7	2	10	6	5
C2	9	1	1	1	10	1	1	11	6
C3	1	1	1	1	1	1	1	1	1
C4A/B	3	1	1	1	1	1	1	1	1
C4BPA	15	1	1	1	1	1	1	1	5
C5	105	50	66	52	94	110	44	67	54
C6	37	27	12	18	3	1	12	15	17
C7	1	1	1	1	1	1	1	1	1
C8A	37	38	11	1	1	1	11	8	1
C8B	1	1	1	1	1	1	2	1	3
C8G	1	1	1	1	1	1	1	1	2
C9	1	1	1	1	1	1	1	1	11
CAMP	113	74	64	1	7	7	27	11	58
CARD9	44	1	38	122	143	169	122	146	119
CASP1	265	223	108	4361	4223	4476	3556	4490	3961
CASP10	1	1	1	1	1	1	9	7	1
CASP2	702	565	663	344	324	347	372	428	330
CASP3	176	109	123	156	142	151	111	188	138
CASP8	2473	1905	1548	918	1580	3193	1879	2485	1148
CCBP2	1	1	1	1	1	1	1	2	1
CCL11	1	1	1	1	3	1	2	1	10
CCL13	1	1	1	1	1	1	1	1	1
CCL15	5	1	1	1	1	1	2	1	1
CCL16	1	1	1	1	1	1	1	1	1
CCL18	1	1	1	1	1	1	1	1	1
CCL19	9	1	1	1	1	1	26	2	11
CCL2	1	1	1	1	1	1	1	1	1

Name	BDCA3^{hi} DCs	BDCA3^{hi} DCs	BDCA3^{hi} DCs	BDCA3^{+} BDCA1^{+} DCs	BDCA3^{+} BDCA1^{+} DCs	BDCA3^{+} BDCA1^{+} DCs	BDCA3^{-} BDCA1^{+} DCs	BDCA3^{-} BDCA1^{+} DCs	BDCA3^{-} BDCA1^{+} DCs
CCL20	1	1	1	1	1	1	1	1	1
CCL22	1	1	1	1	1	5	11	2	1
CCL23	1	1	1	1	1	1	1	1	1
CCL24	1	1	1	1	9	1	1	6	1
CCL26	9	1	1	1	1	1	1	1	1
CCL3	1	1	1	33	1	5	13	1	25
CCL4	10	1	1	9	1	1	11	1	5
CCL5	1	1	1	64	31	20	93	48	115
CCL7	1	1	1	1	1	1	1	1	1
CCL8	1	1	1	1	1	1	1	1	1
CCND3	447	548	572	697	906	775	759	897	1433
CCR1	18	1	5	19	142	110	119	137	48
CCR10	30	1	4	6	5	1	3	11	19
CCR2	1	1	1	31	61	64	73	100	65
CCR5	66	33	20	117	328	147	223	383	106
CCR6	146	164	98	140	152	44	101	179	145
CCR7	1	1	1	1	3	1	1	12	1
CCR8	1	1	1	1	1	1	1	1	1
CCRL1	1	1	1	1	1	1	1	1	1
CCRL2	24	11	1	51	55	51	92	67	61
CD14	1	1	1	23	32	22	9	28	57
CD160	3	1	1	1	1	1	1	1	1
CD163	1	1	1	158	394	638	348	650	387
CD164	5979	4764	3796	4346	4960	5495	4673	5491	4280
CD19	5	1	1	7	12	1	18	64	18
CD1A	1	1	1	65	119	73	37	108	89
CD1D	117	1	15	920	1871	1661	1100	2374	1089
CD2	1	1	1	34	109	115	61	108	73
CD209	1	1	1	4	10	12	11	21	33
CD22	197	227	203	185	94	151	91	190	182
CD24	1	1	1	1	5	1	11	48	25
CD244	128	58	43	513	413	775	610	647	829
CD247	20	1	7	9	3	1	7	3	13
CD27	8	1	1	1	1	1	1	11	1
CD274	1	1	1	1	1	1	1	1	1
CD276	1	1	1	1	1	1	1	1	1
CD28	1	1	1	1	1	1	1	1	1
CD34	1	1	1	1	1	1	1	1	1
CD36	727	582	378	1889	1917	3037	2017	2736	2712
CD3D	1	1	1	1	1	1	1	1	1
CD3E	47	44	26	37	15	20	27	34	45
CD3EAP	1	1	1	1	1	1	1	1	2
CD4	1352	1894	2342	5544	5492	5033	4801	4220	4840
CD40	409	368	368	65	191	88	105	149	39
CD40LG	6	1	1	1	1	1	1	1	1
CD44	2833	2867	2840	4650	6250	6125	6072	5804	5016
CD45R0	2832	1358	1297	1626	2333	2612	1453	2830	1484
CD45RA	814	546	586	1385	1589	1510	1018	1511	906
CD45RB	1289	768	914	1557	2157	2043	1261	1893	1404
CD46	2581	1854	1666	1510	1918	2526	1635	2548	1434
CD48	5964	7929	4989	5967	5259	5285	6934	5178	5630
CD5	554	261	120	394	2316	376	177	732	183
CD53	2123	1312	1406	3128	4405	3838	2802	4291	2975
CD55	1	1	1	1	1	1	1	2	2
CD58	1071	861	850	940	1039	1109	921	1412	997
CD59	2489	2741	2909	810	806	494	989	790	627
CD6	1	1	1	7	26	1	8	12	7
CD7	1	1	1	1	1	1	1	1	1
CD70	1	1	1	1	1	1	1	1	5
CD74	153660	196746	220758	145198	151766	102750	119246	110752	119836
CD79A	38	39	45	1	1	12	163	489	173
CD79B	13	1	5	72	77	61	81	174	119
CD80	11	1	1	2	1	1	2	15	22
CD81	2049	2464	2674	917	944	955	1168	810	962
CD82	37	2	14	1	1	1	13	28	12
CD83	25752	25431	21803	12421	15918	14901	10599	10461	7169
CD86	1261	966	998	2091	2572	2463	1845	2753	2002
CD8A	19	2	2	1	2	1	6	8	2
CD8B	1	1	1	1	1	1	1	1	1
CD9	79	186	19	1	108	7	9	139	21
CD96	11	1	1	1	5	1	4	12	1
CD97	547	474	498	1301	1700	1481	1237	1642	1333
CD99	3409	4070	3415	4569	5003	4755	7748	6301	7539
CDH5	1	1	1	1	1	1	1	1	1
CDKN1A	878	927	294	2620	4868	6931	5363	5331	3340

Name	BDCA3^{hi} DCs	BDCA3^{hi} DCs	BDCA3^{hi} DCs	BDCA3^{+} BDCA1^{+} DCs	BDCA3^{+} BDCA1^{+} DCs	BDCA3^{+} BDCA1^{+} DCs	BDCA3^{-} BDCA1^{+} DCs	BDCA3^{-} BDCA1^{+} DCs	BDCA3^{-} BDCA1^{+} DCs
CEACAM1	61	17	25	2	14	7	24	15	3
CEACAM6	10	1	1	1	1	2	1	1	35
CEACAM8	1	1	1	1	1	1	3	1	30
CEBPB	171	154	94	1387	3142	3843	2468	3914	2092
CFB	167	172	116	36	41	42	63	47	24
CFD	46	8	42	164	49	49	66	64	308
CFH	1	1	1	1	1	1	6	2	1
CFI	28	24	2	17	2	1	18	8	18
CFP	1300	991	1086	4360	4607	5380	4585	4402	5625
CHUK	893	599	445	410	824	1253	686	1068	427
CIITA	2485	1606	2253	2069	2994	2433	2077	3201	2299
CISH	1	1	1	7	14	1	28	21	27
CLEC4A	462	382	369	3566	2727	3169	3051	2646	3433
CLEC4E	1	1	1	1	5	27	1	38	1
CLEC5A	79	107	98	59	68	32	82	59	68
CLEC6A	1	1	1	1	12	29	15	43	1
CLEC7A	1638	1306	867	313	369	645	401	497	158
CLU	6	1	1	1	1	1	1	2	1
CMKLR1	14	1	1	1	1	1	1	1	1
CR1	14	1	1	117	184	129	200	287	113
CR2	9	1	1	1	1	1	1	4	1
CRADD	22	1	10	1	12	5	1	7	14
CSF1	8	1	1	1	1	1	4	1	1
CSF1R	1	1	1	37	92	68	49	80	77
CSF2	1	1	1	1	1	1	1	1	1
CSF2RB	570	625	655	753	1128	1422	814	1166	902
CSF3R	376	373	316	1190	1538	2258	1878	2123	1871
CTLA4_all	492	390	74	96	312	120	101	179	30
CTLA4-TM	84	77	4	10	44	34	3	34	1
CTNNB1	6382	5592	4189	6239	8899	11969	8108	11142	6839
CTSC	575	475	604	1573	1295	1268	1263	1484	1810
CTSG	1	1	1	1	2	1	1	1	44
CTSS	11954	10241	10210	23564	20700	25608	20128	24279	24646
CUL9	138	82	118	110	154	137	133	171	118
CX3CL1	1	1	1	1	1	1	1	1	1
CX3CR1	8	1	1	96	121	88	91	172	96
CXCL1	244	278	171	114	90	73	104	90	74
CXCL10	1	1	1	1	1	1	12	1	1
CXCL11	15	8	24	9	5	22	10	19	20
CXCL12	1	1	1	1	1	1	1	1	1
CXCL13	1	1	1	1	1	1	1	1	1
CXCL2	525	403	90	88	367	208	166	307	90
CXCL9	63	231	31	1	7	7	36	21	1
CXCR1	1	1	1	1	1	1	1	1	1
CXCR2	14	1	1	1	1	2	7	1	1
CXCR3	613	768	576	37	65	49	31	22	25
CXCR4	26819	25941	13507	26600	37951	38474	27786	35839	20939
CXCR6	1	1	1	1	1	1	1	1	1
CYBB	32	16	10	1428	1731	2008	1516	1852	1541
DEFB1	1	1	1	1	1	1	1	1	1
DEFB103A	1	1	1	1	1	1	1	1	1
DEFB103B	1	1	1	1	1	1	1	1	1
DEFB4A	1	1	1	1	1	1	1	1	1
DPP4	1233	1179	1314	84	85	17	61	46	43
DUSP4	7852	4157	1478	332	2142	2047	620	892	116
EBI3	20	27	19	29	26	20	29	39	28
EDNRB	1	1	1	1	1	1	1	1	1
EGR1	1	1	1	10	1	1	11	1	1
EGR2	1	1	1	1	1	1	1	1	1
ENTPD1	105	46	87	158	138	200	110	153	94
EOMES	14	5	1	1	2	1	7	4	1
ETS1	74	17	45	21	26	15	47	138	49
FADD	5	6	1	8	1	10	2	3	5
FAS	1	1	1	1	1	10	13	2	1
FCAR	1	1	1	1	1	1	1	1	1
FCER1A	151	121	19	25017	28343	32749	25735	44590	39350
FCER1G	987	741	825	3275	3545	3056	2905	3084	3218
FCGR1A/B	1	1	1	1	1	1	1	1	1
FCGR2A	1	1	1	1075	1502	2067	913	2017	1365
FCGR2A/C	16	1	1	4669	3917	3293	4278	7397	4984
FCGR2B	1	1	1	4157	1782	2692	2010	2655	4491
FCGR3A/B	1	1	1	1	1	1	1	1	1
FCGRT	6269	7472	8221	7301	7233	6536	7192	6855	8421
FKBP5	1062	727	904	1108	1410	1556	1014	1681	1085
FN1	1	1	1	1	1	1	1	1	1

Name	BDCA3hi DCs	BDCA3hi DCs	BDCA3hi DCs	BDCA3+ BDCA1+ DCs	BDCA3+ BDCA1+ DCs	BDCA3+ BDCA1+ DCs	BDCA3- BDCA1+ DCs	BDCA3- BDCA1+ DCs	BDCA3- BDCA1+ DCs
FOXP3	1	1	1	1	1	1	1	1	1
FYN	2030	1718	1904	1762	1714	1771	1608	1778	1906
GATA3	9	1	1	1	1	1	1	1	1
GBP1	11	1	1	194	338	323	376	402	156
GBP5	1	1	1	5	67	2	32	75	24
GFI1	56	33	45	31	38	39	55	58	47
GNLY	3	1	1	1	1	1	1	1	1
GP1BB	4	1	1	1	1	1	1	14	4
GPI	1114	1127	1130	1513	1601	1371	1375	1259	1557
GPR183	10349	6357	2843	7805	13349	15774	9314	10392	5417
GZMA	4	1	1	1	1	1	3	1	1
GZMB	14	25	1	1	10	10	1	8	1
GZMK	1	1	1	1	1	1	1	1	1
HAMP	5	1	1	1	1	1	1	1	5
HAVCR2	1632	968	1042	580	969	943	527	908	605
HFE	5	1	1	2	17	2	11	15	6
HLA-A	18780	28794	28868	17638	14289	11654	20992	15981	17686
HLA-B	20021	27192	17648	9551	17368	12035	17598	15025	9247
HLA-C	5699	4766	4754	3537	5856	3528	3564	4813	3127
HLA-DMA	14537	15387	16582	15959	14247	10696	12779	12323	13919
HLA-DMB	10704	9118	9835	10063	9191	8835	8961	9793	9298
HLA-DOB	4727	4311	5986	1403	1087	264	706	646	603
HLA-DPA1	217309	271098	303311	255680	156521	94214	152174	128553	176598
HLA-DPB1	216975	235797	251256	229957	174910	114275	144279	140283	157198
HLA-DQA1	31227	87266	46926	53495	40859	621	86353	33012	39551
HLA-DQB1	13793	31082	28144	26612	13385	195	24451	9606	20868
HLA-DRA	137443	162190	170337	180409	139366	94438	130622	114661	123809
HLA-DRB1	32660	64174	69796	62116	18331	7882	50209	15201	47787
HLA-DRB3	66446	87790	97677	81474	58261	40853	58407	43727	60881
HRAS	69	36	42	42	46	73	47	55	55
ICAM1	3126	3861	3879	1874	2084	2319	2661	2234	1834
ICAM2	126	71	76	220	237	217	172	240	245
ICAM3	7860	7935	8397	6214	5047	5048	6247	4410	6850
ICAM4	57	25	27	20	65	2	29	41	61
ICAM5	1	1	1	1	1	1	1	1	1
ICOS	4	1	1	1	1	1	5	1	1
ICOSLG	851	452	673	260	754	926	424	793	314
IDO1	7955	8235	6418	133	265	34	90	73	21
IFI16	1318	640	769	469	732	809	542	964	464
IFI35	197	109	141	145	147	95	107	156	117
IFIH1	256	305	275	169	178	210	259	252	179
IFIT2	24	11	1	22	24	1	98	54	10
IFITM1	134	124	22	983	3248	941	2736	2537	680
IFNA1/13	3	1	1	1	1	1	1	1	1
IFNA2	1	1	1	1	1	1	1	1	1
IFNAR1	37	8	10	16	29	10	22	48	30
IFNAR2	2119	1490	2000	1594	2156	1928	1469	2277	1613
IFNB1	23	1	1	1	1	1	1	1	1
IFNG	1	1	1	1	2	1	1	1	1
IFNGR1	4695	3808	4023	4368	4398	6176	3354	5596	3773
IGF2R	412	444	430	93	174	100	126	187	73
IKBKAP	128	27	102	40	75	68	51	70	65
IKBKB	195	83	124	183	191	318	197	266	207
IKBKE	8	1	1	1	12	17	2	23	24
IKBKG	176	124	162	178	179	151	170	215	194
IKZF1	161	127	162	153	181	132	171	217	171
IKZF2	1	1	1	1	1	1	1	7	1
IKZF3	1	1	1	1	1	1	1	44	6
IL10	1	1	1	1	1	10	5	2	1
IL10RA	4208	4558	4564	4387	5047	4671	4985	5828	4813
IL11RA	11	1	1	1	3	7	5	11	2
IL12A	1	1	1	1	9	12	15	25	4
IL12B	5	1	1	3	1	1	1	3	6
IL12RB1	38	20	24	146	155	144	156	101	113
IL13	25	6	1	4	1	5	21	17	19
IL13RA1	473	444	277	5668	6201	6963	5918	7302	5629
IL15	10	6	1	1	26	27	24	52	10
IL16	3339	2513	3049	2991	2349	2006	1780	2006	2404
IL17A	11	1	1	1	1	1	1	1	1
IL17B	14	1	1	1	1	1	1	1	1
IL17F	1	1	1	1	1	1	1	1	1
IL18	414	345	318	779	739	1205	791	756	782
IL18R1	840	762	538	462	591	384	747	514	313
IL18RAP	36	19	17	9	20	15	11	14	12
IL19	1	1	1	1	1	1	1	1	1

Name	BDCA3^{hi} DCs	BDCA3^{hi} DCs	BDCA3^{hi} DCs	BDCA3^{+} BDCA1^{+} DCs	BDCA3^{+} BDCA1^{+} DCs	BDCA3^{+} BDCA1^{+} DCs	BDCA3^{-} BDCA1^{+} DCs	BDCA3^{-} BDCA1^{+} DCs	BDCA3^{-} BDCA1^{+} DCs
IL1A	1	1	1	1	1	1	1	1	1
IL1B	1	1	1	73	87	318	51	42	43
IL1R1	1	14	1	70	113	44	54	131	59
IL1R2	85	80	53	677	674	320	380	533	369
IL1RAP	57	5	2	61	138	330	102	174	127
IL1RL1	55	57	52	38	53	22	38	31	42
IL1RL2	10	1	4	1	12	1	1	1	1
IL1RN	1	1	1	91	90	164	114	138	226
IL2	1	1	1	1	1	1	4	1	4
IL20	1	1	1	1	1	1	1	1	1
IL21	1	1	1	1	1	1	1	1	1
IL21R	4	1	1	1	20	1	19	38	16
IL22	1	1	1	1	1	1	1	1	1
IL22RA2	1	1	1	1	1	1	1	1	1
IL23A	4	1	1	1	17	17	11	15	7
IL23R	1	1	1	1	1	1	1	1	2
IL26	5	1	1	1	2	1	10	1	1
IL27	1	1	1	1	1	1	1	1	1
IL28A	1	1	1	1	10	1	3	1	4
IL28A/B	1	1	1	1	1	1	1	1	1
IL29	1	1	1	1	1	1	1	1	1
IL2RA	1	1	1	1	1	1	1	1	1
IL2RB	18	1	1	19	3	1	12	21	36
IL2RG	2705	2502	3202	3403	4916	3039	3664	4151	3037
IL3	1	1	1	1	1	1	1	1	1
IL32	357660	481697	304138	119431	192060	168937	181585	105815	47647
IL4	20	1	1	1	1	1	11	4	5
IL4R	250	261	316	446	461	391	449	618	550
IL5	1	1	1	1	1	1	1	1	2
IL6	1	1	1	1	1	1	4	5	6
IL6R	658	560	785	819	739	784	777	806	1060
IL6ST	771	607	310	370	553	987	765	920	303
IL7	1	1	1	10	22	15	48	25	33
IL7R	1	1	1	1	1	1	1	1	1
IL8	1590	1566	317	1084	922	1517	831	495	510
IL9	1	1	1	1	1	1	1	1	1
ILF3	4650	4539	4475	3329	4521	5150	3758	4816	3217
IRAK1	468	532	515	500	628	479	545	535	527
IRAK2	307	456	375	69	48	49	54	67	66
IRAK3	5	3	1	270	372	398	289	526	321
IRAK4	244	253	234	247	321	364	272	376	278
IRF1	1308	1912	341	549	1458	2729	3203	1686	570
IRF3	60	3	57	41	46	17	32	51	47
IRF4	685	824	385	2814	5015	4513	1798	2506	1535
IRF5	766	950	954	645	522	601	795	576	718
IRF7	270	211	197	241	430	335	314	335	258
IRF8	13770	7243	11482	802	992	2099	1515	1172	855
IRGM	1	1	1	1	1	1	1	1	1
ITGA2B	23	1	15	1	5	1	1	11	7
ITGA4	1718	1523	1367	1500	1857	2157	1503	1722	1063
ITGA5	264	230	282	347	526	462	430	694	533
ITGA6	30	1	25	1	1	1	3	1	1
ITGAE	400	592	493	977	659	674	1144	701	1087
ITGAL	2022	1756	1852	1089	1171	1312	1235	903	1133
ITGAM	1	1	1	215	746	347	437	661	282
ITGAX	2103	2868	2926	6538	9143	6778	7320	8474	6308
ITGB1	2126	1842	1771	1196	1323	1669	1311	1552	1074
ITGB2	5846	6664	8761	10511	9860	6712	8529	8412	11010
ITLN1	1	1	1	1	1	1	1	1	2
ITLN2	1	1	1	1	1	1	1	1	1
JAK1	1547	1345	1605	1651	2053	1766	1522	1921	1457
JAK2	1383	994	966	1386	1627	1818	1715	2082	1351
JAK3	308	198	391	465	867	423	455	730	503
KCNJ2	15	1	1	1	14	1	20	20	6
KIR_Activating_Subgroup_1	27	1	19	1	3	1	11	18	21
KIR_Activating_Subgroup_2	3	1	1	1	9	1	2	1	1
KIR_Inhibiting_Subgroup_1	1	1	1	1	1	1	1	1	1

Name	BDCA3^{hi} DCs	BDCA3^{hi} DCs	BDCA3^{hi} DCs	BDCA3^{+} BDCA1^{+} DCs	BDCA3^{+} BDCA1^{+} DCs	BDCA3^{+} BDCA1^{+} DCs	BDCA3^{-} BDCA1^{+} DCs	BDCA3^{-} BDCA1^{+} DCs	BDCA3^{-} BDCA1^{+} DCs
KIR_Inhibiting_Subgroup_2	1	1	1	1	1	1	1	1	1
KIR3DL1	1	1	1	1	1	1	1	3	1
KIR3DL2	1	1	1	1	1	1	1	1	1
KIR3DL3	1	1	1	1	1	1	1	1	1
KIT	556	491	637	260	164	95	127	116	97
KLRAP1	1	1	1	1	1	1	1	1	1
KLRB1	1	1	1	1	1	1	1	1	1
KLRC1	3	1	1	1	1	1	1	14	1
KLRC2	10	2	1	10	9	1	7	8	26
KLRC3	1	1	1	1	1	1	1	1	1
KLRC4	22	1	6	1	1	20	1	5	5
KLRD1	1	1	1	1	1	1	1	1	1
KLRF1	4	1	2	13	1	1	16	1	16
KLRF2	1	1	1	1	1	1	1	1	1
KLRG1	20	80	37	1	1	1	35	7	6
KLRG2	1	1	1	1	1	1	1	1	1
KLRK1	4	14	1	1	1	1	1	1	1
LAG3	25	38	9	10	2	12	13	9	33
LAIR1	13	16	1	120	133	169	93	107	69
LAMP3	23	14	14	80	41	12	78	114	12
LCK	1	1	1	1	1	1	1	1	1
LCP2	926	730	829	1334	1773	2270	2043	1987	1357
LEF1	1	1	1	1	1	1	1	1	1
LGALS3	2279	2368	1874	4595	3840	5114	4639	4504	5192
LIF	1	1	1	1	1	5	1	1	1
LILRA1	33	47	11	339	386	264	303	291	216
LILRA2	363	313	245	1202	942	828	1056	848	1129
LILRA3	1	1	1	17	41	34	36	59	44
LILRA4	235	172	229	59	75	7	2	22	27
LILRA5	458	247	113	1076	2364	1564	801	1895	997
LILRA6	36	9	6	23	55	37	82	97	45
LILRB1	13	25	19	487	611	364	322	454	328
LILRB2	157	138	105	803	1101	926	890	1004	870
LILRB3	28	1	6	835	1101	643	906	1426	836
LILRB4	188	183	99	1057	1628	1410	1539	1718	1373
LILRB5	1	1	1	1	1	1	1	1	6
LITAF	2587	1710	1919	3403	3917	3501	1812	3293	3329
LTA	9	1	1	1	1	1	1	5	1
LTB4R	98	94	115	168	253	213	152	213	214
LTB4R2	100	104	74	71	137	288	147	169	100
LTBR	897	729	897	1070	1074	1036	1025	1158	1222
LTF	16	6	12	1	1	1	7	1	81
LY96	463	393	431	648	628	750	740	725	678
MAF	14	1	1	1	1	1	2	5	1
MALT1	1580	1045	768	1853	3267	4119	2165	3380	1773
MAP4K1	1960	2141	2073	1961	2050	1674	2096	1903	2006
MAP4K2	332	368	385	284	306	247	291	350	305
MAP4K4	967	928	788	679	1166	826	802	1291	598
MAPK1	2020	1646	1805	1832	2023	1752	1458	2058	1876
MAPK11	8	16	2	1	26	1	6	23	9
MAPK14	595	301	403	346	592	792	396	897	450
MAPKAPK2	1800	2011	1469	1311	1755	2087	1886	1566	1379
MARCO	20	2	1	42	77	125	146	132	68
MASP1	1	1	1	1	1	1	1	1	1
MASP2	1	1	1	1	1	1	1	1	1
MBL2	1	1	1	1	1	1	1	1	2
MBP	729	434	653	637	715	332	292	654	594
MCL1	80775	55950	40348	32892	67842	66998	50769	66430	25839
MIF	2395	2848	2288	1858	1937	1412	1797	1437	1572
MME	1	1	1	1	1	1	1	1	1
MR1	53	20	26	75	68	86	84	97	73
MRC1	1	1	1	233	1000	166	391	1363	248
MS4A1	4	1	1	1	1	1	79	414	37
MSR1	20	1	1	213	422	516	39	366	73
MUC1	1	1	1	1	5	1	1	1	1
MX1	1387	1083	687	743	1285	1016	1084	1464	710
MYD88	5388	4480	4258	4548	5404	4083	3789	4428	4026
NCAM1	42	1	1	1	7	5	7	1	1
NCF4	384	307	399	327	213	442	292	286	419
NCR1	1	1	1	1	1	1	1	1	1
NFATC1	82	71	45	40	116	73	93	151	82
NFATC2	1794	1336	1527	787	1360	948	961	1144	670

Name	BDCA3^{hi} DCs	BDCA3^{hi} DCs	BDCA3^{hi} DCs	BDCA3^{+} BDCA1^{+} DCs	BDCA3^{+} BDCA1^{+} DCs	BDCA3^{+} BDCA1^{+} DCs	BDCA3^{-} BDCA1^{+} DCs	BDCA3^{-} BDCA1^{+} DCs	BDCA3^{-} BDCA1^{+} DCs
NFATC3	764	574	803	667	713	628	672	789	608
NFIL3	3319	2875	1701	2446	5417	7146	5989	6388	2649
NFKB1	741	847	509	532	956	1024	827	959	546
NFKB2	444	382	421	334	485	696	386	579	383
NFKBIA	16584	27076	34282	15057	12710	19998	19122	17645	18461
NFKBIZ	1304	1263	1556	1327	2197	2411	1354	2184	986
NLRP3	1102	1084	529	1306	2898	3435	2024	2901	1131
NOD1	30	33	67	59	87	81	78	67	49
NOD2	221	222	229	934	1214	1732	1419	1742	1388
NOS2	1	1	1	1	1	1	1	1	1
NOTCH1	63	25	82	32	106	271	70	84	71
NOTCH2	1311	1023	1381	1045	1500	1104	1169	1457	1020
NT5E	152	104	104	124	44	49	71	103	119
PAX5	1	1	1	1	1	1	7	54	1
PDCD1	1	1	1	1	1	1	1	2	1
PDCD1LG2	1	1	1	1	1	1	1	11	1
PDCD2	123	120	121	131	150	110	115	154	119
PDGFB	1	1	1	1	1	1	1	1	1
PDGFRB	1	1	1	1	1	1	1	1	1
PECAM1	2719	2527	2800	2454	2140	2504	2228	2504	2767
PIGR	1	1	1	22	1	15	1	1	16
PLA2G2A	19	1	9	1	1	1	1	1	1
PLA2G2E	46	44	41	32	17	10	45	28	40
PLAU	22	1	1	202	181	232	239	225	246
PLAUR	1605	1079	774	3424	4547	5294	3411	3954	3902
PML	245	222	177	171	321	310	326	336	218
POU2F2	478	409	456	493	742	650	762	815	526
PPARG	1	1	1	1	1	1	1	1	1
PPBP	5	96	115	189	67	1	81	118	199
PRDM1	69	72	17	254	459	1170	456	444	196
PRF1	5	1	1	1	3	1	6	3	13
PRKCD	324	278	326	575	568	594	678	682	830
PSMB10	1360	1342	1441	1562	1601	1200	1279	1232	1382
PSMB5	435	365	351	314	300	252	291	319	328
PSMB7	4225	4173	4029	4674	3951	4298	4318	4052	4632
PSMB8	5616	5170	4895	2985	2994	2873	3729	2926	3099
PSMB9	4981	4637	5809	2257	2099	1581	2194	1799	2071
PSMC2	1838	1630	1491	1374	1396	1715	1514	1518	1370
PSMD7	3566	3251	3580	3868	3620	3489	3801	4215	3918
PTAFR	1	1	1	71	111	44	53	133	61
PTGER4	879	719	754	994	1398	796	1052	763	616
PTGS2	113	39	15	784	1220	2211	928	894	469
PTK2	1879	1396	1494	178	416	191	299	386	179
PTPN2	1222	983	794	738	997	1065	792	1093	733
PTPN22	1610	991	956	559	541	845	511	714	416
PTPN6	2390	1803	2251	3148	2900	3120	2501	2879	3287
PTPRC_all	12026	5579	5411	7740	10833	13653	7079	13153	6217
PYCARD	454	445	512	444	411	298	358	355	439
RAF1	1144	807	883	730	946	1354	917	1277	819
RAG1	1	1	1	1	1	1	1	1	1
RAG2	41	19	6	10	1	1	1	12	3
RARRES3	65	69	47	390	394	237	382	317	317
RELA	463	562	523	570	645	669	618	656	576
RELB	552	618	652	479	637	594	682	596	543
RORC	1	1	1	1	1	1	4	1	1
RUNX1	926	839	678	541	939	1791	967	1196	735
S100A8	1	1	1	838	840	2211	1527	1721	3281
S100A9	669	702	613	3931	3328	9189	6357	6706	12534
S1PR1	5	1	1	1	12	7	1	11	5
sCTLA4	350	308	128	130	306	134	114	167	84
SELE	1	1	1	1	1	1	1	1	1
SELL	2270	1731	2591	2032	1726	2294	1942	1875	2990
SELPLG	105	38	71	34	48	5	26	48	44
SERPING1	27	27	21	55	79	86	112	118	86
SH2D1A	1	1	1	1	1	1	1	1	1
SIGIRR	70	79	77	133	196	171	206	201	201
SKI	209	170	187	188	544	606	426	696	338
SLAMF1	1	1	1	1	1	1	1	1	1
SLAMF6	1	1	1	1	1	1	1	1	1
SLAMF7	2309	1531	1953	245	234	161	77	108	88
SLC2A1	774	470	468	363	300	491	266	376	476
SMAD3	307	278	234	325	524	652	562	802	436
SMAD5	255	162	183	183	382	369	313	548	194
SOCS1	16	1	1	1	7	2	20	23	10
SOCS3	1023	573	348	1413	3098	2912	2418	2871	1551

Name	BDCA3hi DCs	BDCA3hi DCs	BDCA3hi DCs	BDCA3+ BDCA1+ DCs	BDCA3+ BDCA1+ DCs	BDCA3+ BDCA1+ DCs	BDCA3- BDCA1+ DCs	BDCA3- BDCA1+ DCs	BDCA3- BDCA1+ DCs
SPP1	3	1	1	1	1	1	1	1	1
SRC	415	329	379	240	374	271	184	153	90
STAT1	618	629	224	429	1087	1915	1483	1406	452
STAT2	1667	1317	1562	1033	1444	1490	1025	1441	1185
STAT3	1149	747	948	1842	2620	2385	1809	2847	1864
STAT4	76	33	41	30	63	88	39	92	35
STAT5A	420	326	370	541	502	557	584	581	583
STAT5B	851	689	769	830	814	753	736	824	796
STAT6	1715	1426	1681	1771	2082	2087	1558	1869	2159
SYK	4406	3643	4777	5104	4798	3496	4333	5313	5867
TAGAP	260	200	98	2404	1441	2350	2477	1832	1998
TAL1	8	1	14	1	1	1	1	2	1
TAP1	858	749	713	147	225	156	228	196	170
TAP2	1120	1153	1276	343	558	332	334	441	278
TAPBP	2820	3293	3650	1599	2410	1776	1989	1724	1559
TBK1	491	466	362	381	418	750	596	824	349
TBX21	1	1	2	9	10	2	25	28	20
TCF4	426	385	360	276	427	315	250	431	203
TCF7	5	3	1	1	1	1	4	11	4
TFRC	1535	1245	1296	660	787	1087	611	865	499
TGFB1	1609	1836	1834	2710	3449	2856	3578	3386	3284
TGFBI	7828	9009	7214	5929	5322	4823	5882	3870	5455
TGFBR1	345	274	251	490	362	432	326	478	528
TGFBR2	587	610	596	1159	1511	1217	1371	1443	955
THY1	19	1	1	1	1	1	10	4	1
TICAM1	411	529	547	204	369	286	408	284	246
TIGIT	85	60	82	59	36	12	73	53	64
TIRAP	22	1	1	5	20	1	21	36	19
TLR1	448	271	339	399	522	472	486	762	310
TLR2	121	123	97	325	405	694	509	643	391
TLR3	2318	1872	1621	252	143	457	359	139	175
TLR4	9	16	31	78	126	205	113	162	100
TLR5	18	17	15	79	53	46	69	90	79
TLR7	1	1	1	1	2	1	1	2	1
TLR8	25	88	51	127	56	100	152	78	86
TLR9	39	39	11	16	39	22	34	41	13
TMEM173	65	63	79	866	910	1148	1110	1147	1441
TNF	164	384	499	98	31	34	95	48	140
TNFAIP3	2063	3600	4023	1148	2507	3323	1807	2838	1232
TNFAIP6	109	98	99	76	26	32	50	46	72
TNFRSF10C	1	1	1	1	20	1	1	24	2
TNFRSF11A	359	256	261	1	14	2	12	13	1
TNFRSF13B	1	1	1	1	1	1	1	28	1
TNFRSF13C	16	44	17	1	7	1	44	206	44
TNFRSF14	655	675	627	656	765	572	638	593	506
TNFRSF17	10	1	1	1	1	1	1	1	1
TNFRSF1B	237	126	235	1477	1509	1901	931	1616	2087
TNFRSF4	1	1	1	1	1	1	1	1	1
TNFRSF8	1	1	1	1	1	1	1	1	6
TNFRSF9	1	1	1	1	3	5	1	21	9
TNFSF10	77	91	38	249	278	137	793	399	303
TNFSF11	1	1	1	1	2	17	1	15	8
TNFSF12	75	30	102	650	637	418	532	609	750
TNFSF13B	936	466	441	1169	1828	1850	1105	2309	1239
TNFSF15	5	1	1	1	5	1	10	8	1
TNFSF4	4	1	4	7	1	1	7	24	5
TNFSF8	93	74	81	81	111	120	75	98	86
TOLLIP	245	214	225	149	167	181	199	226	247
TP53	1489	1094	1304	1236	1473	1410	993	1203	1277
TRAF1	147	110	115	5	48	5	21	41	7
TRAF2	60	44	77	44	49	24	48	40	57
TRAF3	170	195	161	310	384	325	453	404	355
TRAF4	948	1221	985	648	628	601	610	531	538
TRAF5	140	65	159	58	73	51	53	105	55
TRAF6	750	738	643	358	618	787	616	705	323
TYK2	1072	809	992	595	1108	1126	717	1191	773
UBE2L3	433	334	295	286	422	374	281	396	305
VCAM1	20	41	233	1	1	1	1	7	1
VTN	15	3	5	3	1	1	1	1	11
XBP1	799	1087	1050	841	671	613	694	727	925
XCL1	23	1	1	1	1	1	1	1	1
XCR1	509	765	533	1	1	1	8	1	1
ZAP70	1	1	1	1	1	1	1	1	1
ZBTB16	1	1	1	1	1	1	1	2	1
ZEB1	268	146	223	46	87	59	13	51	9

Interferon-λ is mainly produced by BDCA3-expressing myeloid dendritic cells and regulated by the NFκB and PI3K-PKB-mTOR pathway

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In preparation

Chapter 6

Abstract

Interferons (IFNs) play a pivotal role in antiviral immunity. Type III IFN (IFN-λ) was most recently discovered, and the pathways regulating its production are still poorly understood. BDCA3^{hi} dendritic cells (DCs), which are characterized by high BDCA3 expression, are excellent IFN-λ-producing cells. However, neither BDCA3 expression nor IFN-λ production is restricted to these cells since both have been described for other cell types. Here, a possible association of BDCA3 expression by immune cells with IFN-λ-producing capacity, and the signaling pathways regulating IFN-λ production were investigated.

Flow cytometric analysis revealed that BDCA3 is highly expressed by peripheral blood BDCA3^{hi} DCs and at intermediate levels by a population of BDCA1^{+} DCs, plasmacytoid DCs (pDCs) and monocytes, but not other immune cells. All DC subsets, but not other immune cells, produced detectable levels of IFN-λ in response to TLR signaling, and highest IFN-λ levels were secreted by BDCA3^{hi} DCs. Enhanced IFN-λ production was associated with increased BDCA3 expression on myeloid DCs (mDCs), but not pDCs, indicating expression of the two is not always linked.

In search of the signaling cascade leading to IFN-λ production by BDCA3^{hi} DCs we demonstrated that intact PI3K-PKB-mTOR pathway signaling and canonical NFκB pathway signaling is required for IFN-λ production by BDCA3^{hi} DCs.

Thus, IFN-λ is produced by all DC subsets, especially by BDCA3^{hi} DCs. We show here for the first time that IFN-λ production by these cells is regulated via the canonical NFκB pathway and PI3K-PKB-mTOR pathway. Insight into the major IFN-λ-producing cells and their regulatory mechanisms underlying IFN-λ production may aid in the development of IFN-λ-manipulating therapies.

Introduction

Interferons (IFNs) play a crucial role in host defense against viral infections. IFNs act in two ways; they have direct antiviral activities inhibiting cell infection and virus replication as well as immunoregulatory functions [1]. IFNs can be distinguished into three subtypes; type I IFNs (IFN-α/β), type II IFNs (IFN-γ) and the most recently discovered type III IFNs (IFN-λ) [2, 3]. The IFN-λ family consists of four members, including IFN-λ1 (IL-29), the 96% homologous isoforms IFN-λ2 (IL-28A) and IFN-λ3 (IL-28B), and IFN-λ4, the latter being only expressed in individuals of a particular genotype [4-6]. All IFN-λ subtypes signal through the heterodimeric IFN-λ receptor, that has similar downstream effectors as the IFN-α receptor. Yet the IFN-λ and IFN-α receptor differ in expression kinetics and bioactivity [2, 7-9]. Like IFN-α/β, IFN-λs have potent antiviral activity against a broad spectrum of viruses and support skewing towards Th1 responses [10]. However, compared to type I and type II IFNs, knowledge on the function and regulation of IFN-λs is still very limited. We know that most cell types are able to express IFN-λ1-3 mRNA in response to viral infections, yet dendritic cells (DCs) seem to be superior IFN-λ-producing cells [11, 12].

DCs are professional antigen presenting cells (APC) that play a pivotal role in the induction and regulation of innate and adaptive immune responses. The DC family comprises different subsets, diverging in ontogeny, phenotype, localization and specialized immune functions [13, 14]. Three main categories of human DC subsets can be distinguished, including BDCA1^{+} myeloid DCs (mDCs), BDCA3^{hi}CLEC9A^{+}XCR1^{+} mDCs and plasmacytoid DCs (pDCs). BDCA3^{hi}CLEC9A^{+}XCR1^{+} DCs (further referred to as BDCA3^{hi} DCs) have been described as the main producers of IFN-λ upon TLR3 stimulation. However, also BDCA1^{+} DCs and pDCs are able to produce IFN-λ upon TLR3 and TLR9 stimulation, respectively [12, 15-18]. The reason why BDCA3^{hi} DCs are such excellent IFN-λ producers compared to other DC subsets is not known. We have previously shown that BDCA3 expression on CD34^{+} hematopoietic precursor cell (HPC)-derived DCs is associated with IFN-λ production [19]. Of course high BDCA3 surface expression is a specific characteristic of BDCA3^{hi} DCs [20, 21], but expression of BDCA3 can also be detected in low levels on other cells, including BDCA1^{+} mDCs and pDCs [22-24], which suggests that the level of BDCA3 expression may be associated with IFN-λ-producing capacity by DCs.

Although the molecular mechanisms regulating TLR ligand and/or virus-induced type I IFN production by DCs are well characterized, the pathways regulating type III IFN production are incompletely understood. TLR3 triggering leads to activation and translocation of NFκB and IRF3, resulting in production of type I IFNs and other pro-inflammatory cytokines. NFκB activation occurs via the TRIF-TRAF6-IKK-α/β cascade, whereas optimal IRF3 phosphorylation requires both TRIF-TRAF3-TBK1 signaling and PI3K-PKB-mTOR signaling [25-27]. So far, most studies on IFN-λ-regulating pathways have been performed in epithelial-like cells, such as hepatocytes, HEK293 cells, airway epithelial cells, and colon epithelial cells, and in vitro-generated DCs [28-32]. Most of these studies used mRNA gene expression instead of protein secretion as a read-out for IFN-λ production. In the cell types analyzed thus far, it seems that regulation of type III IFN production is comparable to that of type I IFN production, since the promotor regions of IFNL1, IFNL2 and IFNL3 contain binding elements for transcription factors IRF3, IRF7 and NFκB, although type III IFN seems more dependent on NFκB than type I IFN [28-36].

The molecular mechanisms regulating IFN-λ production in BDCA3^{hi} DCs, the most potent IFN-λ-producing cells, however, remain to be elucidated. Here, we set out to directly compare

IFN-λ production by naturally occurring human DC subsets, the relation between BDCA3 expression and IFN-λ production, and the signaling pathways involved in the regulation of IFN-λ production in human BDCA3^{hi} DCs.
We confirm that although IFN-λ1 is produced by all DC subsets in response to TLR signaling, BDCA3^{hi}CLEC9A^{+} DCs excel by far. IFN-λ correlated with BDCA3 expression on mDCs, but not pDCs. In BDCA3^{hi}CLEC9A^{+} DCs, both the PI3K-PKB-mTOR and the canonical NFκB pathway are involved in the regulation of IFN-λ production, but their contribution is different for the IFN-λ subtypes. These findings provide insight into the molecular mechanisms underlying regulation of IFN-λ production in naturally occurring DC subsets and may thus contribute to the development of therapies aimed at the manipulation of IFN-λ secretion by these cells.

Materials and Methods

Cell isolation
Peripheral blood mononuclear cells (PBMC) were isolated from heparinized peripheral blood samples or from buffy coats from healthy blood donors using Ficoll density gradient centrifugation. All healthy controls gave written informed consent before blood donation. Cells were enriched for DCs using Dynabeads (Thermo Fisher), and DC subsets were subsequently isolated based on BDCA1, BDCA3 and BDCA4 expression by FACSorting using a FACSAria (BD Biosciences).

Flow cytometric analysis
For phenotypic analysis, cells were washed with PBS containing 1% bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO), 1% heat inactivated human serum and 0.02% NaN_3 (Merck, Darmstadt, Germany; referred to as FACS buffer) and subsequently labeled with fluorochrome-conjugated antibodies (Abs). Labeling was performed during 30 minutes of incubation on ice with Abs recognizing BDCA3/CD141 (AD5-14H12, Miltenyi Biotec, Bergish Gladbach, Germany); BDCA1/CD1c (AD5-8E7, Miltenyi and L161, BioLegend); BDCA4/CD304 (12C2, BioLegend), CD3 (UCHT1, eBioscience, San Diego, CA), CD11c (3.9, eBioscience); CD14 (61D3, eBioscience); CD19 (HIB19, eBioscience), CD56 (MY31, BD Biosciences, Breda, the Netherlands), CD123 (AC145, Miltenyi), CLEC9A (8F9, BioLegend, San Diego, CA). Fluorescence was measured using a FACS Canto II (BD Biosciences).

Cytokine secretion
Cells were resuspended in RPMI 1640 (Invitrogen, Breda, The Netherlands) supplemented with 9% heat-inactivated FCS (Sigma-Aldrich, St. Louis, MO) and penicillin/streptomycin (Invitrogen). Total PBMC were stimulated with polyI:C (20 μg/ml, Invivogen, Toulouse, France), CpG-2336 (class A, 10 μg/ml, Invivogen) or Loxoribin (0.4 mM, Invivogen) for 7 hours at 37°C in the presence of Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF, 10 ng/ml, PeproTech, London, U.K.). PI3K inhibitor LY294002 (10 μM, Biomol), PKB inhibitor VIII (3 μM, Calbiochem, San Diego, CA), mTOR inhibitor rapamycin (0.5 μM, Biomol, Hamburg, Germany), and canonical NFκB signaling pathway inhibitors CAPE (10 μg/ml, Sigma-Aldrich), BAY 11-7082 (1 μM, Calbiochem) and AS602868 (AS, 1 μM, a kind gift from Dr. I. Adcock, Imperial College London, U.K.) were added where indicated. During the last 3 hours, the cells were incubated with 10 mg/ml Brefeldin A (Sigma-Aldrich). Cells were stained with DC-subset specific antibodies as described above, fixed with 2% formaldehyde,

permeabilized with 0.5% saponin and stained with antibodies against TNFα (eBioscience) and IFN-λ (kindly provided by BMS, Uxbridge, U.K.). The frequencies of cytokine-producing cells were determined by flow cytometry (FACS Canto II, BD Biosciences).
Isolated DCs were stimulated for 24 hours with the TLR ligands indicated above with or without signaling pathway inhibitors. The levels of secreted human IFN-λ1 (IL-29, eBioscience) and IFN-λ2 (IL-28A) (R&D systems) were measured in the supernatant using commercially available ELISA kits according to the manufacturer's protocol. Detection limits were 8 pg/ml (IFNλ1) and 30 pg/ml (IFN-λ2).

Statistical analysis
Statistical analysis was performed using Graphpad Prism version 5.01 for Windows (GraphPad Software, San Diego, California USA).

Results

IFN-λ1 is mainly produced by BDCA3-expressing myeloid DCs
To assess whether BDCA3 expression on immune cells derived from peripheral blood in general is associated with IFN-λ production, we first determined which immune cells express BDCA3. NK cells, T cells and B cells did not express any BDCA3 (Figure 1A, B). In contrast, detectable BDCA3 expression levels were found on CD14^{+} monocytes (61±5%), BDCA1^{+} DCs (73±3%) and BDCA2^{+} pDCs (77±2%) , although the expression level on these cells was lower than that on BDCA3^{hi} DCs (Figure 1A, B, Supplementary figure 1). These data show that of the immune cells tested, BDCA3 is only expressed by monocytes and DCs. Next, we investigated the presence of correlation between IFN-λ production and BDCA3 expression. In line with a lack of BDCA3 expression, NK cells, T cells and B cells did not produce any IFN-λ1 upon activation of these cells by commonly used cell-specific activation stimuli (Figure 2A). Although monocytes did express BDCA3, IFN-λ1 production could not be detected for these cells. IFN-λ1 was produced by polyI:C-stimulated BDCA3^{hi} DCs, polyI:C-stimulated BDCA1^{+} DCs and CpG-A-stimulated pDCs. In line with previous studies, BDCA3^{hi} DCs were superior

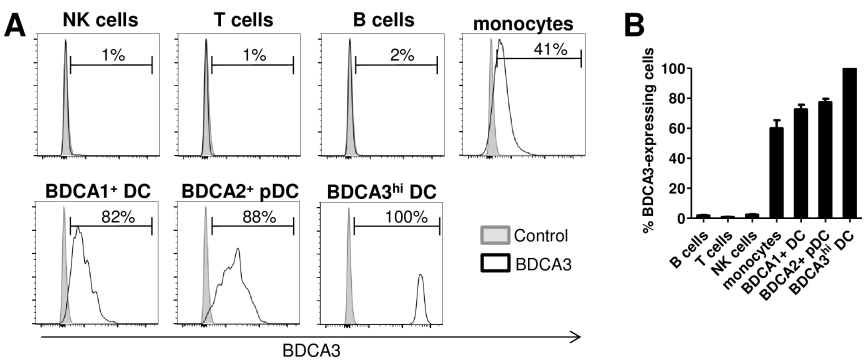

Figure 1. BDCA3 co-expression and IFN-λ1 production by immune cell subsets.
(A-B) PBMC were isolated from healthy controls and BDCA3 expression was analyzed on B cells, T cells, NK cells, monocytes, BDCA1^{+} DCs, BDCA2^{+} pDCs and BDCA3^{hi} DCs. Representative flow cytometry plots (A) and mean±SEM percentage of BDCA3-expressing cells (B) of 10 independent experiments with 15 different donors are shown.

in IFN-λ1 production compared to the other DC subsets (Figure 2B, C, D). This difference in IFN-λ1-producing capacity was not due to a differential response to TLR stimulation, as indicated by comparable TNF-α production by all three DC subsets (Supplementary figure 2). To distinguish between BDCA3^{+} and BDCA3^{-} DCs of the BDCA1^{+} DC and pDC population, cells were sorted as shown in supplementary figure 3. Within the BDCA1^{+} DC population, but not within the pDC population, BDCA3-expressing DCs produced significantly more IFN-λ1 than BDCA3^{-} DCs (Figure 2D). Together these results indicate that IFN-λ production by peripheral blood immune cells is restricted to DCs, preferably BDCA3^{hi} DCs, and that BDCA3 expression on these different immune cells is not consistently predictive for IFN-λ-producing capacity.

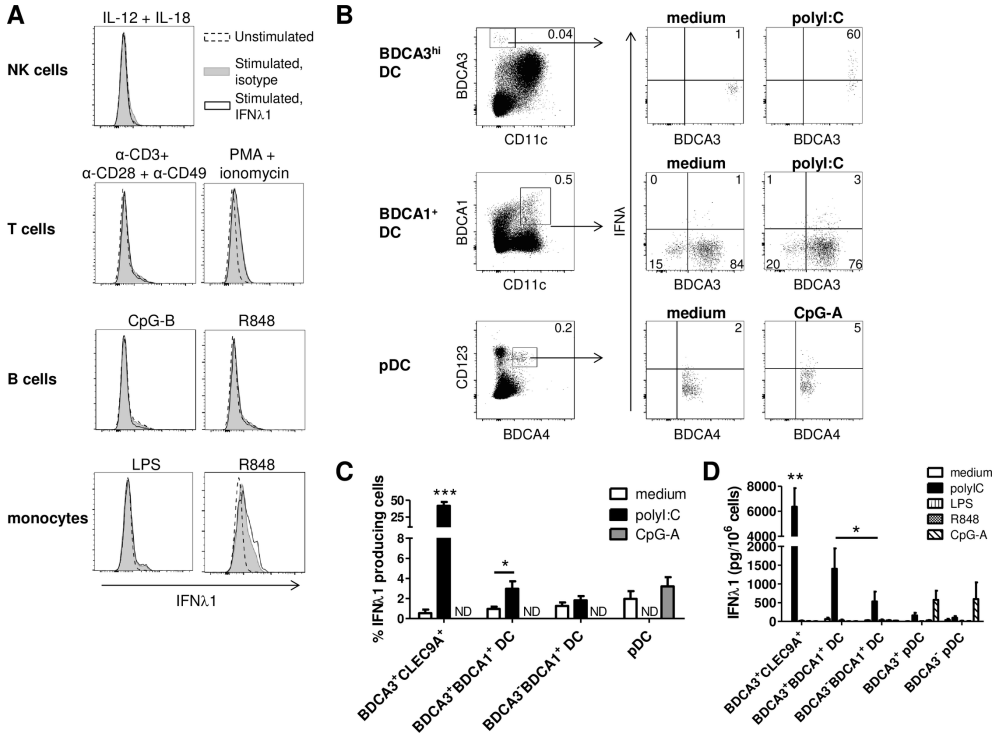

Figure 2. IFN-λ1 is mainly produced by BDCA3-expressing myeloid DCs.
(A-C) PBMC were stimulated for 7 hours with or without IL-12 + IL-18, anti-CD3 + anti-CD28 + anti-CD49, PMA + ionomycin, CpG-B, R848, LPS, polyI:C, or CpG-A. Production of IFN-λ1 by the indicated immune cells was measured by ICS. (A) Representative flow cytometry plots of Aqua^{-} viable NK cells, T cells, B cells and monocytes (n=3). (B) Representative flow cytometry plots of Aqua^{-} viable BDCA3^{hi} DCs, BDCA1^{+} DCs and pDCs (n=7). (C) Mean±SEM percentage IFN-λ1-producing cells (n=7) *p < 0.05, ***p<0.001, paired Student's t-test. Note: the percentage of IFN-λ-producing BDCA3^{hi} DCs upon polyI:C stimulation was significantly higher (***p<0.001) compared to all other conditions. (D) DC subsets were FACSorted and stimulated for 24 hours with or without polyI:C, LPS, R848 or CpG-A. IFN-λ1 concentrations were determined by ELISA. Data are shown as mean±SEM production of IFN-λ1 (n=2-8) and are pooled from 8 independent experiments, each performed with a different donor. *p < 0.05, **p<0.01, paired Student's t-test.

IFN-λ1 production by BDCA3^{hi} DCs is regulated via the NFκB pathway

Although BDCA3^{hi} DCs are superior at production of IFN-λ, the pathways regulating IFN-λ production in these cells have not been clarified. We first investigated the role of the canonical NFκB signaling pathway in the production of the two major isoforms of IFN-λ by BDCA3^{hi} DCs by intracellular cytokine staining (IFN-λ1) and ELISA (IFN-λ1 and IFN-λ2) [31, 32]. Stimulation of PBMC in the presence of AS (IKK-β inhibitor) , BAY (inhibitor of IκBα phosphorylation) and CAPE (inhibitor of translocation of NFκB to the nucleus) showed that these factors of the NFκB pathway are required for IFN-λ1 production by BDCA3^{hi} DCs, as the percentage of IFN-λ1^{+} BDCA3^{hi} DCs was significantly reduced (Figure 3A). Although NFκB is known to be involved in cell survival, neither absolute numbers of BDCA3^{hi} DCs, nor the percentage of early apoptotic or dead cells seemed to be affected by the NFκB inhibitors (Supplementary figure 4A, B). The inhibitors also diminished the secretion of IFN-λ1 into the culture supernatant by isolated BDCA3^{hi} DCs (Figure 3B), and production of the second isoform, IFN-λ2, was even more diminished (Figure 3C). Due to high variation in IFN-λ production between experiments, however, the reduction was significant only for BAY on IFN-λ1 and AS on IFN-λ2. Together, these data demonstrate that canonical NFκB signaling regulates both IFN-λ1 and IFN-λ2 production by BDCA3^{hi} DCs.

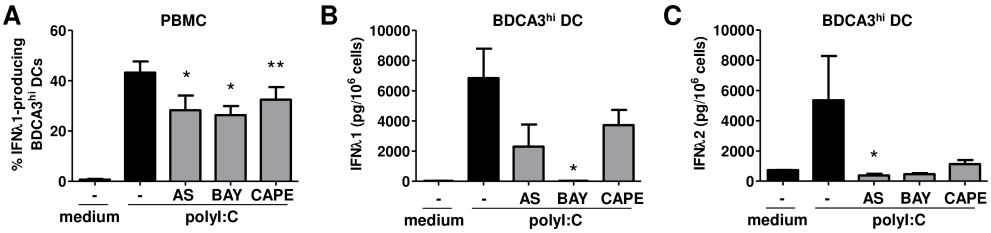

Figure 3. The NFκB pathway is involved in regulation of IFN-λ1 and IFN-λ2 production by BDCA3^{hi} DCs.
(A) PBMC were isolated and stimulated for 7 hours with or without polyI:C in the presence or absence of the NFκB pathway inhibitors AS, BAY and CAPE. Production of IFN-λ1 was measured using ICS. Mean±SEM percentage IFN-λ1-producing BDCA3^{hi} DCs (n=10) are shown. Data are pooled from 8 independent experiments performed with 10 different donors. *p < 0.05, **p<0.01, paired Student's t-test. (B-C) FACSorted BDCA3^{hi} DCs were stimulated for 24 hours with or without polyI:C in the presence or absence of the NFκB pathway inhibitors CAPE, BAY and AS. IFN-λ1 and IFN-λ2 concentrations were determined by ELISA. Data are shown as mean±SEM production of IFN-λ1 (n=4-5) and IFN-λ2 (n=3-4) and are pooled from 5 independent experiments, each performed with a different donor. *p < 0.05, paired Student's t-test.

IFN-λ1 production by BDCA3^{hi} DCs is regulated via the PI3K-PKB-mTOR pathway

NFκB inhibitors could only partially reduce IFN-λ1 production, suggesting also other signaling pathways may be involved. The PI3K-PKB-mTOR signaling pathway is a likely candidate, as it is known to regulate IFN-α production by pDCs and IFN-λ production by *in vitro*-generated monocyte-derived DCs [37, 38]. So far, the involvement of this pathway in the regulation of IFN-λ production has not been addressed in primary DCs. Stimulation of PBMCs in the presence of the PI3K inhibitor LY, PKB inhibitor VIII and mTOR inhibitor rapamycin significantly reduced the percentage of IFN-λ1-producing BDCA3^{hi} cells (Figure 4A). Like NFκB, PI3K-PKB-mTOR signaling is known to be involved in cell survival. VIII and rapamycin did not seem to affect cell death, however, LY induced apoptosis in polyI:C-stimulated cells, resulting in

a complete lack of BDCA3^{hi} DCs in some of the experiments. Therefore, the reduction in IFN-λ production by LY is likely to be due to a decline in cell viability (Supplementary figure 4C, D). Addition of these signaling inhibitors to cultures with purified BDCA3^{hi} DCs almost completely abrogated the production of IFN-λ1, showing a crucial role for PI3K-PKB-mTOR signaling in the production of this cytokine (Figure 4B). Intriguingly, PI3K-PKB-mTOR pathway inhibition much less reduced IFN-λ2 secretion (Figure 4C). These results show that also the PI3K-PKB-mTOR pathway is involved in regulation of IFN-λ1 production in BDCA3^{hi} DCs, but plays only a minor role in regulation of IFN-λ2 production.

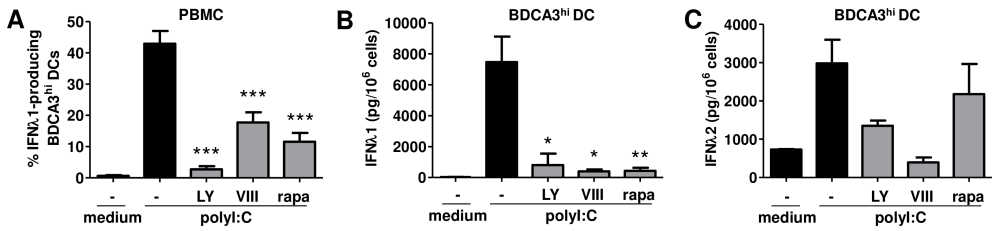

Figure 4. The PI3K-PKB-mTOR pathway is involved in regulation of IFN-λ1 production by BDCA3^{hi} DCs.
(A) PBMC were isolated and stimulated for 7 hours with or without polyI:C in the presence or absence of the PI3K-PKB-mTOR pathway inhibitors LY, inhibitor VIII (VIII) and rapamycin (rapa). Production of IFN-λ1 was measured using ICS. Mean±SEM percentage IFN-λ1-producing BDCA3^{hi} DCs (n=12) are shown. Data are pooled from 10 independent experiments performed with 12 different donors. ***p<0.001, paired Student's t-test. (B-C) FACSorted BDCA3^{hi} DCs were stimulated for 24 hours with or without polyI:C in the presence or absence of the PI3K-PKB-mTOR pathway inhibitors LY, inhibitor VIII and rapamycin. IFN-λ1 and IFN-λ2 concentrations were determined by ELISA. Data are shown as mean±SEM production of IFN-λ1 (n=5) and IFN-λ2 (n=3) and are pooled from 5 independent experiments, each performed with a different donor.

Discussion

BDCA3^{hi} DCs are characterized by high BDCA3 expression and IFN-λ production. Thus far, the pathways regulating IFN-λ production in BDCA3^{hi} DCs as well as the relation between BDCA3 expression and IFN-λ production were unknown. Here, we have shown that although BDCA3 was moderately expressed by BDCA1^{+} DCs, pDCs and monocytes, its expression level was only associated with a higher ability to produce IFN-λ in BDCA1^{+} DCs. Thus, BDCA3 expression on immune cells in itself is not predictive for IFN-λ-producing capacity. Moreover, we have demonstrated that regulation of polyI:C-induced IFN-λ1 production by BDCA3^{hi} DCs involved both the canonical NFκB pathway and PI3K-PKB-mTOR pathway, while IFN-λ2 production involved mostly the NFκB pathway and seemed to depend less on the PI3K-PKB-mTOR pathway (Figure 5).
Apart from the typical BDCA3^{hi} DC subset, we confirm the expression of BDCA3 on other hematopoietic cells, including monocytes, pDCs and BDCA1^{+} DCs. IFN-λ production could be detected for the different DC subsets, but in contrast to previous studies, not for LPS-stimulated monocytes [39]. This discrepancy may be due to the use of mRNA expression instead of protein secretion as read-out for IFN-λ production, which demonstrates the importance of IFN-λ detection at protein level as it reflects actual IFN-λ production.

The enhanced IFN-λ-producing capacity of BDCA3^{+}BDCA1^{+} DCs compared to BDCA3^{-}BDCA1^{+} DCs is in line with our previous work that showed a link between BDCA3 expression and IFN-λ production on CD34^{+} HPC-derived myeloid DCs [19]. This increased IFN-λ production may be due to a more activated status of BDCA3^{+}BDCA1^{+} DCs, as suggested in chapter 5. Nevertheless, the exact function of BDCA3 expression on immune cells remains to be determined.

In contrast to regulation of type I IFN production, regulation of type III IFN production remains poorly understood. Here, we provide for the first time insight into IFN-λ-regulating pathways in the most potent IFN-λ-producing cells, primary BDCA3^{hi} DCs. To establish IFN-λ-manipulating strategies for therapeutic purposes, it is crucial to understand why these cells excel in IFN-λ production and how viruses may interfere with their IFN-λ production. In the present study, inhibitors were used to demonstrate the involvement of NFκB and PI3K-PKB-mTOR pathways in the production of IFN-λ. More solid proof on the involvement of these pathways would come from siRNA-mediated knockdown, but this is unfortunately not a feasible strategy for the very rare BDCA3^{hi} DC subset. Nonetheless, as multiple inhibitors acting on the same pathway were used, and as these all affected IFN-λ production, it is highly likely that these pathways investigated play an important role.

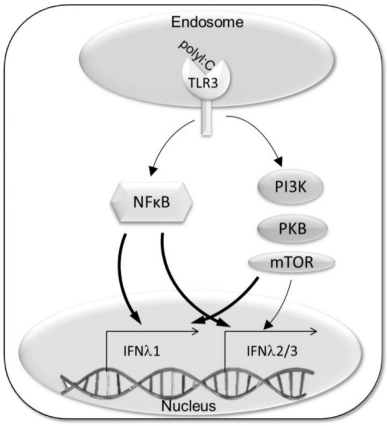

Figure 5. Regulation of IFN-λ production in BDCA3^{hi} DCs. Schematic representation of the involvement of the NFκB pathway and PI3K-PKB-mTOR pathway in polyI:C-induced IFN-λ1 and IFN-λ2 production.

Both the NFκB and PI3K-PKB-mTOR pathway are known to be involved in regulation of DC development and survival. However, two of our previous studies showed that *in vitro*-generated CD34^{+} HPC-derived DCs were rescued from NFκB inhibitor-induced apoptosis by simultaneous presence of activation signals, and that survival of fully differentiated CD34^{+}-derived DCs was unaffected by inhibition of PI3K-PKB-mTOR signaling [40, 41]. In line with this, no loss of BDCA3^{hi} DCs or induction of apoptosis by NFκB and PI3K-PKB-mTOR pathway inhibitors, except LY, was observed, indicating again that the effect on IFN-λ production was specific and not due to cell death.

In addition to IFN-λ production by BDCA3^{hi} DCs, IFN-λ production by pDCs was also regulated by NFκB and PI3K signaling, as all inhibitors except rapamycin significantly reduced the percentage of IFN-λ-producing pDCs (data not shown). What makes BDCA3^{hi} DCs such excellent IFN-λ-producing cells remains elusive. The activation of NFκB and the PI3K-PKB-mTOR signaling pathway is not unique for BDCA3^{hi} DCs. Other factors that specifically stimulate IFN-λ production may contribute to different IFN-λ-producing capacities, i.e. IRF3

and IRF7, and Med23, which enhances IFN-λ1 transcription via interaction with IRF7. Also BLIMP1 and ZEB1 regulate IFN-λ production by repressing IFN-λ1 transcription via binding of the IFN-λ1 promotor [29-32, 34, 42]. Unfortunately, due to experimental limitations, especially the low amount of available BDCA3^{hi} DCs and the lack of specific inhibitors for these molecules, we were not yet able to determine their role.
Differences in expression levels of pattern recognition receptors (PRR) as well as the combined downstream signaling in BDCA3^{hi} DCs compared to other DC subsets may also contribute to differential IFN-λ production. As polyI:C is not only a ligand for TLR3, but also for the cytoplasmic receptors RIG-I and MDA-5, and as RIG-I signaling has been shown to induce IFN-λ production in other cell types, these pathways may also be involved in IFN-λ production [43]. Furthermore, the accessibility and/or availability of pivotal signaling molecules and/or transcription factors may be unique for BDCA3^{hi} DCs, which requires further investigation.

So far, the function and regulation of type I and type III IFNs appear very comparable, except for the cells they target. It is, however, striking that IFNλ production is clearly dependent on canonical NFκB signaling, as demonstrated here and also by others, while IFN-α production seems to depend much less on NFκB [29]. More insight into the pathways regulating type III IFNs will help to understand how type I and type III IFN, as well as subtypes of IFN-λ differ in the location and timing of their secretion, and thus how they each contribute to the final immune response. We showed here that inhibition of the PI3K-PKB-mTOR pathway affected IFN-λ1 more than IFN-λ2 production, whereas inhibition of the NFκB pathway had a more pronounced effect on IFN-λ2 production. Although highly speculative, the subtle difference in regulation of the two subtypes may reflect a different mode of action. Balancing the relative subtype levels may further fine-tune the IFN-λ immune response.

Taken together, we show that among immune cells, IFN-λ production is associated with DCs, in particular with BDCA3^{hi} DCs, and is regulated via the PI3K-PKB-mTOR pathway and NFκB pathway. These data contribute to better understanding of BDCA3^{hi} DC function and the signaling pathways underlying IFN-λ regulation. Insight into the molecular mechanisms that regulate IFN-λ production in these major IFN-λ-producing cells may help to better understand their role in diseases associated with disturbed IFN-λ production and may aid the development of IFN-λ-manipulating therapies.

Acknowledgements

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References

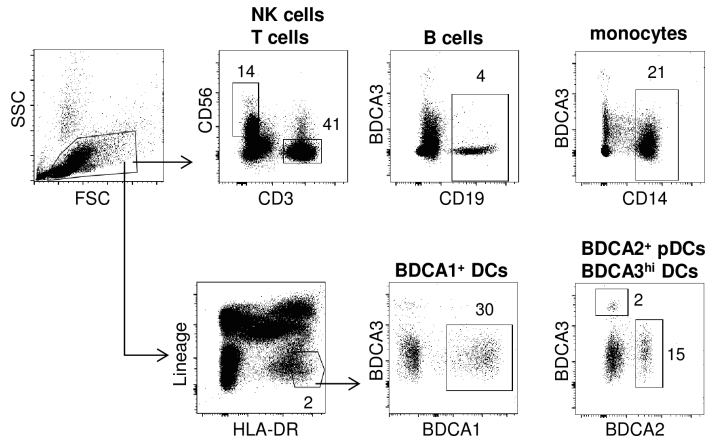
1. McNab, F., Mayer-Barber, K., Sher, A., Wack, A., O'Garra, A. (2015) Type I interferons in infectious disease. Nat Rev Immunol 15, 87-103.
2. Egli, A., Santer, D. M., O'Shea, D., Tyrrell, D. L., Houghton, M. (2014) The impact of the interferon-lambda family on the innate and adaptive immune response to viral infections. Emerg Microbes Infect 3, e51.
3. Platanias, L. C. (2005) Mechanisms of type-I- and type-II-interferon-mediated signalling. Nat Rev Immunol 5, 375-86.
4. Kotenko, S. V., Gallagher, G., Baurin, V. V., Lewis-Antes, A., Shen, M., Shah, N. K., Langer, J. A., Sheikh, F., Dickensheets, H., Donnelly, R. P. (2003) IFN-lambdas mediate antiviral protection through a distinct class II cytokine receptor complex. Nat Immunol 4, 69-77.
5. Sheppard, P., Kindsvogel, W., Xu, W., Henderson, K., Schlutsmeyer, S., Whitmore, T. E., Kuestner, R., Garrigues, U., Birks, C., Roraback, J., Ostrander, C., Dong, D., Shin, J., Presnell, S., Fox, B., Haldeman, B., Cooper, E., Taft, D., Gilbert, T., Grant, F. J., Tackett, M., Krivan, W., McKnight, G., Clegg, C., Foster, D., Klucher, K. M. (2003) IL-28, IL-29 and their class II cytokine receptor IL-28R. Nat Immunol 4, 63-8.
6. Prokunina-Olsson, L., Muchmore, B., Tang, W., Pfeiffer, R. M., Park, H., Dickensheets, H., Hergott, D., Porter-Gill, P., Mumy, A., Kohaar, I., Chen, S., Brand, N., Tarway, M., Liu, L., Sheikh, F., Astemborski, J., Bonkovsky, H. L., Edlin, B. R., Howell, C. D., Morgan, T. R., Thomas, D. L., Rehermann, B., Donnelly, R. P., O'Brien, T. R. (2013) A variant upstream of IFNL3 (IL28B) creating a new interferon gene IFNL4 is associated with impaired clearance of hepatitis C virus. Nat Genet 45, 164-71.
7. Bolen, C. R., Ding, S., Robek, M. D., Kleinstein, S. H. (2014) Dynamic expression profiling of type I and type III interferon-stimulated hepatocytes reveals a stable hierarchy of gene expression. Hepatology 59, 1262-72.
8. Dellgren, C., Gad, H. H., Hamming, O. J., Melchjorsen, J., Hartmann, R. (2009) Human interferon-lambda3 is a potent member of the type III interferon family. Genes Immun 10, 125-31.
9. Liu, B. S., Janssen, H. L., Boonstra, A. (2011) IL-29 and IFNalpha differ in their ability to modulate IL-12 production by TLR-activated human macrophages and exhibit differential regulation of the IFNgamma receptor expression. Blood 117, 2385-95.
10. Lazear, H. M., Nice, T. J., Diamond, M. S. (2015) Interferon-lambda: Immune Functions at Barrier Surfaces and Beyond. Immunity 43, 15-28.
11. Khaitov, M. R., Laza-Stanca, V., Edwards, M. R., Walton, R. P., Rohde, G., Contoli, M., Papi, A., Stanciu, L. A., Kotenko, S. V., Johnston, S. L. (2009) Respiratory virus induction of alpha-, beta- and lambda-interferons in bronchial epithelial cells and peripheral blood mononuclear cells. Allergy 64, 375-86.
12. Lauterbach, H., Bathke, B., Gilles, S., Traidl-Hoffmann, C., Luber, C. A., Fejer, G., Freudenberg, M. A., Davey, G. M., Vremec, D., Kallies, A., Wu, L., Shortman, K., Chaplin, P., Suter, M., O'Keeffe, M., Hochrein, H. (2010) Mouse CD8alpha+ DCs and human BDCA3+ DCs are major producers of IFN-lambda in response to poly IC. J Exp Med 207, 2703-17.
13. Vu Manh, T. P., Bertho, N., Hosmalin, A., Schwartz-Cornil, I., Dalod, M. (2015) Investigating Evolutionary Conservation of Dendritic Cell Subset Identity and Functions. Front Immunol 6, 260.
14. van der Aa, E., van Montfoort, N., Woltman, A. M. (2014) BDCA3CLEC9A human dendritic cell function and development. Semin Cell Dev Biol.
15. Yoshio, S., Kanto, T., Kuroda, S., Matsubara, T., Higashitani, K., Kakita, N., Ishida, H., Hiramatsu, N., Nagano, H., Sugiyama, M., Murata, K., Fukuhara, T., Matsuura, Y., Hayashi, N., Mizokami, M., Takehara, T. (2013) Human blood dendritic cell antigen 3 (BDCA3)(+) dendritic cells are a potent producer of interferon-lambda in response to hepatitis C virus. Hepatology 57, 1705-15.

16. Zhang, S., Kodys, K., Li, K., Szabo, G. (2013) Human type 2 myeloid dendritic cells produce interferon-lambda and amplify interferon-alpha in response to hepatitis C virus infection. Gastroenterology 144, 414-425 e7.
17. Nizzoli, G., Krietsch, J., Weick, A., Steinfelder, S., Facciotti, F., Gruarin, P., Bianco, A., Steckel, B., Moro, M., Crosti, M., Romagnani, C., Stolzel, K., Torretta, S., Pignataro, L., Scheibenbogen, C., Neddermann, P., De Francesco, R., Abrignani, S., Geginat, J. (2013) Human CD1c+ dendritic cells secrete high levels of IL-12 and potently prime cytotoxic T-cell responses. Blood 122, 932-42.
18. Coccia, E. M., Severa, M., Giacomini, E., Monneron, D., Remoli, M. E., Julkunen, I., Cella, M., Lande, R., Uze, G. (2004) Viral infection and Toll-like receptor agonists induce a differential expression of type I and lambda interferons in human plasmacytoid and monocyte-derived dendritic cells. Eur J Immunol 34, 796-805.
19. van der Aa, E., van de Laar, L., Janssen, H. L., van Montfoort, N., Woltman, A. M. (2015) BDCA3 expression is associated with high IFN-lambda production by CD34(+)-derived dendritic cells generated in the presence of GM-CSF, IL-4, and/or TGF-beta. Eur J Immunol 45, 1471-81.
20. Jongbloed, S. L., Kassianos, A. J., McDonald, K. J., Clark, G. J., Ju, X., Angel, C. E., Chen, C. J., Dunbar, P. R., Wadley, R. B., Jeet, V., Vulink, A. J., Hart, D. N., Radford, K. J. (2010) Human CD141+ (BDCA-3)+ dendritic cells (DCs) represent a unique myeloid DC subset that cross-presents necrotic cell antigens. J Exp Med 207, 1247-60.
21. Poulin, L. F., Salio, M., Griessinger, E., Anjos-Afonso, F., Craciun, L., Chen, J. L., Keller, A. M., Joffre, O., Zelenay, S., Nye, E., Le Moine, A., Faure, F., Donckier, V., Sancho, D., Cerundolo, V., Bonnet, D., Reis e Sousa, C. (2010) Characterization of human DNGR-1+ BDCA3+ leukocytes as putative equivalents of mouse CD8alpha+ dendritic cells. J Exp Med 207, 1261-71.
22. Dzionek, A., Inagaki, Y., Okawa, K., Nagafune, J., Rock, J., Sohma, Y., Winkels, G., Zysk, M., Yamaguchi, Y., Schmitz, J. (2002) Plasmacytoid dendritic cells: from specific surface markers to specific cellular functions. Hum Immunol 63, 1133-48.
23. Chu, C. C., Ali, N., Karagiannis, P., Di Meglio, P., Skowera, A., Napolitano, L., Barinaga, G., Grys, K., Sharif-Paghaleh, E., Karagiannis, S. N., Peakman, M., Lombardi, G., Nestle, F. O. (2012) Resident CD141 (BDCA3)+ dendritic cells in human skin produce IL-10 and induce regulatory T cells that suppress skin inflammation. J Exp Med 209, 935-45.
24. Haniffa, M., Shin, A., Bigley, V., McGovern, N., Teo, P., See, P., Wasan, P. S., Wang, X. N., Malinarich, F., Malleret, B., Larbi, A., Tan, P., Zhao, H., Poidinger, M., Pagan, S., Cookson, S., Dickinson, R., Dimmick, I., Jarrett, R. F., Renia, L., Tam, J., Song, C., Connolly, J., Chan, J. K., Gehring, A., Bertoletti, A., Collin, M., Ginhoux, F. (2012) Human tissues contain CD141hi cross-presenting dendritic cells with functional homology to mouse CD103+ nonlymphoid dendritic cells. Immunity 37, 60-73.
25. Akira, S. and Takeda, K. (2004) Toll-like receptor signalling. Nat Rev Immunol 4, 499-511.
26. Sarkar, S. N., Peters, K. L., Elco, C. P., Sakamoto, S., Pal, S., Sen, G. C. (2004) Novel roles of TLR3 tyrosine phosphorylation and PI3 kinase in double-stranded RNA signaling. Nat Struct Mol Biol 11, 1060-7.
27. Zhao, J., Benakanakere, M. R., Hosur, K. B., Galicia, J. C., Martin, M., Kinane, D. F. (2010) Mammalian target of rapamycin (mTOR) regulates TLR3 induced cytokines in human oral keratinocytes. Mol Immunol 48, 294-304.
28. Lee, H. C., Narayanan, S., Park, S. J., Seong, S. Y., Hahn, Y. S. (2014) Transcriptional regulation of IFN-lambda genes in hepatitis C virus-infected hepatocytes via IRF-3.IRF-7.NF-kappaB complex. J Biol Chem 289, 5310-9.
29. Osterlund, P. I., Pietila, T. E., Veckman, V., Kotenko, S. V., Julkunen, I. (2007) IFN regulatory factor family members differentially regulate the expression of type III IFN (IFN-lambda) genes. J Immunol 179, 3434-42.
30. Siegel, R., Eskdale, J., Gallagher, G. (2011) Regulation of IFN-lambda1 promoter activity (IFN-lambda1/IL-29) in human airway epithelial cells. J Immunol 187, 5636-44.
31. Swider, A., Siegel, R., Eskdale, J., Gallagher, G. (2014) Regulation of interferon lambda-1

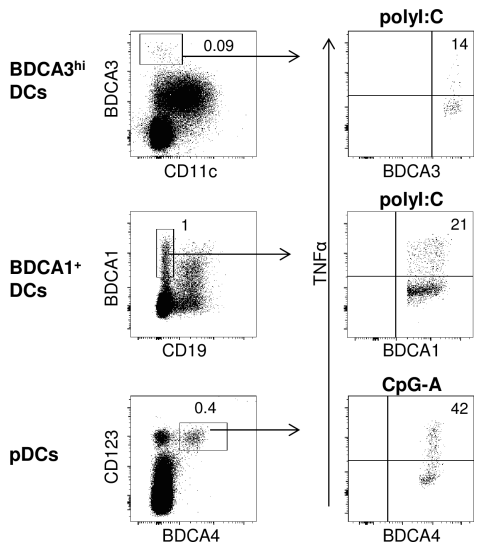
(IFNL1/IFN-lambda1/IL-29) expression in human colon epithelial cells. Cytokine 65, 17-23.

32. Thomson, S. J., Goh, F. G., Banks, H., Krausgruber, T., Kotenko, S. V., Foxwell, B. M., Udalova, I. A. (2009) The role of transposable elements in the regulation of IFN-lambda1 gene expression. Proc Natl Acad Sci U S A 106, 11564-9.
33. Osterlund, P., Veckman, V., Siren, J., Klucher, K. M., Hiscott, J., Matikainen, S., Julkunen, I. (2005) Gene expression and antiviral activity of alpha/beta interferons and interleukin-29 in virus-infected human myeloid dendritic cells. J Virol 79, 9608-17.
34. Onoguchi, K., Yoneyama, M., Takemura, A., Akira, S., Taniguchi, T., Namiki, H., Fujita, T. (2007) Viral infections activate types I and III interferon genes through a common mechanism. J Biol Chem 282, 7576-81.
35. Iversen, M. B., Ank, N., Melchjorsen, J., Paludan, S. R. (2010) Expression of type III interferon (IFN) in the vaginal mucosa is mediated primarily by dendritic cells and displays stronger dependence on NF-kappaB than type I IFNs. J Virol 84, 4579-86.
36. Kawai, T. and Akira, S. (2010) The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat Immunol 11, 373-84.
37. Cao, W., Manicassamy, S., Tang, H., Kasturi, S. P., Pirani, A., Murthy, N., Pulendran, B. (2008) Toll-like receptor-mediated induction of type I interferon in plasmacytoid dendritic cells requires the rapamycin-sensitive PI(3)K-mTOR-p70S6K pathway. Nat Immunol 9, 1157-64.
38. Guiducci, C., Ghirelli, C., Marloie-Provost, M. A., Matray, T., Coffman, R. L., Liu, Y. J., Barrat, F. J., Soumelis, V. (2008) PI3K is critical for the nuclear translocation of IRF-7 and type I IFN production by human plasmacytoid predendritic cells in response to TLR activation. J Exp Med 205, 315-22.
39. Wolk, K., Witte, K., Witte, E., Proesch, S., Schulze-Tanzil, G., Nasilowska, K., Thilo, J., Asadullah, K., Sterry, W., Volk, H. D., Sabat, R. (2008) Maturing dendritic cells are an important source of IL-29 and IL-20 that may cooperatively increase the innate immunity of keratinocytes. J Leukoc Biol 83, 1181-93.
40. van de Laar, L., van den Bosch, A., van der Kooij, S. W., Janssen, H. L., Coffer, P. J., van Kooten, C., Woltman, A. M. (2010) A nonredundant role for canonical NF-kappaB in human myeloid dendritic cell development and function. J Immunol 185, 7252-61.
41. van de Laar, L., Buitenhuis, M., Wensveen, F. M., Janssen, H. L., Coffer, P. J., Woltman, A. M. (2010) Human CD34-derived myeloid dendritic cell development requires intact phosphatidylinositol 3-kinase-protein kinase B-mammalian target of rapamycin signaling. J Immunol 184, 6600-11.
42. Griffiths, S. J., Koegl, M., Boutell, C., Zenner, H. L., Crump, C. M., Pica, F., Gonzalez, O., Friedel, C. C., Barry, G., Martin, K., Craigon, M. H., Chen, R., Kaza, L. N., Fossum, E., Fazakerley, J. K., Efstathiou, S., Volpi, A., Zimmer, R., Ghazal, P., Haas, J. (2013) A systematic analysis of host factors reveals a Med23-interferon-lambda regulatory axis against herpes simplex virus type 1 replication. PLoS Pathog 9, e1003514.
43. Sato, S., Li, K., Kameyama, T., Hayashi, T., Ishida, Y., Murakami, S., Watanabe, T., Iijima, S., Sakurai, Y., Watashi, K., Tsutsumi, S., Sato, Y., Akita, H., Wakita, T., Rice, C. M., Harashima, H., Kohara, M., Tanaka, Y., Takaoka, A. (2015) The RNA sensor RIG-I dually functions as an innate sensor and direct antiviral factor for hepatitis B virus. Immunity 42, 123-32.

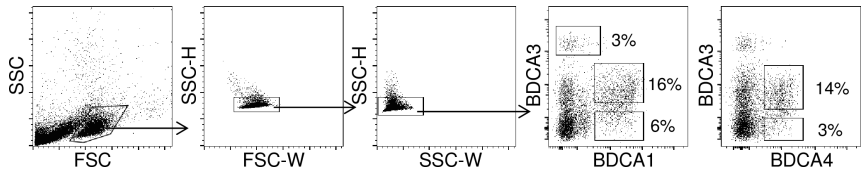
Supplementary information



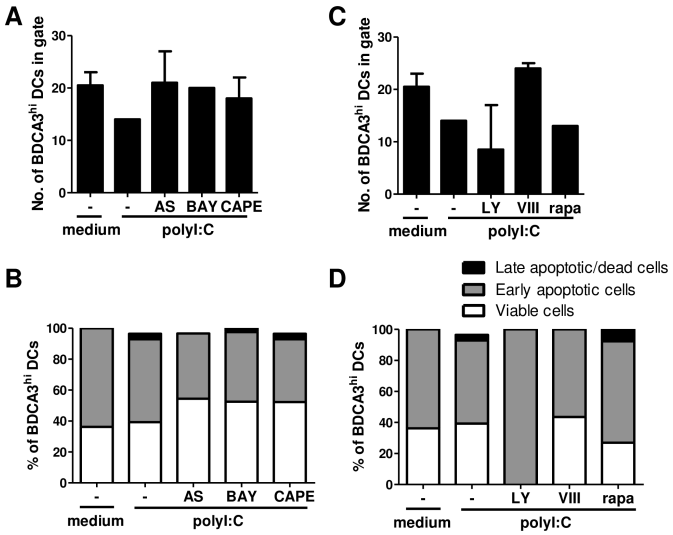
Supplementary figure 1. Gating strategy for analysis of immune cell subsets
PBMC were isolated from healthy controls. FSC/SSC-gated cells were distinguished into $CD56^{+}CD3^{-}$ NK cells, $CD3^{+}CD56^{-}$ T cells, $CD19^{+}$ B cells, $CD14^{+}$ monocytes and $HLA\text{-}DR^{+}Lineage^{-}$ DCs, including $BDCA1^{+}$ DCs, $BDCA3^{hi}$ DCs and $BDCA2^{+}$ pDCs. Representative flow cytometry plots of 10 independent experiments with 15 different donors are shown.



Supplementary figure 2. TNF-α production by DC subsets
PBMC were stimulated for 7 hours with or without polyI:C, or CpG-A. Production of TNF-α by the indicated DC subsets was measured by ICS. Representative flow cytometry plots of $Aqua^{-}$ viable $BDCA3^{hi}$ DCs, $BDCA1^{+}$ DCs and pDCs (n=7).



Supplementary figure 3. Sorting strategy for analysis of BDCA3^{+} and BDCA3^{-} DCs.
PBMC were isolated from healthy controls and enriched for DCs using a Dynabeads Human DC Enrichment kit. Duplicates were excluded based on size, and DC populations were subsequently identified as BDCA3^{hi} cells, BDCA3^{+}BDCA1^{+} cells and BDCA3^{-}BDCA1^{+} cells. Representative FACS plots are shown.



Supplementary figure 4. Effect of inhibitors on cell survival
PBMC were isolated and stimulated for 7 hours with or without polyI:C in the presence or absence of the NFκB pathway inhibitors AS, BAY and CAPE and PI3K-PKB-mTOR inhibitors LY, VIII and mTOR. Mean±SEM percentage of BDCA3^{hi} DCs (A, C; n=2), and the mean percentage of viable cells (Annexin^{-}7AAD^{-}), early apoptotic cells (Annexin^{+}7AAD^{-}) and late apoptotic/dead cells (Annexin^{+}7AAD^{+}) within the BDCA3^{hi} DC population are shown (B, D; n=2). Data are pooled from 1 experiment performed with 2 different donors.

The effect of chronic hepatitis B virus infection on BDCA3^{+} dendritic cell frequency and function

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Chapter 7

Abstract

Chronic hepatitis B virus (HBV) infection results from inadequate HBV-specific immunity. BDCA3$^+$ dendritic cells (DCs) are professional antigen presenting cells considered to be important for antiviral responses because of specific characteristics, including high interferon-λ production. BDCA3$^+$ DCs may thus also have a role in the immune response against HBV, and immunotherapeutic strategies aiming to activate DCs, including BDCA3$^+$ DCs, in patient livers may represent an interesting treatment option for chronic HBV. However, neither the effect of chronic hepatitis B (CHB) infection on the frequency and function of BDCA3$^+$ DCs in liver and blood, nor the effect of the viral surface protein (HBsAg) that is abundantly present in blood of infected individuals are known. Here, we provide an overview of BDCA3$^+$ DC frequency and functional capacity in CHB patients. We find that intrahepatic BDCA3$^+$ DC numbers are increased in CHB patients. BDCA3$^+$ DCs from patient blood are not more mature at steady state, but display an impaired capacity to mature and to produce interferon-λ upon polyI:C stimulation. Furthermore, in vitro experiments exposing blood and intrahepatic BDCA3$^+$ DCs to the viral envelope protein HBsAg demonstrate that HBsAg does not directly induce phenotypical maturation of BDCA3$^+$ DCs, but may reduce IFN-λ production via an indirect unknown mechanism.
These results suggest that BDCA3$^+$ DCs are available in the blood and on site in HBV infected livers, but measures may need to be taken to revive their function for DC-targeted therapy.

Introduction

Hepatitis B virus (HBV) specifically infects hepatocytes and can cause chronic liver infection, often leading to severe liver diseases [1]. Chronic viral infection results from inadequate antiviral immunity, however, the mechanisms underlying ineffective HBV-specific immunity remain poorly understood [2, 3]. Effective viral immunity includes induction of antiviral cytokines such as interferons (IFNs) and virus-specific CD8$^+$ cytotoxic T lymphocytes (CTL). Dendritic cells (DCs) play a crucial role in this process because they can, uniquely, activate virus-specific naïve T cells and produce high type I and type III IFN levels [4, 5]. The DC family comprises several subsets, including plasmacytoid DCs (pDC) and the myeloid DC (mDC) subsets BDCA1$^+$ DCs and BDCA3^{hi}CLEC9A$^+$ DCs [6-8]. These DC subsets differ in ontogeny, localization, phenotype and function.

We and others have previously characterized the frequency and function of BDCA1$^+$ DCs and pDCs in peripheral blood of chronic HBV patients [9]. We demonstrated that although DC frequencies in blood were unaffected, blood BDCA1$^+$ DCs were impaired in their capacity to mature, to produce pro-inflammatory cytokines and to stimulate T cells, and that pDCs were impaired in IFNα-producing capacity [10, 11]. More recently, BDCA3^{hi}CLEC9A$^+$ DCs (further referred to as BDCA3$^+$ DCs) were identified and shown to excel over other DC subsets in cross-presentation of cell-associated antigens (Ag) to CD8$^+$ T cells [12-15]. In mice, the equivalents of BDCA3$^+$ DCs (CD8α$^+$ and CD103$^+$ DCs) have been shown to be crucial for generating optimal virus-specific CD8$^+$ T cell responses to influenza virus and West Nile virus [16, 17]. In addition, BDCA3$^+$ DCs are the most potent producers of IFN-λ in response to viruses that induce TLR3 signaling, or in response to the synthetic RNA polyI:C [18-22]. IFN-λ is an important antiviral cytokine that supports T cell skewing towards Th1 responses and has antiviral activity against multiple viruses, including HBV [23, 24]. Although the effect of IFN-λ on HBV replication in *in vitro* and mouse studies was debatable [25, 26], a recent clinical trial showed that in HBeAg-positive patients, PEG-IFN-λ induced a clear reduction in HBV DNA and viral surface antigen (HBsAg) levels, indicating that this cytokine may be valuable to fight CHB, and we envision that this cytokine could be even more effective when secreted on site [27]. BDCA3$^+$ DCs may thus be a viable target to induce an effective immune response against HBV. BDCA3$^+$ DCs are known to be present in human liver [21, 28, 29], however, it is unknown whether this is altered upon HBV-infection. Furthermore, the actual localization of BDCA3$^+$ DCs within healthy and diseased liver tissue, as well as their functional state in HBV patients, and their response to the abundantly circulating HBsAg remains elusive.

We here assessed the presence of BDCA3$^+$ DCs in liver and blood of HBV-infected patients, as well as the effect of chronic HBV infection and HBsAg on BDCA3$^+$ DC phenotype and function in vitro and ex vivo. We found that although BDCA3$^+$ DCs are present in the liver immune infiltrate of chronic HBV (CHB) patients, their function may be compromised.

Materials and Methods

Patients and controls

Heparinized peripheral blood samples were obtained from CHB patients and control subjects of which the clinical characteristics are listed in Table 1. CHB patients and control subjects used for functional characterization of DCs were matched for age, gender and race. Liver tissue was obtained from HBV-infected livers and non-HBV-infected livers, i.e. donor livers that were used for transplantation, donor livers that were rejected for transplantation, or non-cancerous peri-tumor tissue of donors who had not received chemotherapy in at least three months prior to tissue donation. The clinical characteristics of donors are summarized in Table 2. All patients were negative for antibodies (Abs) against hepatitis C, hepatitis D and human immunodeficiency virus. Patients did not receive antiviral therapy at time of blood or tissue donation. The study was approved by the medical ethical committee of the Erasmus MC University Medical Center and donors gave written informed consent before blood or tissue donation.

Table 1. Characteristics of donors used for peripheral blood DC quantification and functional characterization.

Characteristics	Quantification	Functional characterization
Number	19	18
ALT (IU/L) mean (range)	49 (12-164)	37 (12-78)
HBV DNA (log10 IU/ml) mean (range)	3.9 (1.3-8.6)	3.5 (1.3-8.6)
HBeAg+/HBeAg-	2/17	0/18

Table 2. Characteristics of donors used for intrahepatic DC quantification.

Characteristics	Flow cytometry	Immunohistochemistry
Number	11	14
ALT (IU/L) mean (range)	73 (32-164)	91 (12-370)
HBV DNA (log10 IU/ml) mean (range)	3.8 (1.3-8.6)	4.6 (1.3-9.5)
HBeAg+/HBeAg-	3/8	10/4
Fibrosis		
F0	1	3[a]
F1	6	6
F2	2	2
F3	2	2

[a] Fibrosis status of one patient is unkown.

Cell isolation and stimulation

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized peripheral blood samples or buffy coats from healthy blood donors using Ficoll density gradient centrifugation. PBMC were enriched for DCs using Dynabeads that deplete T cells, B cells, monocytes/macrophages, NK cells, erythrocytes and most granulocytes (Life Technologies),

and DC subsets were sorted based on BDCA3 expression using a FACSAria (BD Biosciences). Liver tissue (>1 cm^3) was digested to obtain a single cell suspension. Briefly, liver tissue was cut into small pieces and digested with 0.5 mg mL^{-1} collagenase (Sigma-Aldrich) and 0.1 mg ml^{-1} DNase (Roche) for 30 minutes at 37°C. The digested material was subsequently filtered through a cell strainer and mononuclear cells were obtained by Ficoll density gradient centrifugation. Core needle-biopsies (14-gauge needle) were only filtered through a cell strainer to obtain a single cell suspension.

Flow cytometric analysis

For phenotypic analysis, cells were labelled with Abs recognizing CD11c (3.9), CD40 (5C3), CD45 (HI30), CD83 (HB15e), HLA-DR (LN3, all eBioscience), BDCA1/CD1c (AD5-8E7, Miltenyi Biotec), BDCA3/CD141 (AD5-14H12, Miltenyi Biotec), CD86 (2331, BD Horizon), CLEC9A (8F9, BioLegend), HBsAg (Acris), a lineage cocktail including CD3 (UCHT1, eBioscience), CD14 (61D3, eBioscience), CD19 (HIB19, eBioscience) and CD56 (MY31, BD Biosciences), and the live/dead marker Aqua (LifeTechnologies). Fluorescence was measured using a FACS Canto II (BD Biosciences).

Cytokine production

1*10^6 PBMC were stimulated with polyI:C (20 µg ml^{-1}, Invivogen) in 250 µl in 96-wells plates (Greiner Bio-one, Alphen aan den Rijn, Netherlands) for 5 or 7 hours at 37°C in RPMI 1640 (Invitrogen) supplemented with 9% heat-inactivated FCS (Sigma-Aldrich) and penicillin/streptomycin (Invitrogen). During the last 3 hours, cells were incubated with 10 µg ml^{-1} Brefeldin A (Sigma-Aldrich). Subsequently, cells were stained for BDCA3 and CD11c, fixed with 2% formaldehyde, permeabilized with 0.5% saponin and stained for tumor necrosis factor α (TNF-α) (eBioscience), IFN-λ1 (kindly provided by Bristol-Myers Squibb and commercial Ab from R&D systems) or polyclonal goat IgG (R&D systems). Cytokine-producing cell frequencies were determined by flow cytometry.

Isolated DCs were stimulated for 24 hours with 20 µg ml^{-1} polyI:C in the presence of 10 ng ml^{-1} GM-CSF. Levels of secreted human IFN-λ1 (interleukin 29; IL-29) were measured using a commercially available ELISA kit (eBioscience) and IL-1β, IL-6, IL-8 and TNFα levels were measured using a BD cytometric bead array (CBA, BD Biosciences). Detection limits were 8 pg ml^{-1} (IFN-λ1), 7.2 pg ml^{-1} (IL-1β), 2.5 pg ml^{-1} (IL-6), 3.6 pg ml^{-1} (IL-8), 3.7 pg ml^{-1} (TNF-α).

HBsAg uptake/binding and stimulation with HBsAg in vitro

For analysis of HBsAg binding/uptake, PBMC were incubated with 1 µg ml^{-1} fluorochrome-labeled HBsAg isolated from pooled serum of patients (pHBsAg; subtype ay; American Research Products, ARP) in 250 µl for 2 hours at 4°C or 37°C. Cells were fixed with 2% formaldehyde and analysed by flow cytometry. For analysis of the effect of HBsAg on BDCA3+ DC function, cells were stimulated with 20 µg ml^{-1} polyI:C in the presence or absence of 5 µg ml^{-1} patient-derived pHBsAg or 5 µg ml^{-1} recombinant CHO-derived HBsAg (rHBsAg; Prospec; determined to be endotoxin free by Endolisa; Hyglos GmbH). Depletion of rHBsAg was performed by immunoprecipitation using protein G sepharose beads (GE Healthcare) that were bound to human anti-HBsAg Abs (Biotest Pharma), as described previously [30].

Immunohistochemistry

Formalin-fixed paraffin-embedded (FFPE) sections (5 µm) of 14 CHB livers and 6 donor livers (Table 2) were deparaffinized and boiled for 10 minutes in citrate buffer (pH 6) for antigen

retrieval. Sections were incubated with 3% H_2O_2, 10% human serum, and Abs against CLEC9A (polyclonal sheep, R&D systems) or non-specific polyclonal sheep Abs (R&D systems) for 1 hour at 37°C. CLEC9A Ab was subsequently bound by horseradish peroxidase (HRP)-labeled donkey(Fab)-anti-sheep Ab (Life Technologies) and the signal was amplified using tyramide-FITC, followed by mouse-anti-FITC (Jackson ImmunoResearch) and DyLight488-labeled goat-anti-mouse (BioLegend). CLEC9A$^+$ cells were manually counted in 5-9 microscopic fields (200x magnification) containing portal tracts and the mean number of cells per microscopic field was calculated.

Statistical analysis

Statistical analysis was performed using Graphpad Prism version 5.01 for Windows (GraphPad Software).

Results

BDCA3$^+$ DCs are prominently present in HBV-infected livers

The frequency and function of BDCA3$^+$ DCs in HBV-infected livers is currently unknown. Therefore, we set out to quantify BDCA3$^+$ DCs in biopsies from HBV-infected livers and control livers from non-HBV infected individuals. The latter included healthy donor livers accepted or rejected for transplantation, as well as non-cancerous peri-tumor tissue. First, to distinguish immune cells from hepatocytes, we used the hematopoietic lineage marker CD45 (Fig 1A). As may be expected in a state of ongoing inflammation, the frequency of total CD45$^+$ immune cells and DCs was significantly higher in livers of HBV-infected individuals compared to those of controls, indicating extensive immune cell infiltration (Fig 1B). Of these CD45$^+$ immune cells, BDCA3$^+$ DCs represented 0.18±0.15% both in control livers and HBV-infected livers, which is in line with previous reports (S1 Fig) [29]. In line with an increase of immune cells in the liver, the percentage of BDCA3$^+$ DCs of total liver cells seemed to be higher in HBV-infected livers, however, this increase was not significant.

In addition to flow cytometry, immunohistochemical (IHC) stainings were used to accurately study BDCA3$^+$ DC frequencies in the liver. This analysis demonstrated that numbers of CLEC9A$^+$ DCs were indeed higher in HBV-infected livers than in control livers (Figs 1C and 1D). The marker CLEC9A, that was used to identify BDCA3$^+$CLEC9A$^+$ DCs by IHC, was uniquely expressed by CD45$^+$HLA-DR$^+$Lineage$^-$BDCA3^{hi} DCs in the liver (S2 Fig). BDCA3$^+$ DCs were predominantly located in portal tracts with immune infiltration. Interestingly, BDCA3$^+$ DC numbers in the liver positively correlated with HBV DNA levels (Spearman r=0.782, p=0.001). However, no difference in BDCA3$^+$ DC numbers could be detected between patients with high or low fibrosis or liver damage, as measured by alanine transaminase (ALT) (Fig 1D), suggesting that mostly active viral replication, and possibly consecutive local production of inflammatory cytokines/chemokines, rather than liver damage induces infiltration of BDCA3$^+$ DCs into the liver. Unfortunately, any association between BDCA3$^+$ DC numbers with HBsAg levels could not be determined as HBsAg levels at the timepoint of biopsy collection were not available for all donors.

Together, these data show that during CHB infection, intrahepatic BDCA3$^+$ DCs are present at a similar frequency with respect to other immune cells as in control livers. The absolute number of BDCA3$^+$ DCs, however, is increased in HBV-infected livers due to the increased inflammatory infiltrate.

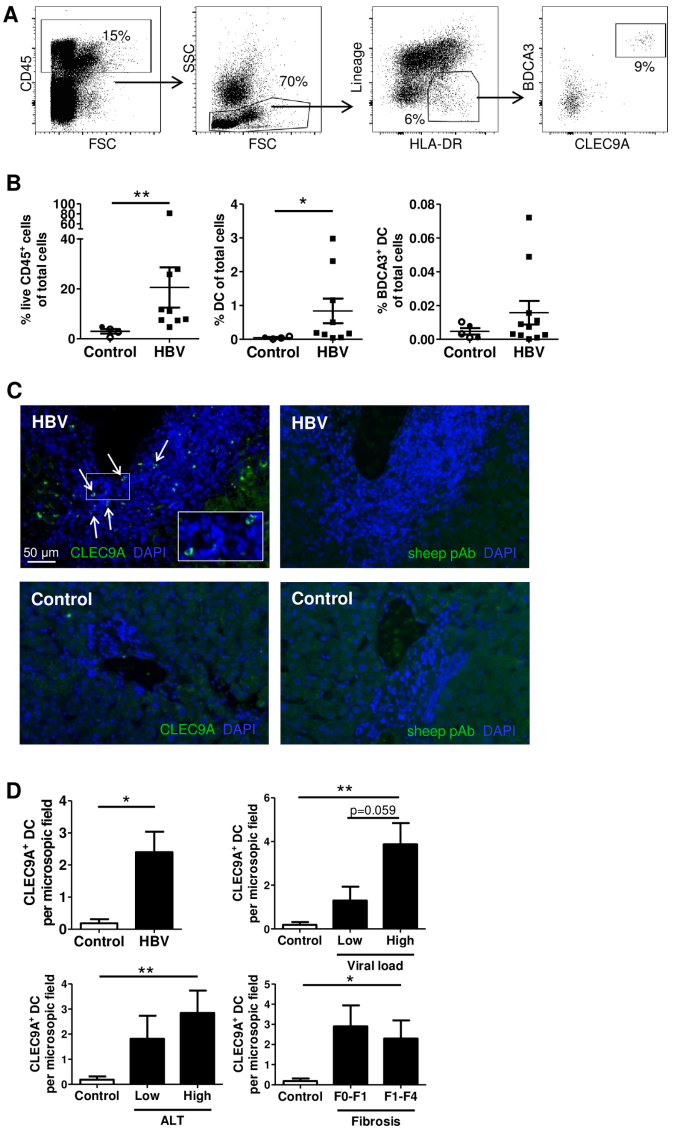

Fig 1. Quantification of intrahepatic BDCA3⁺ DCs from HBV patients and controls.
(A-B) Liver cells were isolated from HBV patients and controls. The DC population was identified as $CD45^+Lineage^-HLA\text{-}DR^+$ mononuclear cells, within which $BDCA3^+CLEC9A^+$ DCs were detected. (A) Representative flow cytometry plots and (B) the percentage $CD45^+$ cells of total cells (control n=4, HBV n=9), percentage DCs of total cells (control n=4, HBV n=9) and percentage $BDCA3^+$ DCs of total cells (control n=5, HBV n=11) in livers of controls and HBV patients. Indicated are the mean percentage and SEM. Open dots represent cells from donor livers and filled dots represent cells from peri-tumor liver tissue. $^{**}p < 0.01$ by Mann-Whitney test. (C-D) FFPE sections of HBV-infected and control livers were stained with anti-CLEC9A Abs or non-specific sheep polyclonal Abs (green) and quantified (see methods). Nuclei were visualized using DAPI (blue). Magnification 200x. (C) Representative pictures of an HBV-infected liver with high ALT (defined as > 60 IU L^{-1}) and high viral load (>10,000 IU ml^{-1}) and a control liver (healthy donor liver accepted for transplantation). White arrows indicate $CLEC9A^+$ DCs. (D) Number of $CLEC9A^+$ DCs per microscopic field in control livers (n=6) and total HBV-infected livers with different levels of viral load (low n=8, high n=6), ALT (low n=8, high n=6), and fibrosis (F0 – F0-1 n=6, F1 – F4 n=7) (mean±SEM). $^*p < 0.05$, $^{**}p < 0.01$ by Mann-Whitney test.

The capacity of blood BDCA3⁺ DCs to mature and produce IFN-λ is reduced in chronic HBV patients

Investigation of the functional state of intrahepatic $BDCA3^+$ DCs in CHB patients was not feasible due to the limited amount of biopsy material. Therefore, the possible effects that HBV infection may have on the function of $BDCA3^+$ DCs were assessed on $BDCA3^+$ DCs from peripheral blood of CHB patients and healthy controls. $BDCA3^+$ DCs were equally present in blood of HBV patients and healthy controls (both 0.04%, Figs 2A and 2B). Blood $BDCA3^+$ DCs of both HBV patients and controls were largely immature, as indicated by low expression

of the maturation markers CD40, CD83 and CD86 (Figs 2C and 2D). Subsequent in vitro maturation by polyI:C induced upregulation of maturation markers both in healthy control DCs and DCs from HBV patients, however, this was much less pronounced in BDCA3$^+$ DCs from HBV patients (Fig 2D and S3 Fig). Assessment of cytokine secretion showed that blood-derived BDCA3$^+$ DCs produced TNF-α and IFN-λ1, but no IFN-α or IFN-β, upon polyI:C stimulation (Figs 2E and 2F, S4A Fig, data not shown). Most IFN-λ-producing BDCA3$^+$ DCs co-produced TNF-α, and IFN-λ production correlated with TNF-α production (S4B and S4C Figs). However, only the secretion of IFN-λ1 by blood BDCA3$^+$ DCs from HBV patients was significantly impaired (Figs 2E and 2F). The frequency of IFN-λ1-producing BDCA3$^+$ DCs did neither correlate with serum HBV DNA or serum ALT levels nor age (Fig 2G, data not shown). Together, these results indicate that, although BDCA3$^+$ DCs are not matured by chronic HBV infection, the capacity of CHB patient-derived blood BDCA3$^+$ DCs to mature and produce IFN-λ1 upon TLR activation is impaired.

HBsAg can affect BDCA3$^+$ DC function via an indirect effect

HBsAg, an HBV-derived protein which is abundantly present in patient's circulation, has previously been shown to functionally impair pDC function [11, 31]. We therefore investigated whether HBsAg affected BDCA3$^+$ DC function by incubating BDCA3$^+$ DCs with HBsAg in vitro. Using fluorochrome-labeled patient-derived HBsAg (pHBsAg) we observed that BDCA3$^+$ DCs readily internalized HBsAg via active endocytosis (Fig 3A). As we used a concentration comparable to that found in vivo, these results indicate that a direct interaction between BDCA3$^+$ DCs and HBsAg is also likely to occur in vivo.

Incubation of PBMCs with either pHBsAg or recombinant HBsAg (rHBsAg) for 6 hours increased CD40, CD83 and CD86 expression on BDCA3$^+$ DCs, showing that under these circumstances the viral antigen can induce maturation (Fig 3B). Contamination of endotoxins in the HBsAg preparations, which might affect BDCA3$^+$ DC function, was excluded by Endolisa, a specific and sensitive method to detect endotoxins (data not shown). Next, we investigated whether HBsAg affected polyI:C-induced cytokine production. IFN-λ1-producing capacity, but not TNF-α-producing capacity, of both peripheral blood and intrahepatic BDCA3$^+$ DCs upon polyI:C stimulation of PBMCs or liver cells, respectively, was significantly decreased by rHBsAg (Figs 3C-3E, data not shown). This effect was diminished upon depletion of rHBsAg by immunoprecipitation using anti-HBsAg-coated beads, and restored upon addition of rHBsAg to the depleted fraction (S5A Fig), indicating that the effect is HBsAg-specific. In addition, patient-derived HBsAg (pHBsAg) also reduced IFN-λ1 production by peripheral blood BDCA3$^+$ DCs (S5B Fig). The HBsAg-induced maturation and functional impairment of BDCA3$^+$ DCs, however, was only observed when these cells were exposed to HBsAg in the presence of other PBMCs. Incubation of isolated BDCA3$^+$ DCs alone with rHBsAg neither induced DC maturation, nor affected polyI:C or TNFα and IL-1β-induced maturation, suggesting that HBsAg acts on BDCA3$^+$ DCs only via other immune cells (Fig 4A). In addition, rHBsAg did not affect polyI:C-induced production of IL-1β, IL-6, IL-8, TNFα and IFN-λ by isolated BDCA3$^+$ DCs and also had no effect on the viability of BDCA3$^+$ DCs (Figs 4B and 4C, data not shown). Addition of PBMC to isolated BDCA3$^+$ DCs increased the production of IFN-λ, which was reduced by rHBsAg, confirming that rHBsAg affected BDCA3$^+$ DCs only indirectly (Fig 4C).

Together, these results demonstrate that HBsAg does not directly mature or impair BDCA3$^+$ DC function, but may have an indirect effect via other immune cells.

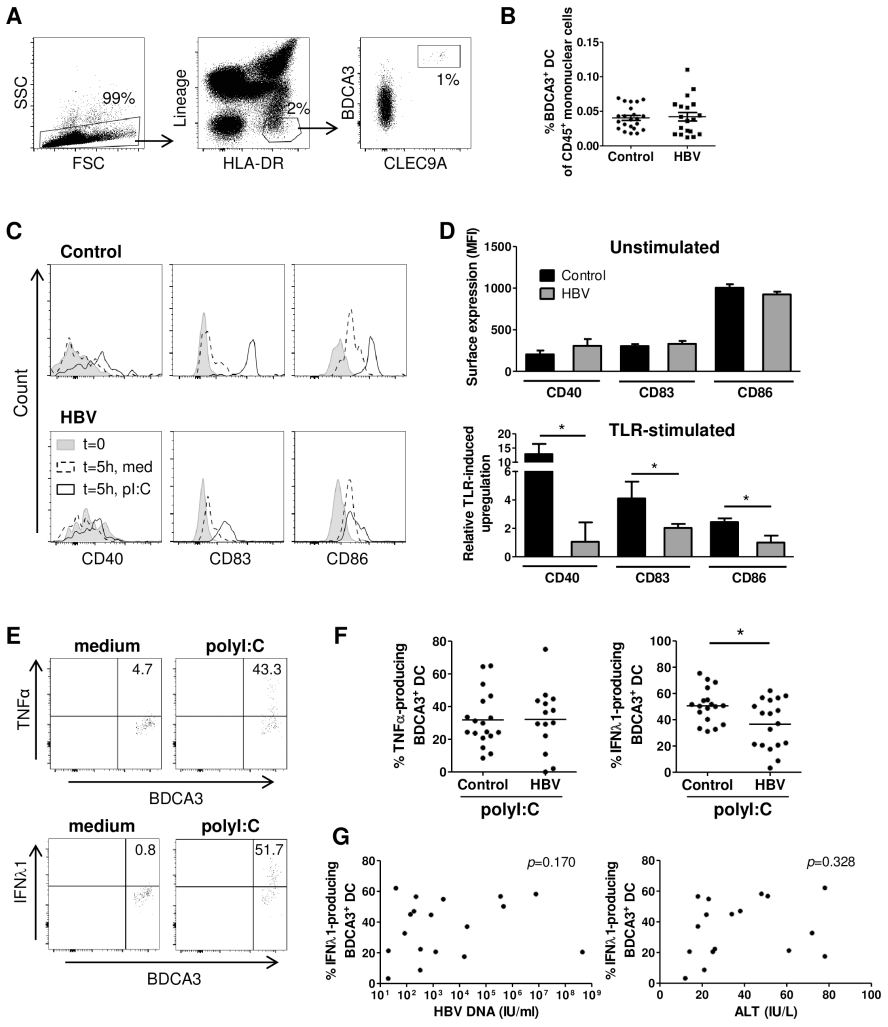

Fig 2. Blood BDCA3⁺ DCs from CHB patients are impaired in their capacity to mature and produce IFN-λ.
PBMC were isolated from CHB patients and healthy controls. (A-B) The DC population was identified as Lineage⁻ HLA-DR⁺ mononuclear cells, within which BDCA3⁺CLEC9A⁺ DCs were detected. (A) Representative flow cytometry plots and (B) the percentage of BDCA3⁺ DCs of CD45⁺ mononuclear cells (control n=22, HBV n=19; mean±SEM). PBMCs were stimulated for 5 hours with or without polyI:C. Expression of the maturation markers CD40, CD83 and CD86, and cytokine production by BDCA3⁺ DCs was measured by flow cytometry. (C) Representative histograms of maturation marker expression by BDCA3⁺ DCs. (D) Collected expression data (MFI) for each marker after isolation at t=0 (Unstimulated), and relative upregulation after stimulation compared to the medium control at t=5 (TLR-stimulated) (control n=15, HBV n=8) (mean±SEM). (E) Representative flow cytometry plots of TNFα and IFN-λ1 production by FSC/SSC gated viable BDCA3⁺ DC. (F) Collected percentages of TNFα-producing and IFN-λ1-producing BDCA3⁺ DCs in controls (n=18) and HBV patients (n=18) (mean±SEM). *$p < 0.05$ by Mann-Whitney test. (G) Spearman's correlation between the frequency of IFN-λ1-producing BDCA3⁺ DCs from HBV patients and serum HBV DNA or serum ALT levels (n=18).

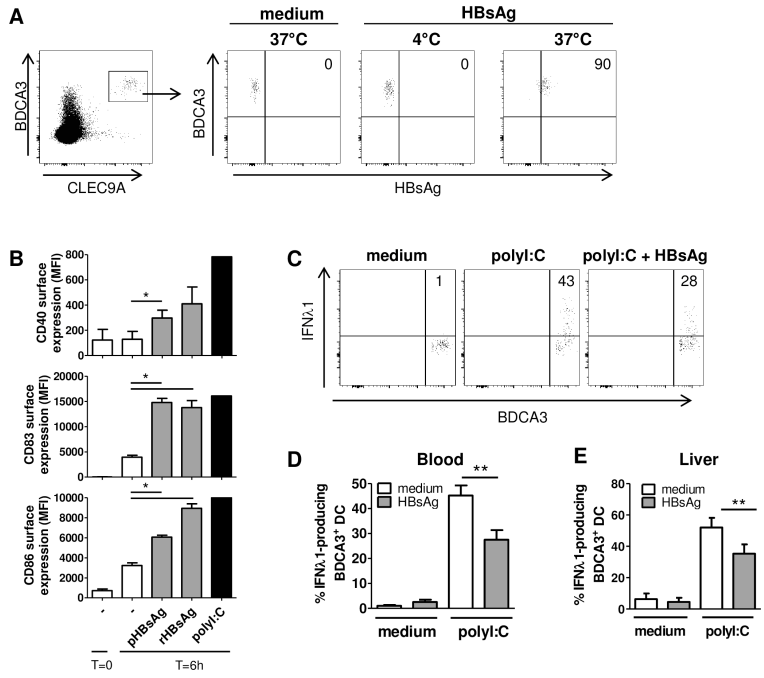

Fig 3. HBsAg diminishes IFN-λ1 production by BDCA3⁺ DCs.
(A) PBMC from healthy subjects were incubated with or without fluorescently-labeled HBsAg for 2 hours at indicated temparatures and HBsAg binding/uptake by BDCA3⁺ DCs was measured by flow cytometry. Representative plots of 3 independent experiments and donors are shown. (B) PBMC from healthy subjects were stimulated for 6 hours with or without rHBsAg, pHBsAg or polyI:C and maturation marker-expression on BDCA3⁺ DCs was analyzed by flow cytometry (n=3; mean±SEM; * p<0.05 by paired Student's *t*-test). (C-D) PBMC from healthy subjects were stimulated for 7 hours with polyI:C in the presence or absence of rHBsAg and the production of IFN-λ1 by BDCA3⁺ DCs was measured by ICS. Representative flow cytometry plots (C) and the summarized percentage of IFN-λ1-producing BDCA3⁺ DCs (D; n=7; mean±SEM) are shown. To determine the percentage of IFN-λ-producing BDCA3⁺ DCs in blood, a minimum threshold of 70 BDCA3⁺ DCs was used. ** $p < 0.01$ by paired Student's *t*-test. (E) Liver cells from peri-tumor liver tissue were stimulated for 5 hours with or without polyI:C in the presence or absence of rHBsAg and the production of IFN-λ1 by BDCA3⁺ DCs was measured by ICS. The percentage of IFN-λ1-producing BDCA3⁺ DCs is shown (n=3; mean±SEM). ** $p < 0.01$ by paired Student's *t*-test.

Discussion

BDCA3⁺ DCs are professional APCs that excel in IFN-λ production. In this study, we report on the intrahepatic presence and localization of BDCA3⁺ DCs in healthy and HBV-infected livers. We showed that BDCA3⁺ DCs reside in inflamed portal tracts and that their numbers are increased in HBV-infected livers compared to controls. In addition, blood BDCA3⁺ DCs of CHB patients displayed an impaired maturation and IFN-λ1 response upon ex vivo stimulation compared to controls. Furthermore, we demonstrated that the most prominent HBV protein, HBsAg, does neither directly induce BDCA3⁺ DC maturation, nor affects their function, but may exert an effect indirectly via an unknown mechanism.
Previous studies have shown that absolute numbers of BDCA1⁺ DCs and pDCs are increased in HBV-infected livers [32]. A result we here confirm and complement by demonstrating that

also BDCA3+ DC numbers are increased in the liver upon HBV infection. IHC stainings showed that intrahepatic BDCA3+ DCs predominantly reside in portal tracts, and especially in those with high immune infiltration. Since these areas accommodate many other immune cells, including T cells, this suggests that BDCA3+ DCs may regulate immunity not only in the liver draining lymph nodes, but can also do so locally.

The impaired functional capacity of BDCA3+ DCs from CHB patients adds up to our previous findings for pDCs and mDCs, which demonstrated that the function of these DC subsets is also diminished in CHB patients [10, 11]. The reduced maturation capacity of BDCA3+ DCs together with a reduced IFN-λ production may impair T cell activation or skewing in these patients, and may thus affect the induction of effective adaptive immune responses [23].

We here find HBsAg was able to reduce IFN-λ production in vitro via an indirect mechanism. Therefore HBsAg may have a systemic effect that can contribute to the impaired IFN-λ production we observed in BDCA3+ DCs ex vivo, possibly via a monocyte-mediated mechanism [11, 33].

In contrast to the reduced maturation we observed for CHB patient-derived BDCA3+ DCs in response to polyI:C, maturation of BDCA3+ DCs in vitro was rather enhanced by HBsAg, suggesting the presence of alternative mechanisms and/or viral components that act on BDCa3+ DCs in these patients. Although BDCA3+ DCs may have become refractory to maturation as a result of continued HBsAg exposure, it is likely that the state of chronic inflammation itself plays a major role in reduction of BDCA3+ DC function as well. The exact contribution of viral proteins/particles or chronic inflammation in the impairment of BDCA3+ DC function during CHB remains to be determined.

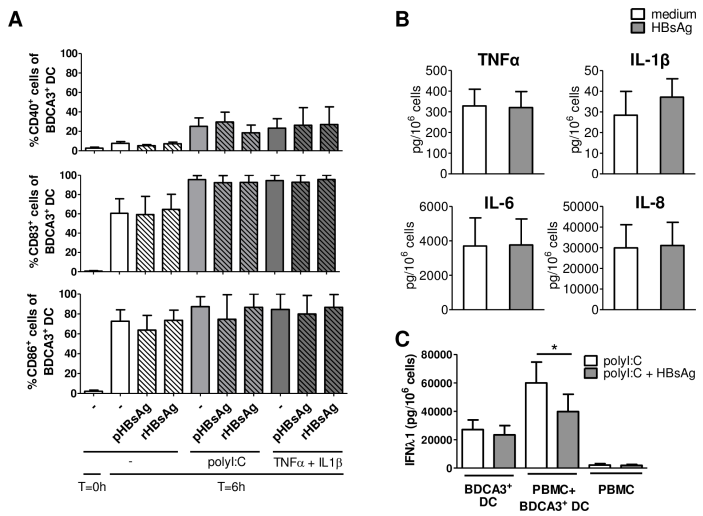

Fig 4. HBsAg does not have a direct effect on BDCA3+ DC function.

(A) Isolated BDCA3+ DCs from healthy subjects were stimulated for 6 hours with or without polyI:C or TNFα and IL-1β in the presence or absence of rHBsAg or pHBsAg. Mean±SEM percentages of maturation marker-expressing BDCA3+ DCs are shown (n=2-3). (B) Isolated BDCA3+ DCs from healthy subjects were stimulated for 24 hours with polyI:C in the presence or absence of rHBsAg. Data are shown as mean±SEM cytokine levels determined by CBA (n=5). (C) Isolated blood BDCA3+ DCs from healthy subjects were stimulated for 24 hours with polyI:C in the presence (n=6) or absence (n=7) of PMBC and/or rHBsAg, and cytokine levels in the culture supernatant were determined by ELISA (mean±SEM). *$p < 0.05$ by paired Student's *t*-test.

Like blood BDCA3$^+$ DCs, we and others showed that intrahepatic BDCA3$^+$ DCs are able to produce IFN-λ [21]. In case of HCV infection, high IFN-λ levels have been detected in the liver, which may in part derive from BDCA3$^+$ DCs [34]. CHB livers in contrast hardly contained IFN-λ transcripts [34]. Furthermore, IFN-λ levels in serum of CHB patients are comparable to those of controls [34-37]. One explanation may be that HBV by itself does not induce an effective IFN-λ response during its natural course of infection. However, our finding that IFN-λ1-producing capacity of BDCA3$^+$ DCs is impaired in HBV patients, together with recent data showing the inhibition of IFN-λ production in infected hepatocytes by HBV virions, also open up the possibility that HBV may actively diminish IFN-λ1-production [38, 39]. These latter studies demonstrated that in hepatocytes, HBV can induce but concurrently suppress host innate responses, in particular the TLR3/RIG-I/MDA5-induced response, and that it does so by factors present in the viral inoculum and via pgRNA [38]. We here already demonstrate that HBsAg has an indirect effect on IFN-λ production by BDCA3$^+$ DCs, but more research is required to find out how it does so and whether other viral factors and/or the state of chronic inflammation may also contribute to the defect observed in patients' blood BDCA3$^+$ DCs. A pressing remaining question now is whether in HBV-infected livers also intrahepatic BDCA3$^+$ DCs show a reduced IFN-λ-producing capacity. Unfortunately, the scarceness of BDCA3$^+$ DCs in the liver makes the functional experiments on biopsy BDCA3$^+$ DCs extremely challenging. In addition, the availability of liver biopsies for such studies is limited, especially since implementation of the fibroscan to determine fibrosis stage. Therefore, performing such studies is at this moment beyond our possibility.

In conclusion, we demonstrate for the first time that BDCA3$^+$ DCs are increased in HBV-infected livers and that the function of BDCA3$^+$ DCs of HBV patients is impaired. These results suggest that BDCA3$^+$ DCs are available on site to be exploited to improve/redirect HBV-specific immune responses. For example, by targeting local BDCA3$^+$ DCs with TLR3 ligands to achieve local IFN-λ production, possibly even in combination with HBV antigens for simultaneous cross-presentation of viral antigens. To achieve optimal effect however, our study suggests that measures may need to be taken to overcome the impaired maturation and IFN-λ-producing capacity of BDCA3$^+$ DCs in CHB patients.

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References

1. Dandri, M. and Locarnini, S. (2012) New insight in the pathobiology of hepatitis B virus infection. Gut 61 Suppl 1, i6-17.
2. Boonstra, A., Woltman, A. M., Janssen, H. L. (2008) Immunology of hepatitis B and hepatitis C virus infections. Best Pract Res Clin Gastroenterol 22, 1049-61.
3. Bertoletti, A. and Gehring, A. J. (2006) The immune response during hepatitis B virus infection. J Gen Virol 87, 1439-49.
4. Steinman, R. M. (2008) Dendritic cells in vivo: a key target for a new vaccine science. Immunity 29, 319-24.
5. van Montfoort, N., van der Aa, E., Woltman, A. M. (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.
6. van der Aa, E., van Montfoort, N., Woltman, A. M. (2014) BDCA3CLEC9A human dendritic cell function and development. Semin Cell Dev Biol.
7. Reynolds, G. and Haniffa, M. (2015) Human and Mouse Mononuclear Phagocyte Networks: A Tale of Two Species? Front Immunol 6, 330.
8. Vu Manh, T. P., Bertho, N., Hosmalin, A., Schwartz-Cornil, I., Dalod, M. (2015) Investigating Evolutionary Conservation of Dendritic Cell Subset Identity and Functions. Front Immunol 6, 260.
9. Gehring, A. J. and Ann D'Angelo, J. (2014) Dissecting the dendritic cell controversy in chronic hepatitis B virus infection. Cell Mol Immunol.
10. van der Molen, R. G., Sprengers, D., Binda, R. S., de Jong, E. C., Niesters, H. G., Kusters, J. G., Kwekkeboom, J., Janssen, H. L. (2004) Functional impairment of myeloid and plasmacytoid dendritic cells of patients with chronic hepatitis B. Hepatology 40, 738-46.
11. Woltman, A. M., den Brouw, M. L. O., Biesta, P. J., Shi, C. C., Janssen, H. L. A. (2011) Hepatitis B Virus Lacks Immune Activating Capacity, but Actively Inhibits Plasmacytoid Dendritic Cell Function. Plos One 6.
12. Poulin, L. F., Salio, M., Griessinger, E., Anjos-Afonso, F., Craciun, L., Chen, J. L., Keller, A. M., Joffre, O., Zelenay, S., Nye, E., Le Moine, A., Faure, F., Donckier, V., Sancho, D., Cerundolo, V., Bonnet, D., Reis e Sousa, C. (2010) Characterization of human DNGR-1+ BDCA3+ leukocytes as putative equivalents of mouse CD8alpha+ dendritic cells. J Exp Med 207, 1261-71.
13. Jongbloed, S. L., Kassianos, A. J., McDonald, K. J., Clark, G. J., Ju, X., Angel, C. E., Chen, C. J., Dunbar, P. R., Wadley, R. B., Jeet, V., Vulink, A. J., Hart, D. N., Radford, K. J. (2010) Human CD141+ (BDCA-3)+ dendritic cells (DCs) represent a unique myeloid DC subset that cross-presents necrotic cell antigens. J Exp Med 207, 1247-60.
14. Bachem, A., Guttler, S., Hartung, E., Ebstein, F., Schaefer, M., Tannert, A., Salama, A., Movassaghi, K., Opitz, C., Mages, H. W., Henn, V., Kloetzel, P. M., Gurka, S., Kroczek, R. A. (2010) Superior antigen cross-presentation and XCR1 expression define human CD11c+CD141+ cells as homologues of mouse CD8+ dendritic cells. J Exp Med 207, 1273-81.
15. Crozat, K., Guiton, R., Contreras, V., Feuillet, V., Dutertre, C. A., Ventre, E., Vu Manh, T. P., Baranek, T., Storset, A. K., Marvel, J., Boudinot, P., Hosmalin, A., Schwartz-Cornil, I., Dalod, M. (2010) The XC chemokine receptor 1 is a conserved selective marker of mammalian cells homologous to mouse CD8alpha+ dendritic cells. J Exp Med 207, 1283-92.
16. Waithman, J., Zanker, D., Xiao, K., Oveissi, S., Wylie, B., Ng, R., Togel, L., Chen, W. (2013) Resident CD8(+) and migratory CD103(+) dendritic cells control CD8 T cell immunity during acute influenza infection. Plos One 8, e66136.
17. Hildner, K., Edelson, B. T., Purtha, W. E., Diamond, M., Matsushita, H., Kohyama, M., Calderon, B., Schraml, B. U., Unanue, E. R., Diamond, M. S., Schreiber, R. D., Murphy, T. L., Murphy, K. M. (2008) Batf3 deficiency reveals a critical role for CD8alpha+ dendritic cells in cytotoxic T cell immunity. Science 322, 1097-100.

18. Lauterbach, H., Bathke, B., Gilles, S., Traidl-Hoffmann, C., Luber, C. A., Fejer, G., Freudenberg, M. A., Davey, G. M., Vremec, D., Kallies, A., Wu, L., Shortman, K., Chaplin, P., Suter, M., O'Keeffe, M., Hochrein, H. (2010) Mouse CD8alpha+ DCs and human BDCA3+ DCs are major producers of IFN-lambda in response to poly IC. J Exp Med 207, 2703-17.

19. Nizzoli, G., Krietsch, J., Weick, A., Steinfelder, S., Facciotti, F., Gruarin, P., Bianco, A., Steckel, B., Moro, M., Crosti, M., Romagnani, C., Stolzel, K., Torretta, S., Pignataro, L., Scheibenbogen, C., Neddermann, P., De Francesco, R., Abrignani, S., Geginat, J. (2013) Human CD1c+ dendritic cells secrete high levels of IL-12 and potently prime cytotoxic T-cell responses. Blood 122, 932-42.

20. Zhang, S., Kodys, K., Li, K., Szabo, G. (2013) Human type 2 myeloid dendritic cells produce interferon-lambda and amplify interferon-alpha in response to hepatitis C virus infection. Gastroenterology 144, 414-425 e7.

21. Yoshio, S., Kanto, T., Kuroda, S., Matsubara, T., Higashitani, K., Kakita, N., Ishida, H., Hiramatsu, N., Nagano, H., Sugiyama, M., Murata, K., Fukuhara, T., Matsuura, Y., Hayashi, N., Mizokami, M., Takehara, T. (2013) Human blood dendritic cell antigen 3 (BDCA3)(+) dendritic cells are a potent producer of interferon-lambda in response to hepatitis C virus. Hepatology 57, 1705-15.

22. Schulte, B. M., Gielen, P. R., Kers-Rebel, E. D., Schreibelt, G., van Kuppeveld, F. J., Adema, G. J. (2015) Enterovirus-infected beta-cells induce distinct response patterns in BDCA1+ and BDCA3+ human dendritic cells. PLoS One 10, e0121670.

23. Egli, A., Santer, D. M., O'Shea, D., Tyrrell, D. L., Houghton, M. (2014) The impact of the interferon-lambda family on the innate and adaptive immune response to viral infections. Emerg Microbes Infect 3, e51.

24. Wack, A., Terczynska-Dyla, E., Hartmann, R. (2015) Guarding the frontiers: the biology of type III interferons. Nat Immunol 16, 802-9.

25. Robek, M. D., Boyd, B. S., Chisari, F. V. (2005) Lambda interferon inhibits hepatitis B and C virus replication. J Virol 79, 3851-4.

26. Pagliaccetti, N. E., Chu, E. N., Bolen, C. R., Kleinstein, S. H., Robek, M. D. (2010) Lambda and alpha interferons inhibit hepatitis B virus replication through a common molecular mechanism but with different in vivo activities. Virology 401, 197-206.

27. Chan, H. L., Ahn, S. H., Chang, T. T., Peng, C. Y., Wong, D., Coffin, C. S., Lim, S. G., Chen, P. J., Janssen, H., Marcellin, P., Serfaty, L., Zeuzem, S., Cohen, D., Critelli, L., Xu, D., Wind-Rotolo, M., Cooney, E., Team, L.-B. S. (2015) Peginterferon Lambda for the Treatment of HBeAg-Positive Chronic Hepatitis B: A Randomized Phase 2b Study (LIRA-B). J Hepatol.

28. Haniffa, M., Shin, A., Bigley, V., McGovern, N., Teo, P., See, P., Wasan, P. S., Wang, X. N., Malinarich, F., Malleret, B., Larbi, A., Tan, P., Zhao, H., Poidinger, M., Pagan, S., Cookson, S., Dickinson, R., Dimmick, I., Jarrett, R. F., Renia, L., Tam, J., Song, C., Connolly, J., Chan, J. K., Gehring, A., Bertoletti, A., Collin, M., Ginhoux, F. (2012) Human tissues contain CD141hi cross-presenting dendritic cells with functional homology to mouse CD103+ nonlymphoid dendritic cells. Immunity 37, 60-73.

29. Kelly, A., Fahey, R., Fletcher, J. M., Keogh, C., Carroll, A. G., Siddachari, R., Geoghegan, J., Hegarty, J. E., Ryan, E. J., O'Farrelly, C. (2014) CD141(+) myeloid dendritic cells are enriched in healthy human liver. J Hepatol 60, 135-42.

30. van Montfoort, N., van der Aa, E., van den Bosch, A., Brouwers, H., Vanwolleghem, T., Janssen, H. L., Javanbakht, H., Buschow, S. I., Woltman, A. M. (2016) Hepatitis B Virus Surface Antigen Activates Myeloid Dendritic Cells via a Soluble CD14-Dependent Mechanism. J Virol 90, 6187-99.

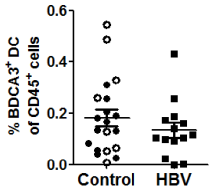
31. Xu, Y., Hu, Y., Shi, B., Zhang, X., Wang, J., Zhang, Z., Shen, F., Zhang, Q., Sun, S., Yuan, Z. (2009) HBsAg inhibits TLR9-mediated activation and IFN-alpha production in plasmacytoid dendritic cells. Mol Immunol 46, 2640-6.

32. Nattermann, J., Zimmermann, H., Iwan, A., von Lilienfeld-Toal, M., Leifeld, L., Nischalke, H. D., Langhans, B., Sauerbruch, T., Spengler, U. (2006) Hepatitis C virus E2 and CD81 interaction may be associated with altered trafficking of dendritic cells in chronic hepatitis C. Hepatology

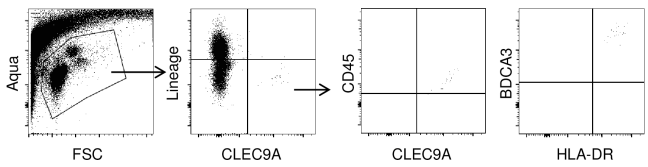
44, 945-54.

33. Boltjes, A., Groothuismink, Z. M., van Oord, G. W., Janssen, H. L., Woltman, A. M., Boonstra, A. (2014) Monocytes from chronic HBV patients react in vitro to HBsAg and TLR by producing cytokines irrespective of stage of disease. Plos One 9, e97006.
34. Duong, F. H., Trincucci, G., Boldanova, T., Calabrese, D., Campana, B., Krol, I., Durand, S. C., Heydmann, L., Zeisel, M. B., Baumert, T. F., Heim, M. H. (2014) IFN-lambda receptor 1 expression is induced in chronic hepatitis C and correlates with the IFN-lambda3 genotype and with nonresponsiveness to IFN-alpha therapies. J Exp Med 211, 857-68.
35. de Groen, R. A., McPhee, F., Friborg, J., Janssen, H. L., Boonstra, A. (2014) Endogenous IFNlambda in viral hepatitis patients. J Interferon Cytokine Res 34, 552-6.
36. Mihm, S., Frese, M., Meier, V., Wietzke-Braun, P., Scharf, J. G., Bartenschlager, R., Ramadori, G. (2004) Interferon type I gene expression in chronic hepatitis C. Lab Invest 84, 1148-59.
37. Park, H., Serti, E., Eke, O., Muchmore, B., Prokunina-Olsson, L., Capone, S., Folgori, A., Rehermann, B. (2012) IL-29 is the dominant type III interferon produced by hepatocytes during acute hepatitis C virus infection. Hepatology 56, 2060-70.
38. Luangsay, S., Gruffaz, M., Isorce, N., Testoni, B., Michelet, M., Faure-Dupuy, S., Maadadi, S., Ait-Goughoulte, M., Parent, R., Rivoire, M., Javanbakht, H., Lucifora, J., Durantel, D., Zoulim, F. (2015) Early inhibition of hepatocyte innate responses by hepatitis B virus. J Hepatol 63, 1314-22.
39. Sato, S., Li, K., Kameyama, T., Hayashi, T., Ishida, Y., Murakami, S., Watanabe, T., Iijima, S., Sakurai, Y., Watashi, K., Tsutsumi, S., Sato, Y., Akita, H., Wakita, T., Rice, C. M., Harashima, H., Kohara, M., Tanaka, Y., Takaoka, A. (2015) The RNA sensor RIG-I dually functions as an innate sensor and direct antiviral factor for hepatitis B virus. Immunity 42, 123-32.

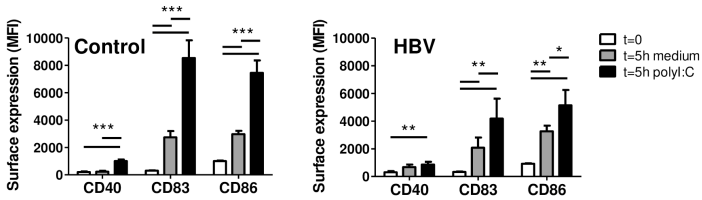
Supplementary information



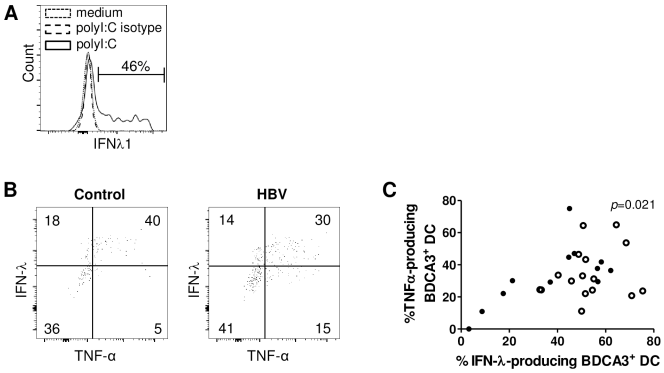
S1 Fig. Quantification of intrahepatic BDCA3$^+$ DCs from HBV patients and controls.
Liver cells were isolated from HBV patients and controls. The percentage BDCA3$^+$ DCs of CD45$^+$ cells (control n=20, HBV n=14) in livers of controls and HBV patients were determined. Indicated are the mean percentage and SEM. Open dots represent cells from donor livers and filled dots represent cells from peri-tumor liver tissue.



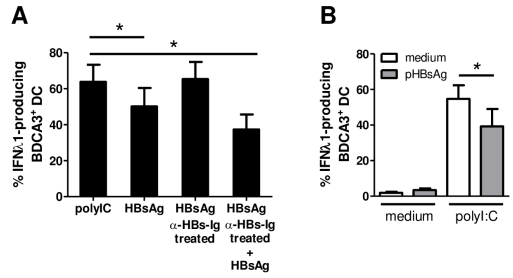
S2 Fig. Specific expression of CLEC9A on BDCA3$^+$ DC.
Representative flow cytometry plots of CLEC9A expression on lineage$^-$CD45$^+$HLA-DR$^+$BDCA3^{hi} liver cells.



S3 Fig. Upregulation of maturation markers upon stimulation.
PBMC were isolated from CHB patients and healthy controls and stimulated for 5 hours with or without polyI:C. Expression of the maturation markers CD40, CD83 and CD86 by BDCA3$^+$ DCs was measured by flow cytometry. Collected expression data (MFI) for each marker after isolation at t=0 and stimulation at t=5 is shown (control n=15, HBV n=8) (mean±SEM). *p<0.05, **p<0.01, ***p<0.001 by Wilcoxon signed rank test.



S4 Fig. Correlation between IFN-λ and TNF-α production.
PBMCs of CHB patients and healthy controls were stimulated for 5 hours with or without polyI:C. (A) Representative histogram including the isotype control of IFN-λ1 production by FSC/SSC-gated viable BDCA3+ DCs. (B) Representative flow cytometry plots of TNF-α and IFN-λ1 production by FSC/SSC-gated viable BDCA3+ DC. (C) Pearson's correlation between TNF-α and IFN-λ1 production by BDCA3+ DCs from controls (open dots) and HBV patients (filled dots) (n=28).



S5 Fig. Inhibition of IFN-λ production by pHBsAg and rHBsAg
(A) PBMCs of healthy controls were stimulated for 7 hours with polyI:C in the presence or absence of rHBsAg (HBsAg), a fraction from which rHBsAg was depleted (α-HBs-Ig treated), or a HBsAg-depleted fraction to which (5 μg ml^{-1}) rHBsAg was added (α-HBs-Ig treated + HBsAg). The mean ±SEM percentage of IFN-λ1-producing BDCA3+ DCs is shown. * p<0.05 by paired Student's *t*-test (B) PBMC of healthy controls were stimulated for 7 hours with or without polyI:C in the presence or absence of pHBsAg. The production of IFN-λ1 by BDCA3+ DCs was measured by ICS. The mean±SEM percentage of IFN-λ1 BDCA3+ DCs from 5 different donors is shown. * p<0.05 by paired Student's *t*-test.

Hepatitis B surface antigen activates myeloid dendritic cells via a soluble CD14-dependent mechanism

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Chapter 8

Abstract

Hepatitis B virus (HBV) infection can cause chronic liver disease which is associated with increased risk of liver cirrhosis, liver failure and liver cancer. Clearance of HBV infection requires effective HBV-specific immunity, however the immunological mechanisms that determine the development of effective HBV-specific immunity are poorly understood. Dendritic cells (DC) play a pivotal role in the regulation of anti-viral immunity. Here, we investigated the interaction between HBV surface antigen (HBsAg), the main envelop glycoprotein of HBV, and BDCA1+ myeloid dendritic cells (mDC).

Exposure of peripheral blood-derived BDCA1+ mDC to HBsAg resulted in strong DC maturation, cytokine production and enhanced capacity to activate antigen-specific CTL. By using neutralizing antibodies, crucial roles for CD14 and TLR4 in HBsAg-mediated BDCA1^{+} mDC maturation were identified. Concordantly, HBsAg-mediated DC maturation required FCS or human plasma, naturally containing soluble CD14 (sCD14). Intriguingly, HBsAg-induced DC maturation was significantly reduced in umbilical cord blood-plasma that contained less sCD14 than adult plasma, indicating that sCD14 is an important host factor for recognition of HBsAg by DC and subsequent DC activation. A direct interaction between sCD14 and HBsAg was demonstrated by using ELISA. Moreover, sCD14-HBsAg complexes were detected both in vitro and in serum of HBV-infected patients. Abundance of sCD14-HBsAg complexes varied between chronic HBV disease stages and correlated with activation of BDCA1+ mDC in vivo.

We conclude that HBsAg activates BDCA1+ DC via a sCD14-dependent mechanism. These findings provide important novel insights into initiation of HBV-specific immunity and facilitate development of effective immunotherapeutic interventions for HBV.

Importance

Hepatitis B virus (HBV) infection is a significant health problem as it causes progressive liver injury and liver cancer in patients with chronic HBV infection, which affects approximately 250 million individuals worldwide. Part of adults and the majority of neonates fail to mount an effective immune response and consequently develop chronic infection. The viral and host factors involved in the initiation of effective HBV-specific immune responses remain poorly understood. Here we identified CD14 and TLR4 as receptors for HBsAg, the main HBV envelop antigen. HBsAg induced strong maturation of dendritic cells (DC), which have a central role in regulation of virus-specific immunity. These results provide essential novel insights into the mechanisms underlying initiation of HBV-specific immunity. Intriguingly, since neonates have naturally low sCD14, the finding that serum-derived sCD14 is a crucial host factor for recognition of HBsAg by DC may have implications for immunity of neonates to HBV infection.

Introduction

Hepatitis B virus (HBV) is a dsDNA virus that is transmitted via blood and specifically infects hepatocytes. It can cause chronic liver disease and progressive liver injury leading to increased risk of liver cirrhosis, liver failure and liver cancer [1]. The current estimated prevalence of HBV infection is 248 million individuals globally [2]. Although the initiation of an effective anti-viral immune response is paramount for resolving HBV infection [3], the early steps in the recognition of the virus by immune cells and the functional consequences of this interaction remain to be resolved.

A pivotal role for dendritic cells (DC) is anticipated, because these cells play a central role in the orchestration of anti-viral immunity due to the expression of a wide variety of different pathogen-recognition receptors and their unique capacity to initiate virus-specific cytotoxic T cell (CTL) responses [4,5]. Among the different DC subsets, BDCA1+ myeloid DC (mDC) are of particular interest for HBV-specific immunity, since Hepatitis B surface antigen (HBsAg) positive mDC were detected in liver [6] and peripheral blood [7] of HBV patients. This suggests that interaction between BDCA1+ mDC and HBsAg, the main envelop glycoprotein present on HBV infectious particles and sub-viral particles, occurs in vivo. Although initially it was suggested that HBsAg has immune-regulating capacities (reviewed in [8]), more recently, also immune-stimulatory effects of HBsAg on myeloid cells including monocytes [9], monocyte-derived DC [10] and Kupffer cells [11] have been reported. Nevertheless, whether HBsAg is able to directly activate mDC and which receptor(s) are involved is currently unknown.

In the present study we investigated the interaction of HBsAg with BDCA1+ myeloid DC and its functional consequences. We demonstrated that HBsAg can induce maturation/activation and cytokine production in these mDC. By using neutralizing antibodies, we showed that HBsAg-dependent maturation of DC, but not uptake of HBsAg, is dependent on CD14 and TLR4. Since DC hardly express membrane-bound CD14, we postulated that HBsAg-induced DC maturation is mediated via serum component soluble CD14 (sCD14). Interestingly, by using serum from umbilical cord blood that naturally contains low sCD14, we showed that sCD14 concentration is associated with the strength of HBsAg-mediated DC maturation. In conclusion, we identified CD14 and TLR4 as PRR for HBsAg and sCD14 as important host factor for activation of BDCA1^{+} DC by HBsAg.

Materials and Methods

Isolation of monocytes and BDCA1+ myeloid DC

Monocytes and BDCA1+ myeloid DC (mDC) were isolated from buffy coats of healthy blood donors. First, PBMC were isolated by using Ficoll-Paque (GE Healthcare) density gradient centrifugation. Monocytes were isolated from PBMC by positive selection using anti-CD14 microbeads (Milteny Biotec). BDCA1+ mDC were isolated from PBMC by CD19+ cell depletion followed by positive selection using anti–BDCA1-PE and PE–conjugated microbeads (Miltenyi Biotec). Acceptable purity was minimally 95%, as assessed by flow cytometry. Standard culture medium was RPMI 1640 (Lonza), with 8% heat-inactivated fetal calf serum (FCS, Sigma), penicillin/streptomycin (Invitrogen), L-glutamin (Lonza) and 10 ng/ml GM-CSF (Leukine, genzyme).

Hepatitis B surface antigen (HBsAg) and control proteins

HBsAg isolated from pooled serum of patients (pHBsAg; subtype ay; purity 98%) was purchased from American Research Products (ARP). Recombinant HBsAg (rHBsAg) and beta Nerve Growth Factor (beta-NGF), both produced in mammalian cells, and recombinant HBsAg produced in yeast cells (yHBsAg) were purchased from Prospec. Since many commercially obtained recombinant proteins contain endotoxin impurities [12], we carefully selected preparations with the highest purity. Furthermore, all proteins were tested at used concentrations for endotoxin impurities by Endolisa (Hyglos GmbH, Bernried, Germany), a specific and sensitive method to detect endotoxins [12], according to manufacturer's instructions. Recombinant HBsAg and beta-NGF had endotoxin levels <0.1 EU/ml, which is below detection limit for BDCA1+ DC [12]. Unexpectedly, patient-derived HBsAg used at 1 µg/ml contained low endotoxin impurities (Endolisa result 1.4 EU/ml), yet at 100 ng/ml, which was used in most experiments, pHBsAg endotoxin levels were < 0.1 EU/ml, which is below the reported detection limit for BDCA1+ DC [12]. Heat-treatment of 3x concentrated preparations of pHBsAg and LPS (Ultrapure, from S. Minnesota, Invivogen) was performed by boiling for 30 minutes at 100 degrees in a heat block [13]. Depletion of pHBsAg was performed via 2 different methods, starting with a stock of pHBsAg of 100 ug/ml diluted in medium. First, heparin depletion was performed by injecting HBsAg stock (untreated fraction) into a HiTrap Heparin HP column (GE Healthcare). Flowthrough was collected (depleted fraction), column was washed with PBS and bound HBsAg was eluted with TRIS buffer (350 mM NaCl, 20mM Tris, pH 7.4). Efficiency of HBsAg depletion, as measured by HBsAg ELISA, was 95.6% whereas recovery after elution was 52.2%. For specific immune depletion, pHBsAg was incubated with protein G sepharose beads (GE Healthcare) pre-coated with either Hepatect (Biotest), a pool of human IgG specific for HBsAg, or mouse IgG1 (Abcam) for 2h at 4 degrees followed by centrifugation to obtain a HBsAg depleted supernatant. Delipidation of HBsAg was performed as described previously [14]. In brief, 1 mg/ml of HBsAg was incubated for 2h at RT with the non-denaturing detergent β-D-octyl glucoside (OG, Sigma-Aldrich) at 0% (control delipidation) or 2% [wt/vol in PBS] and subsequently dialysed 2x against PBS.

Exposure of mDC to HBsAg

Isolated mDC were incubated with medium or HBsAg at indicated concentrations. To assess the role of CD14 or TLR4, cells were pre-incubated with 0.2 µg/ml neutralizing antibodies to CD14 (clone MEM-18, mouse IgG1, Abcam), TLR4 (clone HT52, mouse IgG1, eBioscience) or mouse IgG1 isotype control (clone MG1-45, Abcam) for 1 hr at 37°C. After 20h of exposure at 37°C, mDC supernatants were harvested and stored at -20°C and cell surface markers were analyzed by flow cytometry. Antibodies used were mouse anti-BDCA1-PE (Miltenyi Biotec), mouse anti-CD14-eFluor450 (clone 61D3), mouse anti-CD83-Fi (clone HB15e) and mouse anti-CD40-APC (clone 5C3, all eBioscience). Samples were stained for 30 minutes at 4˚C in flow buffer (PBS containing 1% BSA, 1% heat-inactivated human serum and 0.02% NaN3). Samples were acquired on a FACS Canto II cytometer (BD Biosciences) and data was analyzed by FlowJo software (Treestar). Concentrations of human IL-6 and IL-12p40 were measured in culture supernatants by ELISA (eBioscience).

For experiments assessing the role of serum, standard medium was replaced by X-Vivo (Lonza) with 10 ng/ml GM-CSF, supplemented or not with 8% heat-inactivated FCS or 1% or 2% human serum/plasma, as indicated. Human plasma, from adult peripheral blood or umbilical cord blood was collected after Ficoll-Paque density gradient centrifugation and

stored at -80˚C. Plasma was centrifuged for 1 min at 3000 RPM before use in experiments.

Exposure of monocytes to HBsAg
Monocytes were pre-incubated with αCD14 or isotype control for 1 hr and subsequently exposed to medium, 1 µg/ml pHBsAg or rHBsAg for 18 hr at 37˚C. During the last 16 hours of culture, brefeldin A (10 µg/ml; Sigma-Aldrich) was added. The cells were harvested, fixed with 2% formaldehyde (Merck), permeabilized with 0.5% saponin (VWR) in flow buffer and stained for CD14, IL-6 and TNFα (all from eBioscience).

In vitro uptake/binding experiments
pHBsAg was conjugated to DyLight 650 sulfhydryl-reactive dye (FL-HBsAg; Thermo Fisher Scientific). After conjugation, unbound conjugate was removed by 2x dialysis against PBS. For confocal microscopy analysis, mDC were seeded in 35mm poly-d-lysine coated glass bottom petri dishes (MatTek corporation) and exposed for 2 or 20h to 5 µg/ml FL-HBsAg. After exposure, DC were washed with PBS, stained for HLA-DR (clone LN3, eBioscience) and goat anti-mouse Dylight 488 (clone Poly4053, Biolegend) as a secondary Ab and fixed with 2% formaldehyde. DC were acquired on a Zeiss LSM 510 inverted confocal microscope with Argon (488nm) and HeNe (633nm) lasers and 63x oil-immersed objective. Digital zoom 3x. Image J software was used to merge images. For flow cytometry analysis, PBMC depleted for CD14+ cells by MACS were pre-treated with medium, 1 µg/ml αCD14 or isotype control Ab, and subsequently exposed to 1 µg/ml FL-HBsAg for 2 hr, either at 4°C or 37°C. Cells were washed with cold PBS, stained with mouse anti-BDCA1-PE (Miltenyi Biotec) and mouse anti-CD20-eFluor450 (eBioscience), fixed with 2% formaldehyde and analysed by flow cytometry.

T cell activation and cross-presentation experiments
To examine the potency of HBsAg to enhance T cell stimulatory activity by DC, 25.000/well freshly isolated HLA-A2+ BDCA1+ mDC were pre-incubated for 30 minutes at 37 °C with 1 µg/ml pHBsAg or medium as control and subsequently incubated with 100 ng/ml HBV $Core_{18-27}$ peptide, for 20h at 37 °C. Subsequently, DC were washed 2 times and co-cultured with 50.000 HLA-A2-restricted HBV $Core_{18-27}$-specific CD8+ T cells [15]. To examine cross-presentation of HBsAg by DC, 25.000/well freshly isolated HLA-A2+ BDCA1+ mDC were pre-incubated for 1h at 37 °C with 5 µg/ml rHBsAg or medium as a control. After 2 washes, DC were co-cultured with 50.000 HLA-A2-restricted $HBsAg_{183-192}$-specific CD8+ T cells [15,16]. HBV-specific CTL were kindly provided by Prof. Dr. A. Bertoletti, Emerging Infectious Diseases, Duke-Nus Graduate Medical School, Singapore). After 20h, supernatants were collected and IFNγ was measured by ELISA (eBioscience).

Analysis of sCD14 concentration and sCD14-HBsAg complexes by ELISA
CD14 concentration and sCD14-HBsAg complexes were analyzed by sandwich ELISA. For sCD14 and sCD14 – HBsAg complexes in serum, samples were diluted 1000x and 100x, respectively. Coating antibody was rat anti-human sCD14 (clone 55-3, BD Biosciences). Detection antibodies were biotinylated anti-human sCD14 (clone 3-C39, BD Biosciences) or biotinylated anti-HBsAg (clone 9H9)[17]. Assay diluent, used for blocking and sample dilution, streptavidin-HRP and TMB solution were all from eBioscience. Wash buffer was PBS + 0.05% Tween20. Reaction was stopped with 1:3 v/v of H_2SO_4. Optical density (OD) at 450 nm was measured on Biorad imager.

Patients
Serum of sixty patients with HBV infection that visited our outpatient clinic (ErasmusMC) and fifteen age- and sex-matched healthy control individuals was collected and stored at -80°C. The sixty patients were all seropositive for HBsAg and represented a well characterized cohort belonging to different clinical phases of chronic HBV infection according to standardized criteria [18]. Serum HBV DNA was determined by realtime PCR using a Cobas 48 (Roche). HBsAg levels were determined on a Cobas 411 analyzer (Roche). Serum ALT was measured on an automated analyzer. Qualitative HBeAg and anti-HBeAg were measured on an Abbot Architect analyzer. For a subgroup of these patients, PBMCs were also collected and stored at -150°. The institutional ethical review board of the Erasmus MC, Rotterdam approved the clinical protocols, and written informed consent was obtained from all individuals prior to their donation of blood.

Analysis of DC phenotype ex vivo
1 million thawed PBMC from patients with CHB (n=4/group) and healthy controls (n=7) were analyzed for mDC-specific activation markers by flow cytometry. Samples were stained for 30 minutes at 4˚C in flow buffer. Antibodies used were mouse anti-BDCA1-PE (Miltenyi Biotec), mouse anti-CD20-eFluor450, anti-CD80-FITC (mIgG1) or isotype control and anti-CD83-APC (mIgG1) or isotype control (all eBioscience). Dead cells were excluded with Aqua dead cell stain (eBioscience). mDC were gated as BDCA1+/CD20-. Isotype controls were used to discriminate positive cells from non-specific background staining.

Results

HBsAg induces maturation, cytokine production and enhanced T cell activation capacity in myeloid DC
The interaction between BDCA1+ mDC and patient-derived HBsAg (pHBsAg) was studied in vitro by exposing freshly isolated mDC to fluorescent pHBsAg (FL-HBsAg). After 2 hours of incubation with FL-HBsAg and subsequent co-staining for HLA-DR to visualize the cell membrane, confocal microscopy showed intracellular HBsAg positivity in the majority of DC, indicating that mDC can efficiently take up HBsAg (Fig. 1A). After 20 hours of incubation with FL-HBsAg, HBsAg+ DC formed clusters, indicating that HBsAg induced activation of DC. Therefore, the functional consequence of this interaction was further investigated by exposing BDCA1+ mDC isolated from healthy donors to different concentrations of HBsAg. Exposure of mDC to pHBsAg resulted in dose-dependent upregulation of the typical DC maturation marker CD83 and the co-stimulatory molecule CD40 (Fig. 1B), and dose-dependent secretion of L-6 and IL-12 (Fig. 1C). In addition to DC maturation and cytokine production, pre-incubation of mDC with pHBsAg enhanced peptide-specific IFNγ production by HBV core-specific CTL (Fig. 1D). Interestingly, HBsAg-positive DC were able to cross-present HBsAg-derived epitope to the cognate CTL, in the absence of any additional maturation stimulus (Fig. 1E).
Since antibodies capable of neutralizing the immune stimulatory effect of HBsAg are not available, we addressed the specific role of HBsAg by several approaches. Heat treatment at 100 °C completely abrogated the stimulatory activity of LPS whereas the stimulatory activity of HBsAg was maintained (Fig. 1F). Depletion of HBsAg from the preparation via either heparin chromatography or specific immune precipitation significantly reduced

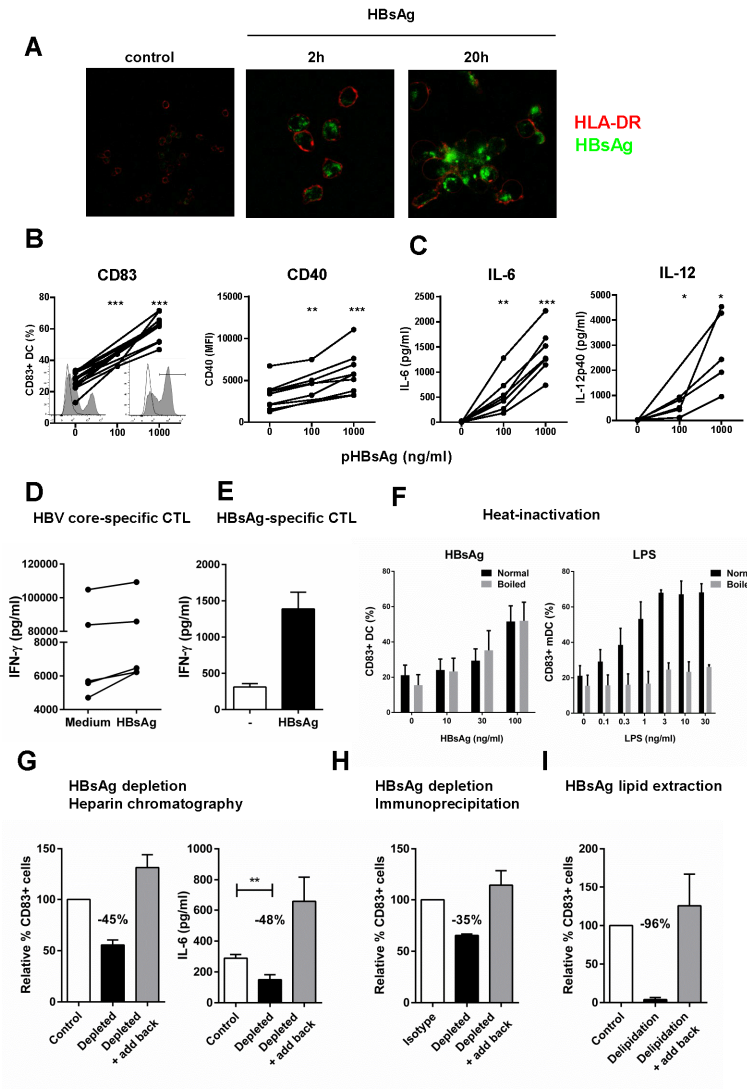

Figure 1: Interaction with HBsAg induces mDC maturation and function. (A) BDCA1+ mDC were incubated with medium (control) or fluorescently-conjugated HBsAg for 2 or 20h and binding/uptake was measured by confocal microscopy after co-staining for HLA-DR. Representative merged confocal images of 2 independent experiments are shown. (B/C) BDCA1+ mDC were incubated for 20h with medium, 100 ng/ml or 1000 ng/ml patient-derived HBsAg (pHBsAg). (B) CD83 and CD40 expression was determined by flow cytometry and displayed as % positive DC or mean fluorescense intensity (MFI), respectively. Representative FACS histograms show mDC incubated with medium (left) or 1000 ng/ml pHBsAg (right) and stained for CD83 (grey) or isotype control (white). (C) Concentrations of IL-6 and IL-12p40 in mDC supernatants were determined by ELISA. Paired Student's t-test was used to compare means of 6-15 experiments with different donors. * P<0.05, ** P<0.01, *** P<0.001. (D) IFNγ production by HBV Core-specific CTL after 20h co-culture with HLA-A2+ BDCA1+ mDC pre-incubated with pHBsAg or medium and subsequently loaded with peptide HBV $Core_{18-27}$. Summary of five experiments with DC from different subjects. (E) IFNγ production by HBsAg-specific CTL after 20h co-culture with HLA-A2+ BDCA1+ mDC incubated with rHBsAg or medium control (-). Shown is mean±SD of IFNγ from 2 experiments with DC from different subjects. (F) BDCA1+ mDC were exposed to increasing concentrations of pHBsAg (left graph) or LPS (right graph), either untreated (normal, black bars) or heated at 100°C (boiled, grey bars). Shown is mean±SD of %CD83+ DC from 2 experiments with DC from different subjects. (G-I) BDCA1+ mDC were exposed to (G) untreated pHBsAg (control) or a preparation depleted for pHBsAg via heparin affinity chromatography (depleted) or the depleted preparation supplemented with normal pHBsAg (depleted + add-back); (H) pHBsAg after immunoprecipitation with protG-isotype (isotype) or protG-Hepatect (depleted) or the latter with added normal pHBsAg (depleted + add-back); (I) pHBsAg that was treated with 2% OG (delipidation) or 0% OG (control) and pHBsAg treated with 2% OG and complemented with normal HBsAg (delipidation + add-back). Mean±SEM of relative %CD83+ mDC (G-I) and IL-6 concentration in mDC supernatant (G) based on three to five pooled experiments with DC from different subjects. % decrease of second bar compared to first bar is indicated. Paired Student's t-test was used to compare means of IL-6 production (G). ** P<0.01

upregulation of CD83 and secretion of IL-6 (Fig. 1G/H). When HBsAg was added-back to the depleted preparations, DC maturation and secretion of IL-6 was restored (Fig. 1 G/H) showing that the treatment by itself did not cause the reduced DC maturation and function. Heparin depletion of LPS did not reduce its ability to stimulate BDCA1+ DC (data not shown). Together, these experimental approaches demonstrated that the observed DC maturation and function was specifically dependent on HBsAg.

In addition to patient-derived HBsAg, DC maturation and function was also induced by recombinant HBsAg (rHBsAg) produced in Chinese Hamster Ovary (CHO) cells, but neither by a control CHO-derived glycoprotein from the same company, recombinant Nerve Growth Factor beta (beta-NGF), nor by recombinant HBsAg produced in yeast (yHBsAg) (data not shown). The immune-stimulatory effect observed by both rHBsAg and pHBsAg, but not yHBsAg, suggests that the mammalian nature of the host-derived lipids and/or glycosylation may be important for its immune stimulatory effect. The role of lipids in the HBsAg particle was further addressed by exposing mDC to pHBsAg that was treated with 2% β-D-octyl glucoside (OG) to extract lipids from the particle, as described previously [14]. OG-treated but not sham-treated HBsAg totally lost its capacity to induce DC maturation (Fig. 1I), suggesting that the lipids play a major role in the immune-stimulatory effect of HBsAg on DC. In contrast, similar OG treatment of LPS only partly reduced its capacity to induce DC maturation and function (data not shown). Thus, exposure of freshly isolated mDC to HBsAg induces DC maturation, cytokine production and enhanced capacity to activate virus-specific CTL. Furthermore, the immune-stimulatory effect on myeloid DC is specific for HBsAg and is restricted to HBsAg particles from a mammalian host.

HBsAg-induced DC maturation is dependent on CD14 and TLR4

Since monocytes are described to bind HBsAg via CD14 [19], we investigated the role of this molecule in cellular activation by HBsAg. First, we examined the role of CD14 in HBsAg-induced activation of monocytes. pHBsAg efficiently induced secretion of IL-6 and TNFα in the majority of monocytes, which was largely absent when monocytes were pre-treated with αCD14 but not isotype control antibodies (data not shown). Next, we examined the role of CD14 in HBsAg-dependent activation of BDCA1+ mDC. To exclude a possible interference of endotoxin contamination, we used HBsAg concentrations that were tested free of endotoxin contamination by Endolisa (see Methods section). Pre-incubation of mDC with αCD14, but not isotype control antibodies completely abrogated pHBsAg-dependent upregulation of CD83, CD40 (Fig. 2A) and secretion of IL-6 and IL-12 (Fig. 2B), but did not affect pHBsAg uptake (Fig. 2C), suggesting a crucial role for CD14 in HBsAg-induced maturation and function of mDC. Given the essential role of CD14 as co-receptor for TLR4 [20], we also investigated the role of TLR4 in HBsAg-induced DC maturation. Interestingly, neutralizing antibodies to TLR4 also blocked HBsAg-mediated upregulation of CD83 (Fig. 2D), suggesting that HBsAg-induced DC maturation is dependent on both CD14 and TLR4. HBsAg-induced DC maturation by rHBsAg was also dependent on CD14 and partially dependent on TLR4 (Fig. 2E). The observation that only a sub-population of about 11% BDCA1+ DC express CD14 on their cell membrane (Fig. 2F) was not in line with the crucial observed role of CD14 in HBsAg-induced DC maturation. Additional depletion of CD14+ cells revealed that purified CD14- BDCA1+ DC were also activated by HBsAg in a CD14-dependent manner (Fig. 2G). Based on these results, the role of membrane-expressed CD14 (mCD14) in HBsAg-mediated DC maturation was brought into question and the data pointed towards a role for soluble CD14 (sCD14), which is naturally present in FCS and human serum and can facilitate cellular

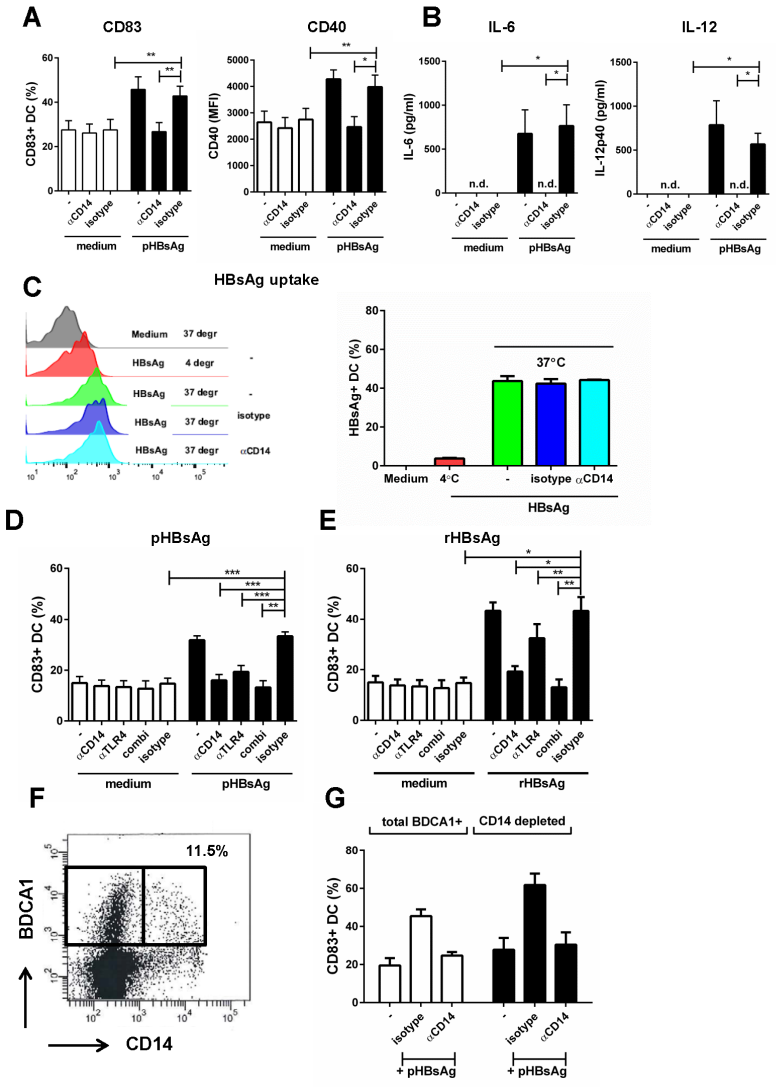

Figure 2: HBsAg-induced DC maturation is dependent on CD14 and TLR4.
(A/B) mDC were pre-incubated with αCD14 or isotype control and subsequently incubated with medium or 100 ng/ml pHBsAg for 20 hr. Mean±SEM of %CD83+ DC and CD40 MFI (A) and IL-6 or IL-12p40 (B) of 4-5 experiments with different donors. (C) PBMC depleted for CD14+ cells were pre-incubated with medium (-), αCD14 or isotype control and subsequently exposed to 1 µg/ml FL-HBsAg for 2 hr at 37°C. mDC exposed to medium and FL-HBsAg at 4°C were used as controls. %HBsAg+ cells of BDCA1+CD19-DC was determined by flow cytometry. Representative FACS histograms and summary of mean±SD of duplicate showing %HBsAg+ DC. Data is representative for three experiments with different donors. (D/E) mDC were pre-incubated with medium (-), αCD14, αTLR4, a combination of αCD14 and αTLR4 (combi) or isotype control and subsequently exposed to medium or 100 ng/ml pHBsAg (D) or recombinant HBsAg (rHBsAg) (E) for 20 hr. Mean±SEM of %CD83+ DC of four (D) or three (E) independent experiments with different donors. Paired Student's t-test: * P<0.05, ** P<0.01, *** P<0.001. (F) Example of flow cytometry analysis of CD14 and BDCA1 expression on PBMC. Indicated percentage represents %CD14+ of BDCA1+ cells. Representative plot of five experiments with different donors. (G) mDC, either isolated by standard procedure (white bars) or with additional CD14 depletion (black bars), were pre-incubated with αCD14 or isotype control and subsequently exposed to medium (-) or pHBsAg. Data represents mean±SEM %CD83+ mDC of three experiments with different donors.

activation of cells that do not express mCD14 [21,22].

HBsAg-mediated DC maturation is dependent on sCD14 naturally present in FCS and human serum

To investigate the role of sCD14, HBsAg-dependent DC maturation was compared between serum-free cultures or cultures supplemented with FCS (Fig. 3A/B). Serum-free cultures neither impaired cell viability, nor induced a general defect in DC maturation, as TNFα/IL-1β-induced DC maturation was still intact (data not shown). HBsAg-induced DC maturation and cytokine production was absent in serum-free cultures, but was restored by addition of FCS (Fig. 3A/B), suggesting that a serum component is needed to support HBsAg-induced DC maturation. Similar as with FCS, HBsAg-induced DC maturation and cytokine production was restored by adding human serum (Fig. 3C/D). Furthermore, in the presence of human serum, HBsAg-dependent DC maturation was totally blocked by pre-incubation with CD14 neutralizing antibodies (Fig. 3C/D), suggesting a crucial role for serum factor sCD14 in facilitating HBsAg-dependent DC maturation.

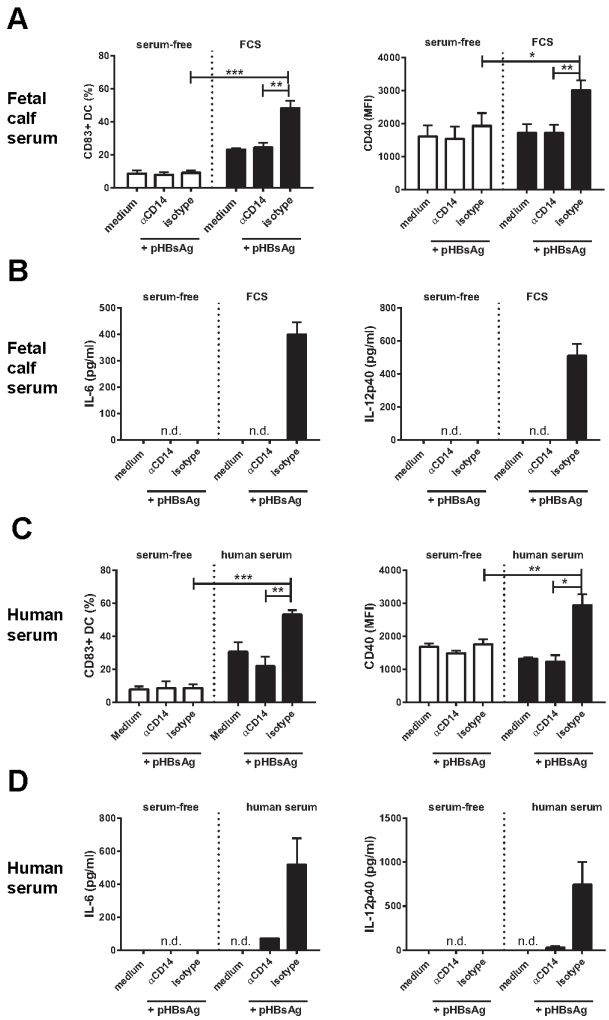

Figure 3: HBsAg-mediated DC maturation is dependent on sCD14 naturally present in FCS and human serum.

mDC were pre-incubated with αCD14 or isotype control and subsequently incubated with 100 ng/ml pHBsAg or medium in Xvivo serum-free medium with or without 8% FCS (A/B) or 1% human serum (C/D) for 20 hr. Mean±SEM of %CD83+ mDC or CD40 MFI (A/C) and mean±SEM of IL-6 and IL-12p40 (B/D) is displayed. Paired Student's t-test was used to compare means of five (A/B) and three (C/D) experiments with different donors. Paired Student's t-test: * P<0.05, ** P<0.01, *** P<0.001.

Low CD14 in umbilical cord blood-derived plasma correlates with reduced HBsAg-induced DC maturation

Interestingly, plasma from umbilical cord blood contains significantly lower sCD14 levels than plasma from adults (Fig. 4A and [23]. To further establish the role of sCD14 in BDCA1+ DC activation by HBsAg, we tested if neonatal serum consequently has a reduced capacity to facilitate HBsAg-induced DC maturation compared to adult serum. HBsAg-dependent maturation of healthy control DC was compared in serum-free medium supplemented

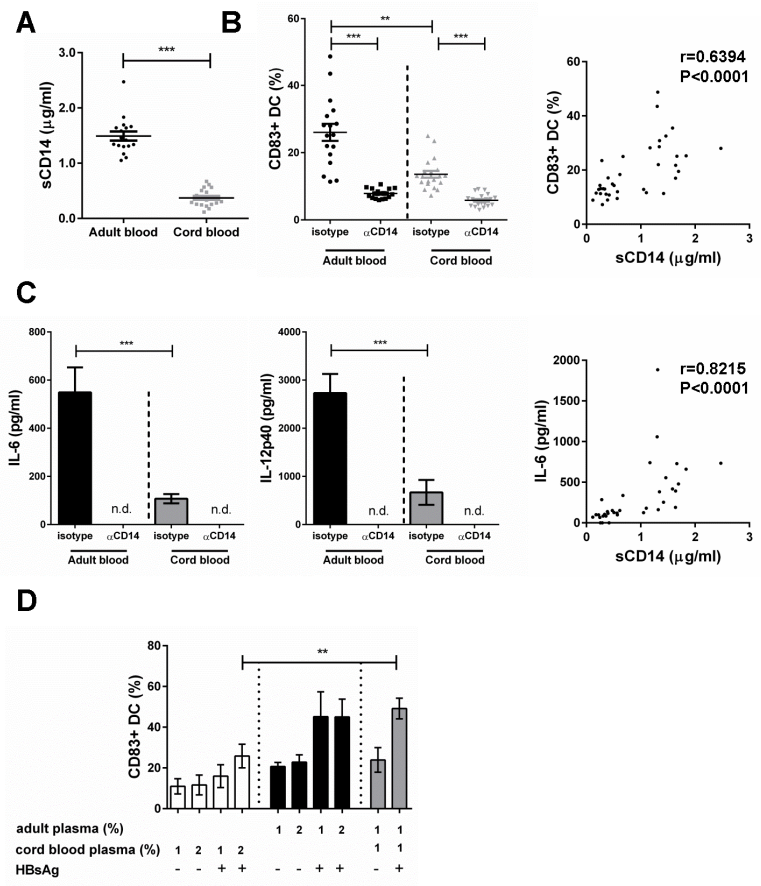

Figure 4: Low CD14 in neonates correlates with reduced HBsAg-induced DC maturation.

(A) Mean±SEM of sCD14 concentrations in plasma obtained from peripheral blood of healthy adults (n=17) or umbilical cord blood (n=20). Student's t-test ***P <0.001. (B/C) mDC obtained from healthy adult donor were pre-treated with isotype control or αCD14 and subsequently exposed to 100 ng/ml pHBsAg in the presence of plasma from adult blood (n=17) or cord blood (n=20) for 20 hr. (B) Summary of mean±SEM of %CD83+ mDC (left) and correlation between sCD14 concentration and %CD83+ mDC (right) after exposure to HBsAg for each individual plasma sample. (C) Summary of mean±SEM of IL-6 or IL-12p40 in mDC supernatants and correlation between sCD14 concentration and IL-6 concentration after exposure to HBsAg for each individual plasma sample. Student's t-test: ** P<0.01, *** P<0.001. Spearman correlation coefficient and P-value is shown. Data shown in B/C is representative for 3 independent experiments with DC of different donors. (D) mDC obtained from healthy adult donors were exposed to medium (-) or 100 ng/ml pHBsAg (+) in the presence of Xvivo medium supplemented with 1 or 2% plasma from adult blood, cord blood or both. Mean±SD of %CD83+ mDC from 2 independent experiments with different mDC donors in which 3 different cord blood samples were separately tested. Paired Student's t-test: ** P<0.01.

with plasma samples either derived from different adult individuals or different cord blood plasma samples. Upregulation of CD83 (Fig. 4B), CD40 (data not shown) and production of IL-6 and IL-12 (Fig. 4C) in response to HBsAg was indeed significantly reduced when cultures were supplemented with cord blood plasma samples compared to adult plasma samples. In both cultures, HBsAg-induced maturation and function were completely abrogated in the presence of CD14 neutralizing antibodies. Furthermore, the level of sCD14 in each individual plasma sample highly correlated with its capacity to mediate HBsAg-induced DC maturation and cytokine production (Fig. 4B/C). Increasing concentrations of cord blood plasma enhanced HBsAg-induced DC maturation (Fig. 4D), however, HBsAg-induced DC maturation never reached the level as observed with adult plasma (data not shown). Furthermore, addition of adult plasma together with neonatal plasma in a 1:1 ratio resulted in HBsAg-induced DC maturation similar to the level induced by adult plasma alone (Fig. 4D). These data suggest that the reduced capacity of cord blood plasma to facilitate HBsAg-dependent DC activation is not caused by the presence of a dominant negative/inhibitory factor in cord blood plasma, but can be mainly attributed to a suboptimal concentration of sCD14. Together these results indicate that strength of HBsAg-induced maturation and function of DC is regulated by the concentration of sCD14.

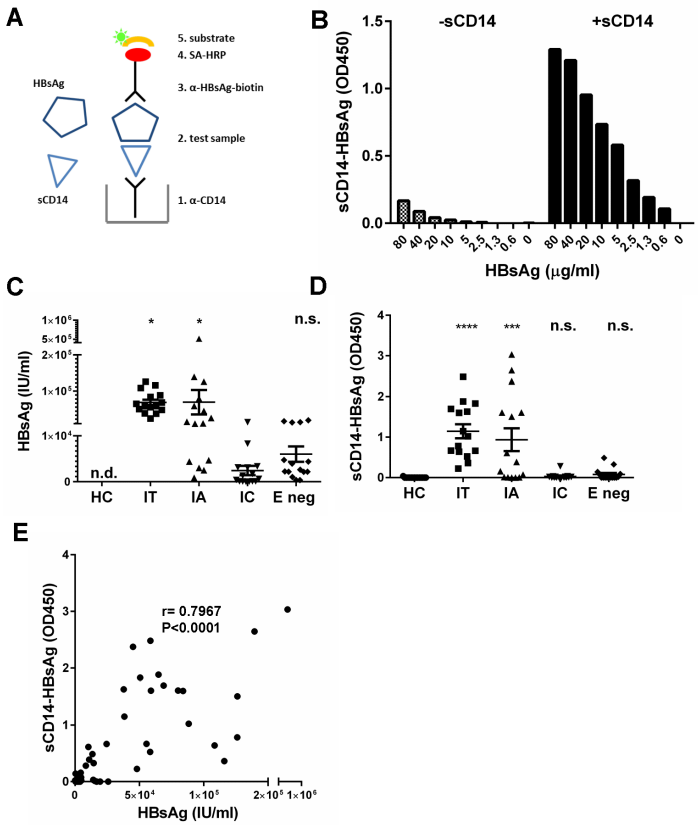

Figure 5: sCD14-HBsAg complexes are detected in vitro and in serum of HBV-infected patients.
(A) Schematical representation of sandwich ELISA to measure complexes of soluble CD14 (sCD14) and HBsAg. (B) Detection of sCD14-HBsAg complexes (optical density, OD) by ELISA after 20 hr incubation of indicated concentrations pHBsAg with medium (-sCD14) or 630 ng/ml recombinant sCD14 (+sCD14). Data is representative of 3 similar experiments. (C/D) Concentration of HBsAg (C) and abundance (OD) of sCD14-HBsAg complexes (D) in sera of healthy controls and HBV-infected patients categorized by clinical disease stages (HC= healthy controls, IT=immune tolerant, IA=immune active, IC=inactive carrier, E neg=E negative CHB patients). Data represent individual values and mean of n=15/group. Means were compared by ANOVA followed by Dunnett multiple comparison test to compare means of different groups to reference group, which was IC group (C) or HC group (D): **** P<0.0001, *** P<0.001, * P<0.05. n.s. not significant. (E) Correlation between OD of sCD14-HBsAg complexes and HBsAg level. Spearman r=0.7967, P<0.0001.

sCD14-HBsAg complexes are detected in vitro and in serum of HBV-infected patients
Next, it was important to know whether HBsAg and sCD14 directly interact. To investigate this, we developed a sandwich ELISA to detect complexes of HBsAg and sCD14 (Fig. 5A). The ELISA specifically detected in vitro-formed complexes of pHBsAg and recombinant sCD14 but not pHBsAg or sCD14 alone (Fig. 5B). The detection of sCD14 – HBsAg complexes demonstrated that HBsAg and CD14 can directly interact. Furthermore, the dose-dependent detection of sCD14 – HBsAg complexes (Fig. 5B) indicated that the ELISA is also suitable for semi-quantification of these complexes. To investigate whether these complexes are also present in vivo, we tested serum samples from HBV-infected patients and healthy control subjects for the presence and abundance of sCD14-HBsAg complexes. The patients represented different clinical disease stages, based on viral load, serum HBsAg (Fig. 5C) and ALT (Table 1). The HBsAg concentrations measured in serum of these patients could reach concentration of more than 5x10e5 IU/ml, which corresponds to approximately 1000 µg/ml (Table 1). This level exceeds more than 500x the concentrations used in our in vitro experiments which were thus well within the range found in patients. sCD14-HBsAg complexes were readily detected in serum of HBV-infected patients, but were non-detectable in serum of healthy control individuals (Fig. 5D). In addition, the sCD14-HBsAg complexes varied substantially in different HBV clinical disease phases; soluble CD14-HBsAg complexes were detected in serum of patients in immune tolerant (IT) and immune active (IA) stages, but were low in serum of patients in inactive carrier (IC) and E negative CHB stages (Fig. 5D, $P < 0.001$). The abundance of sCD14-HBsAg complexes correlated with the serum level of HBsAg (Fig. 5E) and the viral load but not with ALT (data not shown).

sCD14-HBsAg complexes are associated with a more matured DC phenotype in vivo
To investigate whether the immune stimulatory effect of HBsAg on BDCA1+ DC is also relevant in vivo, we compared cell surface expression of maturation markers CD80 and CD83 on BDCA1+ DC directly ex vivo between CHB patients and healthy control individuals (Fig. 6A/B). We found that in agreement with our *in vitro* findings, CHB patients had higher percentages of CD80+ and CD83+ BDCA1+ DC compared to healthy controls (Fig. 6B). Moreover, percentages of CD80+ and CD83+ DC were positively correlated with the concentration of HBsAg in serum (Fig. 6C) and with the abundance of sCD14 – HBsAg complexes (Fig. 6D), suggesting that HBsAg can activate BDCA1+ mDC, also *in vivo*.

Discussion

Dendritic cells (DC) are considered important players in the regulation of anti-viral immunity. Proper activation of DC is a pre-requisite for the induction of effective virus-specific immunity. In the present study, we demonstrated that the main HBV envelop protein HBsAg can activate BDCA1+ myeloid DC leading to enhanced DC maturation and T cell stimulatory capacity including cross-presentation of HBsAg by DC. Patient-derived HBsAg was used at concentrations also frequently found in serum of HBV-infected individuals. A positive correlation between HBsAg levels and percentage of activated BDCA1+ DC in a well-defined cohort of patients with chronic HBV infection (CHB) suggests that the observed immune stimulatory-effect of HBsAg is also present in vivo. Only a limited number of studies addressed DC phenotype of myeloid DC in CHB patients ex vivo, as reviewed in [8,24]. Enhanced mDC maturation in CHB patients compared to healthy control individuals was

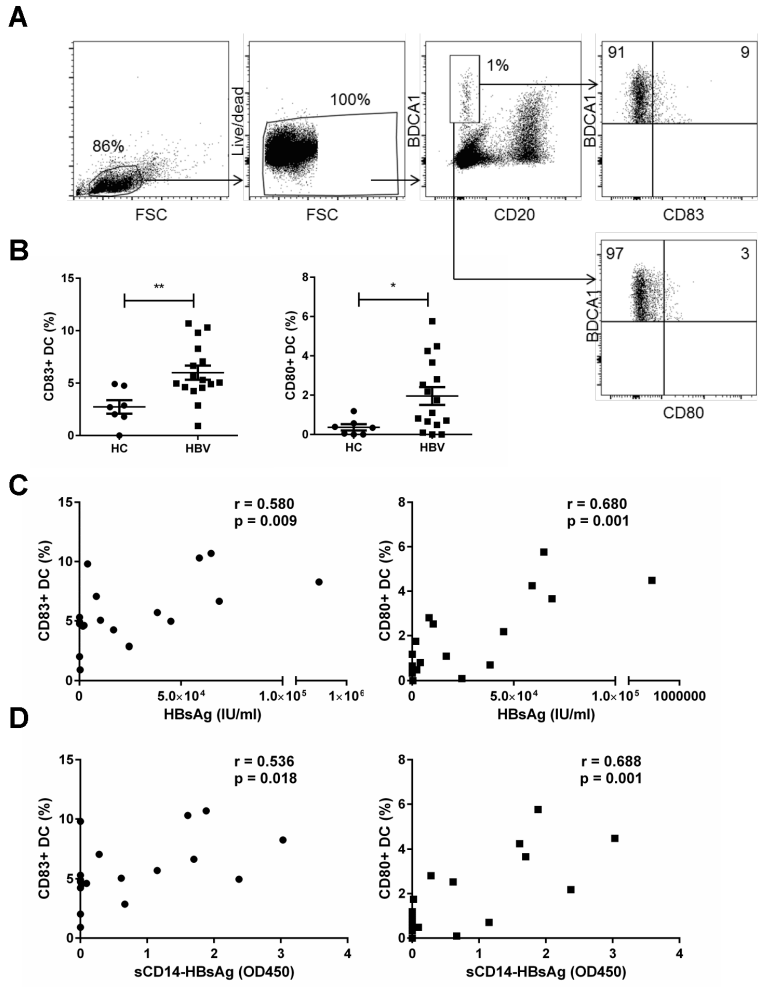

Figure 6: HBsAg concentration sCD14-HBsAg complexes are associated with matured DC phenotype in vivo.
(A) Gating strategy for analysis of CD80 and CD83 expression on gated BDCA1+ DC in total PBMC. Isotype control antibody was used to define background for CD80 and CD83. (B) Mean percentage of CD83+ (left) and CD80+ (right) BDCA1+ DC between patients with chronic HBV infection (HBV, n=16) and healthy control individuals (HC, n=7). Student's t-test: * P<0.05, ** P<0.01. (C) Correlation between HBsAg concentration and percentage CD83+ (left) or CD80+ (right) BDCA1+ DC. Spearman r and P is indicated. (D) Correlation between sCD14-HBsAg complexes and percentage CD83+ (left) or CD80+ (right) BDCA1+ DC. Spearman r and P is indicated.

previously reported by a single study from our group [25], whereas the majority of these studies did not find enhanced DC maturation in CHB patients [8,24]. We found a lot of variation between patients however, and because HBsAg levels were not taken into account in these previous studies it is possible that the effect on DC maturation may have been overlooked. In addition, our cohort consisted solely of untreated patients without certain co-morbidities or advanced fibrosis, factors that could influence DC maturation.
Interestingly, the immune-stimulatory effect of HBsAg observed here was specific and

restricted to patient-derived HBsAg or recombinant HBsAg produced in mammalian cells, suggesting that the type of glycosylation and/or host-derived lipids/factors may be important for its immune stimulatory effect. A role for specific glycosylation would be in line with other viral glycoproteins that can activate DC, including the Fusion protein of Respiratory Syncytial Virus and HIV-derived gp120 as previously described [26,27].

In addition to the immune stimulatory effect of HBsAg, we demonstrated a novel and crucial role for CD14 in HBsAg-mediated activation of DC and monocytes, thus identifying CD14 as a receptor for HBV. CD14 is a glycoprotein expressed on the cell surface of cells of the myeloid lineage and is most well-known for its role as PRR [20,28]. In contrast to HBsAg-mediated DC activation, CD14 blockade did not reduce binding of HBsAg to DC. Based on our previous work showing a role for the mannose receptor in binding of HBsAg [6], it is likely that multiple receptors can facilitate HBsAg binding to DC and CD14 is redundant for binding, but not activation.

CD14 serves as a co-receptor for several TLRs, including TLR4, and contributes to ligand recognition and cellular activation [20], but an autonomous signaling function on DC has also been described [29]. Since both TLR4 blockade and CD14 blockade resulted in abrogation of HBsAg-induced DC maturation, we conclude that recognition of HBsAg by mDC is mediated by the TLR4/CD14 receptor complex. In addition to the well-known role of CD14/TLR4 in recognition of bacterial products [20], this study and previous work together show that these receptors also have an emerging role in recognition of viral glycoproteins [26,27]. Similar to BDCA1+ DC, HBsAg can also activate other cells of myeloid origin including monocytes and Kupffer cells (KC) [9,11], but not BDCA3+ myeloid DC (data not shown) and plasmacytoid DC [30], which may relate to the absence of TLR4 on these cells [31,32]. The identification of CD14 and TLR4 as receptors for HBsAg may also have implications for HBV-associated liver pathology in the chronic phase of HBV infection. Activated hepatic stellate cells, the most fibrinogenic cell type in the liver, have a functional CD14/TLR4 response [33] and may therefore be activated by interaction with HBsAg in the liver.

In addition to membrane-bound CD14 (mCD14), CD14 exists in soluble form, which is either released from CD14+ cells [34,35] or secreted by hepatocytes [36,37] and can also be blocked by our antibody. Based on the findings that HBsAg-induced DC maturation could be abrogated by blocking CD14, DC maturation was not dependent on expression of mCD14 and required the presence of FCS or human serum, naturally containing soluble CD14 (sCD14), we concluded that HBsAg-dependent maturation of mDC is driven by sCD14. The pivotal role of sCD14 was further supported by the observation that sCD14 and HBsAg directly interact, both in vitro and in vivo. The abundance of sCD14 – HBsAg complexes correlated with percentage of activated DC in these patients.

In the present study we observed that umbilical cord-blood derived plasma contained low levels of sCD14, as was previously reported in newborns [23,38], and had reduced capacity to support HBsAg-induced DC maturation and function. This low sCD14 concentration coincides with a high risk of developing chronic hepatitis B (CHB) upon HBV infection in newborns [39]. The immunological mechanisms underlying the high risk for neonates to develop CHB upon perinatal HBV infection are poorly understood. Based on our results, it is tempting to speculate that insufficient DC activation due to low sCD14 levels may be one of the factors contributing to inadequate HBV specific immunity in HBV-infected newborns. Seemingly paradoxical, Hong and colleagues recently showed that monocytes of HBV-exposed newborns had an enhanced activation state compared to those from unexposed neonates [40]. Monocytes however, in contrast to the BDCA1+ DC we studied here, are not

able to induce activation of naïve virus-specific T cells and express membrane CD14 and thus do not depend on sCD14 for their activation. Thus, reduced sCD14 may in particular impact the initiation of HBV-specific immunity in these newborns via myeloid DC. Taken together, despite the recent work of Hong et al. and the work we present here, further research is required to identify the definite factors that determine the high risk to develop CHB in newborns.

We conclude that HBsAg can induce maturation of BDCA1^{+} mDC via a sCD14-dependent mechanism. These findings help to comprehend the early steps in development of HBV-specific immune responses, which is essential to understand the inadequate HBV-specific immune responses in patients with chronic HBV infection and to facilitate the development of effective immuno-therapies for this disease.

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References

1. H.J. Yim, A.S.-F. Lok, Natural history of chronic hepatitis B virus infection: what we knew in 1981 and what we know in 2005., Hepatology (Baltimore, Md.). 43 (2006) S173–81. doi:10.1002/hep.20956.
2. Schweitzer, J. Horn, R.T. Mikolajczyk, G. Krause, J.J. Ott, Estimations of worldwide prevalence of chronic hepatitis B virus infection: a systematic review of data published between 1965 and 2013., Lancet. 386 (2015) 1546–55. doi:10.1016/S0140-6736(15)61412-X.
3. Bertoletti, C. Ferrari, Innate and adaptive immune responses in chronic hepatitis B virus infections: towards restoration of immune control of viral infection., Gut. 61 (2012) 1754–64. doi:10.1136/gutjnl-2011-301073.
4. N. van Montfoort, E. van der Aa, A.M. Woltman, Understanding MHC class I presentation of viral antigens by human dendritic cells as a basis for rational design of therapeutic vaccines., Frontiers in Immunology. 5 (2014) 182. doi:10.3389/fimmu.2014.00182.
5. M. Dalod, R. Chelbi, B. Malissen, T. Lawrence, Dendritic cell maturation: functional specialization through signaling specificity and transcriptional programming., The EMBO Journal. 33 (2014) 1104–16. doi:10.1002/embj.201488027.
6. M.L. Op den Brouw, R.S. Binda, T.B.H. Geijtenbeek, H.L.A. Janssen, A.M. Woltman, The mannose receptor acts as hepatitis B virus surface antigen receptor mediating interaction with intrahepatic dendritic cells., Virology. 393 (2009) 84–90. doi:10.1016/j.virol.2009.07.015.
7. Untergasser, U. Zedler, A. Langenkamp, M. Hösel, M. Quasdorff, K. Esser, et al., Dendritic cells take up viral antigens but do not support the early steps of hepatitis B virus infection., Hepatology (Baltimore, Md.). 43 (2006) 539–47. doi:10.1002/hep.21048.
8. A.M. Woltman, A. Boonstra, H.L.A. Janssen, Dendritic cells in chronic viral hepatitis B and C: victims or guardian angels?, Gut. 59 (2010) 115–25. doi:10.1136/gut.2009.181040.
9. Boltjes, Z.M. Groothuismink, G.W. van Oord, H.L.A. Janssen, A.M. Woltman, A. Boonstra, Monocytes from chronic HBV patients react in vitro to HBsAg and TLR by producing cytokines irrespective of stage of disease., PloS One. 9 (2014) e97006. doi:10.1371/journal.pone.0097006.
10. R.-H. Jan, Y.-L. Lin, C.-J. Chen, T.-Y. Lin, Y.-C. Hsu, L.-K. Chen, et al., Hepatitis B virus surface antigen can activate human monocyte-derived dendritic cells by nuclear factor kappa B and p38 mitogen-activated protein kinase mediated signaling., Microbiology and Immunology. 56 (2012) 719–27. doi:10.1111/j.1348-0421.2012.00496.x.
11. Boltjes, N. van Montfoort, P.J. Biesta, M.L. Op den Brouw, J. Kwekkeboom, L.J.W. van der Laan, et al., Kupffer cells interact with HBsAg in vivo and in vitro leading to pro-inflammatory cytokine production and NK cell function., The Journal of Infectious Diseases. 211 (2015) 1268–78. doi:10.1093/infdis/jiu599.
12. H. Schwarz, M. Schmittner, A. Duschl, J. Horejs-hoeck, Residual Endotoxin Contaminations in Recombinant Proteins Are Sufficient to activate human CD1c+ dendritic cells, PloS One. 9 (2014) e0113840. doi:10.1371/journal.pone.0113840.
13. B. Gao, Y. Wang, M.-F. Tsan, The heat sensitivity of cytokine-inducing effect of lipopolysaccharide., Journal of Leukocyte Biology. 80 (2006) 359–66. doi:10.1189/jlb.1205738.
14. Desombere, A. Willems, Y. Gijbels, G. Leroux-roels, Partial Delipidation Improves the T-Cell Antigenicity of Hepatitis B Virus Surface Antigen, Journal of Virology. 80 (2006) 3506–3514. doi:10.1128/JVI.80.7.3506.
15. A.J. Gehring, S.-A. Xue, Z.Z. Ho, D. Teoh, C. Ruedl, A. Chia, et al., Engineering virus-specific T cells that target HBV infected hepatocytes and hepatocellular carcinoma cell lines., Journal of Hepatology. 55 (2011) 103–10. doi:10.1016/j.jhep.2010.10.025.
16. M. Haniffa, A. Shin, V. Bigley, N. McGovern, P. Teo, P. See, et al., Human Tissues Contain CD141(hi) Cross-Presenting Dendritic Cells with Functional Homology to Mouse CD103(+) Nonlymphoid Dendritic Cells., Immunity. 37 (2012) 1–14. doi:10.1016/j.immuni.2012.04.012.

17. R.A. Heijtink, J. Kruining, Y.A. Weber, R.A. de Man, S.W. Schalm, Anti-hepatitis B virus activity of a mixture of two monoclonal antibodies in an “inhibition in solution” assay., Hepatology (Baltimore, Md.). 22 (1995) 1078–83. http://www.ncbi.nlm.nih.gov/pubmed/7557854 (accessed June 4, 2015).
18. T. Vanwolleghem, J. Hou, G. van Oord, A.C. Andeweg, A.D.M.E. Osterhaus, S.D. Pas, et al., Re-evaluation of hepatitis B virus clinical phases by systems biology identifies unappreciated roles for the innate immune response and B cells., Hepatology (Baltimore, Md.). 62 (2015) 87–100. doi:10.1002/hep.27805.
19. P. Vanlandschoot, F. Van Houtte, A. Roobrouck, A. Farhoudi, F. Stelter, D.L. Peterson, et al., LPS-binding protein and CD14-dependent attachment of hepatitis B surface antigen to monocytes is determined by the phospholipid moiety of the particles., The Journal of General Virology. 83 (2002) 2279–89.
20. Zanoni, F. Granucci, Role of CD14 in host protection against infections and in metabolism regulation., Frontiers in Cellular and Infection Microbiology. 3 (2013) 32. doi:10.3389/fcimb.2013.00032.
21. Z. Yang, M.A. Breider, R.C. Carroll, M.S. Miller, P.N. Bochsler, Soluble CD14 and lipopolysaccharide-binding protein from bovine serum enable bacterial lipopolysaccharide-mediated cytotoxicity and activation of bovine vascular endothelial cells in vitro., Journal of Leukocyte Biology. 59 (1996) 241–7. http://www.ncbi.nlm.nih.gov/pubmed/8603996 (accessed November 23, 2014).
22. V. Verhasselt, C. Buelens, F. Willems, D. De Groote, N. Haeffner-Cavaillon, M. Goldman, Bacterial lipopolysaccharide stimulates the production of cytokines and the expression of costimulatory molecules by human peripheral blood dendritic cells: evidence for a soluble CD14-dependent pathway., Journal of Immunology (Baltimore, Md. : 1950). 158 (1997) 2919–25. http://www.ncbi.nlm.nih.gov/pubmed/9058830 (accessed July 22, 2014).
23. T.D. LeVan, S. Guerra, W. Klimecki, M.M. Vasquez, I.C. Lohman, F.D. Martinez, et al., The impact of CD14 polymorphisms on the development of soluble CD14 levels during infancy., Genes and Immunity. 7 (2006) 77–80. doi:10.1038/sj.gene.6364276.
24. A.J. Gehring, J. Ann D’Angelo, Dissecting the dendritic cell controversy in chronic hepatitis B virus infection., Cellular & Molecular Immunology. 12 (2015) 283–91. doi:10.1038/cmi.2014.95.
25. E.T.T.L. Tjwa, G.W. van Oord, P.J. Biesta, A. Boonstra, H.L.A. Janssen, A.M. Woltman, Restoration of TLR3-activated myeloid dendritic cell activity leads to improved natural killer cell function in chronic hepatitis B virus infection., Journal of Virology. 86 (2012) 4102–9. doi:10.1128/JVI.07000-11.
26. E.A. Kurt-Jones, L. Popova, L. Kwinn, L.M. Haynes, L.P. Jones, R.A. Tripp, et al., Pattern recognition receptors TLR4 and CD14 mediate response to respiratory syncytial virus., Nature Immunology. 1 (2000) 398–401. doi:10.1038/80833.
27. Nazli, J.K. Kafka, V.H. Ferreira, V. Anipindi, K. Mueller, B.J. Osborne, et al., HIV-gp120 induces TLR2- and TLR4-mediated innate immune activation in human female genital epithelium., Journal of Immunology (Baltimore, Md. : 1950). 191 (2013) 4246–58. doi:10.4049/jimmunol.1301482.
28. J. Pugin, I.D. Heumann, A. Tomasz, V. V Kravchenko, Y. Akamatsu, M. Nishijima, et al., CD14 is a pattern recognition receptor., Immunity. 1 (1994) 509–16. http://www.ncbi.nlm.nih.gov/pubmed/7534618 (accessed August 5, 2014).
29. Zanoni, R. Ostuni, G. Capuano, M. Collini, M. Caccia, A.E. Ronchi, et al., CD14 regulates the dendritic cell life cycle after LPS exposure through NFAT activation., Nature. 460 (2009) 264–8. doi:10.1038/nature08118.
30. A.M. Woltman, M.L. Op den Brouw, P.J. Biesta, C.C. Shi, H.L.A. Janssen, Hepatitis B virus lacks immune activating capacity, but actively inhibits plasmacytoid dendritic cell function., PloS One. 6 (2011) e15324. doi:10.1371/journal.pone.0015324.
31. S.L. Jongbloed, A.J. Kassianos, K.J. McDonald, G.J. Clark, X. Ju, C.E. Angel, et al., Human CD141+ (BDCA-3)+ dendritic cells (DCs) represent a unique myeloid DC subset that cross-

presents necrotic cell antigens., The Journal of Experimental Medicine. 207 (2010) 1247–60. doi:10.1084/jem.20092140.

32. G. Schreibelt, J. Tel, K.H.E.W.J. Sliepen, D. Benitez-Ribas, C.G. Figdor, G.J. Adema, et al., Toll-like receptor expression and function in human dendritic cell subsets: implications for dendritic cell-based anti-cancer immunotherapy., Cancer Immunology, Immunotherapy : CII. 59 (2010) 1573–82. doi:10.1007/s00262-010-0833-1.
33. Y.-H. Paik, R.F. Schwabe, R. Bataller, M.P. Russo, C. Jobin, D.A. Brenner, Toll-like receptor 4 mediates inflammatory signaling by bacterial lipopolysaccharide in human hepatic stellate cells., Hepatology (Baltimore, Md.). 37 (2003) 1043–55. doi:10.1053/jhep.2003.50182.
34. V. Bazil, J.L. Strominger, Shedding as a mechanism of down-modulation of CD14 on stimulated human monocytes., Journal of Immunology (Baltimore, Md. : 1950). 147 (1991) 1567–74.
35. R. Landmann, A.E. Fisscher, J.P. Obrecht, Interferon-gamma and interleukin-4 down-regulate soluble CD14 release in human monocytes and macrophages., Journal of Leukocyte Biology. 52 (1992) 323–30.
36. Z. Pan, L. Zhou, C.J. Hetherington, D.E. Zhang, Hepatocytes contribute to soluble CD14 production, and CD14 expression is differentially regulated in hepatocytes and monocytes., The Journal of Biological Chemistry. 275 (2000) 36430–5. doi:10.1074/jbc.M003192200.
37. G.L. Su, K. Dorko, S.C. Strom, A.K. Nüssler, S.C. Wang, CD14 expression and production by human hepatocytes., Journal of Hepatology. 31 (1999) 435–42.
38. R. Berner, B. Fürll, F. Stelter, J. Dröse, H.-P. Müller, C. Schütt, Elevated levels of lipopolysaccharide-binding protein and soluble CD14 in plasma in neonatal early-onset sepsis., Clinical and Diagnostic Laboratory Immunology. 9 (2002) 440–5. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=119928&tool=pmcentrez&rendertype=abstract (accessed March 27, 2016).
39. Y.-H. Ni, Natural history of hepatitis B virus infection: pediatric perspective., Journal of Gastroenterology. 46 (2011) 1–8. doi:10.1007/s00535-010-0304-7.
40. M. Hong, E. Sandalova, D. Low, A.J. Gehring, S. Fieni, B. Amadei, et al., Trained immunity in newborn infants of HBV-infected mothers., Nature Communications. 6 (2015) 6588. doi:10.1038/ncomms7588.

Summary and discussion

Chapter 9

Hepatitis B virus infection is a major global health problem, as worldwide 250 million people are chronic carriers of HBV and at risk to develop severe liver diseases and hepatocellular carcinoma. To resolve HBV infection, induction of adequate HBV-specific immune responses is crucial. Thus far, it is poorly understood why the immune system of chronic hepatitis B (CHB) patients has failed to generate effective immunity against HBV.
DCs can bridge innate and adaptive immune responses by antigen presentation, and in addition can produce high levels of IFNs, which are essential for induction of effective antiviral immunity. Therefore, differential DC function could contribute to the lack of adequate HBV-specific immune responses in CHB patients. In addition, the central role of DCs in initiating and regulating antiviral immune responses suggests that these cells may be exploited for immunotherapy against CHB.
We and others have previously studied the function of BDCA1$^+$ DCs and pDCs in CHB patients. More recently, an additional DC subset, BDCA3$^+$CLEC9A$^+$XCR1$^+$ DCs (further referred to as BDCA3^{hi} DCs), was identified. However, the role this DC subset may have in antiviral immunity has been less well investigated compared to other DC subsets, and its functional state in chronic HBV infection is still unknown. In this thesis, the functional characteristics of BDCA3^{hi} DCs that make them relevant for HBV immunology and therapy design, in particular their IFN-λ-producing capacity and how they are affected by CHB, were studied.

BDCA3^{hi} DC development

BDCA3^{hi} DCs represent a distinct DC subset with specific phenotypic and functional characteristics that are extensively described in chapter 3. However, knowledge about this DC subset is still limited, and as the frequency of BDCA3^{hi} DCs in blood and tissues is very low, extensive study of these cells is difficult. Over the years, *in vitro* generation of DCs from progenitor cells has provided a major contribution to understanding of DC biology. For this reason, attempts have also been made to develop culture systems to generate BDCA3^{hi}-like DCs in large amounts. The first system for *in vitro* generation of BDCA3^{hi} DCs was described by Poulin *et al.* (1). In this system, BDCA3^{hi}CLEC9A$^+$ DCs are generated from Lineage$^-$ cord blood cells in the presence of SCF, GM-CSF, IL-4 and Flt3L. However, the yield of this system was low. Therefore, we and others have attempted to develop a more efficient system to generate an *in vitro* counterpart. We have used the well-known system described by Caux *et al.* that generates DC-SIGN$^+$ interstitial DCs and Langerin$^+$ Langerhans cells from cord blood-derived CD34$^+$ hematopoietic progenitor cells (HPC) in the presence of SCF, TNF-α and GM-CSF, and tested cultures with or without TGF-β and/or IL-4 (2, 3). Using this system, BDCA3$^+$ DCs were generated, which not only upregulated BDCA3 expression but also co-expressed CLEC9A in the presence of both IL-4 and TGF-β, and produced large amounts of IFN-λ in response to TLR ligation (chapter 4). However, expression profiling of immune related genes showed that in other aspects these BDCA3$^+$CLEC9A$^+$ DCs were not identical to naturally occurring blood BDCA3^{hi}CLEC9A$^+$ DCs.
In parallel to our study, three other systems to generate BDCA3^{hi} DCs were presented by Thordardottir *et al.*, Proietto *et al.* and Balan *et al.* (4-7). Although the cells in the first two systems (Thordardottir *et al.* and Proietto *et al.*), like in our model system, exhibited phenotypic and functional characteristics of BDCA3^{hi} DCs, these studies did not determine to what extent the cells resembled blood DCs at the transcriptome level. Balan *et al.* on the other hand used an optimized version of the protocol described by Poulin *et al.*,

which yielded higher BDCA3^{hi} DC numbers, and using whole genome expression profiling demonstrated that these cells closely resembled blood BDCA3^{hi} DCs (6). Because of the different quantitative and qualitative success of each model, comparison of these different culture systems yielding BDCA3^{hi} DCs now also provides insight into the factors that contribute to development of *bona fide* BDCA3^{hi} DCs. Balan *et al*. for example described that reduction of GM-CSF and IL-4 concentrations used by Poulin *et al*. enhanced the yield of BDCA3^{hi} DCs. The concentration of GM-CSF and IL-4 in our system was twice as high as the concentration used by Poulin *et al.*, and this may thus have caused the lack of *bona fide* BDCA3^{hi} DCs. The negative effect of high GM-CSF concentrations on BDCA3^{hi} DC development may be explained by the activation of STAT5 by GM-CSF, which inhibits induction of IRF8. IRF8 is a transcription factor that is highly expressed by blood BDCA3^{hi} DCs and presumed to be required for their development, in analogy to their murine counterpart, CD8α^{+} DCs (8-11). Together, these studies indicate that although GM-CSF is indispensable for generation of myeloid DCs, a too high concentration of GM-CSF prohibits development of BDCA3^{hi} DCs.
Expression profiling revealed that the BDCA3^{+} DCs in our system do not completely resemble BDCA3^{hi} DCs *in vivo*, and may lack certain functional aspects attributed to this cell type. Nonetheless, similar to naturally occurring BDCA3^{hi} DCs, they do excel in the production of IFN-λ. Therefore, our system may represent a valuable model to study regulation of IFN-λ production by (BDCA3^{+}) DCs. Although a side-by-side comparison of these two systems, to exclude differences between laboratories, should still be made, it seems that the use of cells from the system of Balan *et al.* is more relevant, as these BDCA3^{hi} DCs also produce high levels of IFN-λ and do resemble blood DCs.

Interaction between HBV and DCs, and subsequent DC activation

In order to induce an adequate HBV-specific immune response, DCs need to be activated by the virus via their pattern recognition receptors (PRR). If and how HBV is recognized by DCs is still poorly understood. For BDCA1^{+} DCs it was previously, and in this thesis, shown that they can interact with HBV or viral proteins *in vitro* and *in vivo* (chapter 8) (12, 13). The exact receptor involved in this interaction however remains to be determined. Previously, a role for the mannose receptor in the recognition and uptake of HBsAg by BDCA1^{+} DCs was identified (12). In addition, in chapter 8 is demonstrated that HBsAg can activate BDCA1^{+} DCs and that this occurs via a soluble CD14 (sCD14) and TLR4-dependent mechanism (Figure 1). sCD14 that is present in serum and secreted by hepatocytes may thus allow activation of cells that do not express membrane-bound CD14 themselves. Interestingly, we also show that in neonates the serum levels of sCD14 are much lower than in adults and that these low CD14 levels are associated with impaired activation of BDCA1^{+} DCs by HBsAg *in vitro*. This suggests that the activation of BDCA1^{+} DCs upon HBV infection may be inefficient in neonates, which may be one of the contributing factors leading to the inadequate immune responses and an immunotolerant state observed in neonates.
The role for CD14 in BDCA1^{+} DC activation by HBsAg is in line with CD14-dependent activation of monocytes by HBsAg (14). In addition, the enhanced activation status of BDCA1^{+} DCs of CHB patients indicates that activation of these cells by HBsAg also occurs in vivo. However, except for a study from our group, most previous studies showed that BDCA1^{+} DCs or monocytes isolated from CHB patients do not have an enhanced activated phenotype compared to healthy control individuals (15-18). This discrepancy may be due to variation

in patient population, especially variation in HBsAg levels, the use of frozen versus fresh material or other differences in experimental setup or laboratories. On the other hand, the sCD14-HBsAg complexes found in blood of CHB patients may serve a different or additional role *in vivo*. sCD14 could reduce binding of HBsAg to membrane-bound CD14 on monocytes by capturing HBsAg, thus preventing systemic activation of $CD14^+$ cells such as Kupffer cells and monocytes (14). Such a mechanism may contribute to a lack of initial anti-HBV immune responses, but concurrently and on the long run may prevent induction of severe systemic inflammatory responses. Taken together, although our work demonstrates a role for sCD14 in the recognition of HBV by the immune system, further research is required to understand the exact role of CD14-HBsAg complexes during the course of HBV infection and their potential clinical or diagnostic importance.

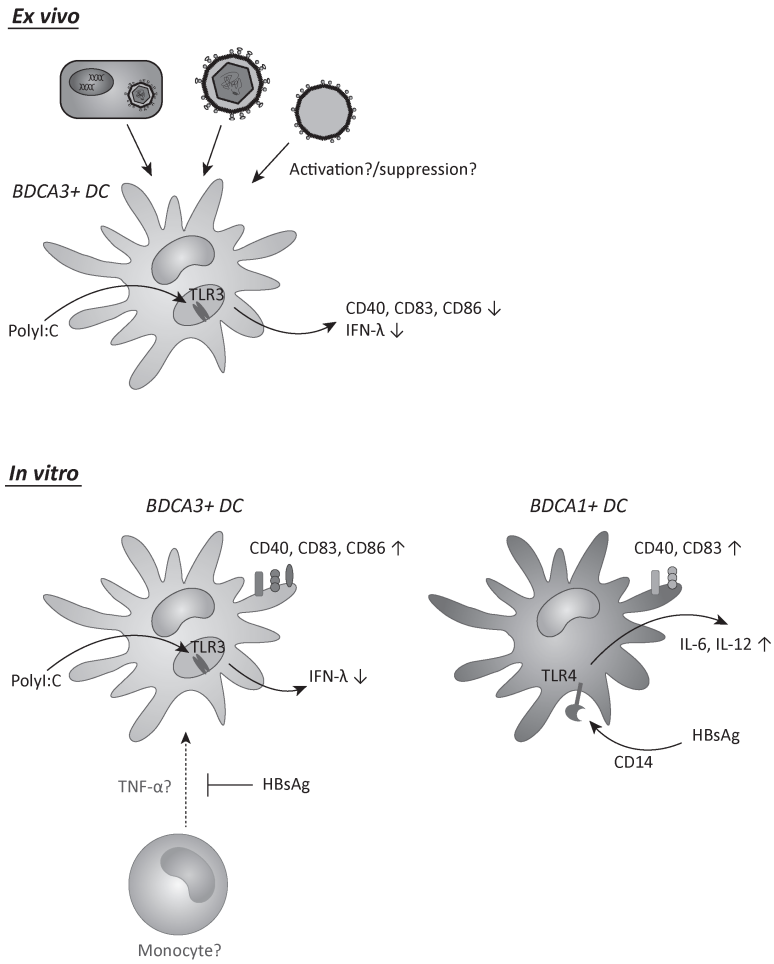

Figure 1. Effects of chronic HBV infection *ex vivo* and of HBsAg *in vitro* on $BDCA3^{hi}$ DCs and $BDCA1^+$ DCs.
Chronic HBV infection reduces the capacity of $BDCA3^{hi}$ DCs to mature and produce IFN-λ upon TLR3 stimulation. On $BDCA3^{hi}$ DCs, HBsAg enhances expression of maturation markers CD40, CD80, and CD86, but reduces polyI:C-induced IFN-λ production, via an indirect mechanism. HBsAg activates $BDCA1^+$ DCs via a CD14- and TLR4-dependent mechanism, which results in upregulation of CD40 and CD83, as well as induction of IL-6 and IL-12 production.

In chapter 7 is demonstrated that also BDCA3^{hi} DCs acquire HBsAg when exposed to concentrations found in serum of patients, indicating that an interaction between BDCA3^{hi} DCs and HBV/HBsAg is likely to occur *in vivo*. In depth analysis of the uptake of HBsAg by BDCA3^{hi} DCs with inhibitors for different endocytic pathways demonstrated that this involved divalent cation-dependent receptor-mediated endocytosis, as well as phagocytosis (Figure 2).

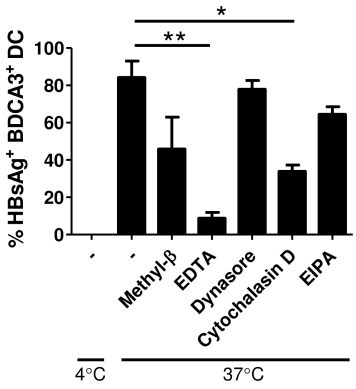

Figure 2. BDCA3^{hi} DCs take up HBsAg *in vitro* via receptor-mediated endocytosis and phagocytosis. CD14^{+}-depleted PBMC were incubated with APC-labelled patient-derived HBsAg for 2 hours at 4°C or 37°C after 1 hour pre-incubation with or without inhibitors of endocytosis (Methyl-β), divalent cation-dependent receptor-mediated uptake (EDTA), phagocytosis (Cytochalasin D) or macropinocytosis (EIPA). HBsAg uptake by BDCA3^{hi} DCs was analysed by FACS. Mean±SEM percentages of BDCA3^{hi} DCs containing HBsAg are depicted in *(n*=3). * $p<0.05$, ** $p<0.01$ by paired Student's *t*-test.

However, in contrast to BDCA1^{+} DCs, we found no evidence that BDCA3^{hi} DCs are directly activated by HBsAg *in vivo* or *in vitro* (chapter 7). The fact that BDCA3^{hi} DCs do not express TLR4 seems a reasonable explanation for this finding (19, 20). Although our experiments indicate that HBsAg is taken up but does not activate BDCA3^{hi} DCs, it is at this moment not clear whether whole viral particles are taken up by BDCA3^{hi} DCs, or whether BDCA3^{hi} DCs are activated at all upon HBV infection and play a role in induction of HBV-specific immune responses during the natural course of infection. BDCA3^{hi} DCs express the dsRNA receptor TLR3, but not the DNA receptors TLR7 or TLR9 (19). Although other viral products, such as HBeAg and HBcAg, may activate BDCA3^{hi} DCs, it remains questionable whether BDCA3^{hi} DCs become directly activated by HBV DNA-containing virions during (acute) infection. TLR3-mediated activation of BDCA3^{hi} DCs requires access to TLR3 ligands such as pregenomic RNA, however, this is only present in infected hepatocytes. Since HBV is a non-cytolytic virus, internalization of dead virus-infected cells by DCs may occur upon liver damage mediated by NK cells in acute HBV infection. Because this innate response may be low or even lacking in patients that evolve into CHB (21), it is an intriguing thought that low exposure of BDCA3^{hi} DCs to material from dead infected cells arising from this initial response could also contribute to inadequate induction of adaptive HBV immunity in these patients. During the chronic phases, that are associated with tissue damage caused by chronic inflammation, such material may also reach DCs, but a high viral burden and/or ongoing chronic inflammation may prevent the induction of an effective response at this stage of the disease. Thus, it will be very interesting to determine if and how BDCA3^{hi} DCs become activated during the different phases of HBV infection.

Effect of HBV infection on BDCA3^{hi} DCs in vivo and in vitro

In chapter 7 we show that polyI:C-stimulated blood BDCA3^{hi} DCs of CHB patients have an impaired capacity to become activated and to produce IFN-λ, but demonstrate normal TNF-α production (Figure 1). This may be the consequence of chronic inflammation and/or long-term exposure to viral antigens in these patients. In support of the latter, exposure to HBsAg was shown to inhibit IFN-λ-producing capacity of BDCA3^{hi} DCs from both blood and liver *in vitro* (chapter 7, Figure 1). The exact mechanism underlying impairment of IFN-λ production by HBsAg remains to be determined. We and others have previously shown that HBV and HBsAg can inhibit IFN-α production by pDCs *in vitro* in part indirectly by interfering with pDC-monocyte interaction (22, 23). Monocytes produce IL-6, TNF-α and IL-10 in response to HBsAg, and TNF-α was shown to inhibit IFN-α production by pDCs (15, 23). Shi *et al.* found that also IL-10 may inhibit IFN-α production by pDCs, but we did not detect such an effect (22, 23). The effect of these cytokines on IFN-λ production by BDCA3^{hi} DCs, however, remains to be investigated.
Besides a possible (indirect) action of HBsAg, chronic inflammation likely plays a major role in reduction of BDCA3^{hi} DC function as well. During a chronic infection, regulatory mechanisms are present that balance immune activation to control viral replication on the one hand, and immune suppression to avert excessive collateral damage on the other hand. These regulatory mechanisms, such as elevated levels of IL-10 and TGF-β, may also impair DC function (24, 25). We have previously shown that the function of DCs from HBV patients is enhanced upon reduction of liver inflammation and/or viral load by antiviral therapy (26, 27). However, the IFN-α-producing capacity of pDCs inversely correlates with ALT, but not with viral load (16, 22). These findings suggest that the chronic inflammatory status in CHB patients rather than the virus itself affect BDCA1^{+} mDCs and pDCs, and a similar scenario may be true for BDCA3^{hi} DCs. Additional studies should be performed to determine the contribution of viral proteins/particles and chronic inflammation in the impairment of DC function during CHB. Experimental work so far however indicates that diminished DC function is present in some CHB patients, and that it thus may be necessary to reduce HBsAg levels and/or chronic inflammation by antiviral therapy prior to DC-targeted immunotherapy in order to enhance DC function and achieve an optimal effect.

Due to experimental difficulties and the low availability of patient liver material, most of our studies were performed on BDCA3^{hi} DCs from blood. However, the situation may be different for liver DCs. Analysis of intrahepatic DC indicates that BDCA3^{hi} DCs of CHB patients can produce IFN-λ upon polyI:C stimulation. Preliminary results, however, indicate that in contrast to blood BDCA3^{hi} DCs, the number of BDCA3^{hi} DCs from patient livers producing IFN-λ in response to *ex vivo* stimulation is even increased compared to those from control livers (Figure 3). It is very well possible that this discrepancy arises from the pro-inflammatory intrahepatic environment in these patients, which may prime the DCs and make them more responsive to stimulation. This hypothesis is supported by our preliminary observation that also DCs from livers with non-HBV related inflammation are more responsive (Figure 3). Likewise, polyI:C-induced IFN-λ production by BDCA3^{hi} DCs from perfusate of HCV-infected livers was also enhanced compared to healthy donor livers (28). Nevertheless, despite the increased percentage of IFN-λ-producing cells in HBV-infected livers, these cells may still produce less IFN-λ on a per-cell base and be subject to inhibition by HBsAg or other viral factors. A more in-debt side-by-side comparison of BDCA3^{hi} DCs from inflamed HBV and

non-HBV livers is thus required to shed more light on this matter.
Regardless of the role of BDCA3^{hi} DCs during the natural course of infection, the observations that BDCA3^{hi} DCs are present in HBV-infected livers and able to produce IFN-λ makes these cells viable targets for immunotherapy. Especially because BDCA3^{hi} DCs are not only a source of high amounts of IFN-λ, but have also been described to be excellent cross-presenting cells. Targeting these cells with HBV antigens/peptides in combination with TLR3 ligands may thus boost HBV-specific adaptive immune responses and deliver local IFN-λ at the same time. As a next step towards such therapy we need to assess the capacity of (intrahepatic) BDCA3^{hi} DCs to (cross)present HBV antigens.

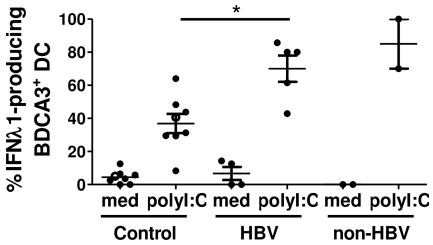

Figure 3. IFN-λ production by intrahepatic BDCA3^{hi} DCs.
Liver cells were isolated and stimulated for 5 hours with or without 20 μg/ml polyI:C. Production of IFN-λ1 by BDCA3^{hi} DCs was measured by ICS. Mean±SEM percentage IFN-λ1-producing BDCA3^{hi} DCs of control livers (n=8), HBV-infected livers (n=5), and livers with inflammation not caused by a virus (n=2) are shown. Open dots represent cells from donor livers and filled dots represent cells from peri-tumor liver tissue. $^{**}p < 0.01$, Mann-Whitney test.

As BDCA3^{hi} DCs can take up HBsAg (Figure 2), we anticipate that these cells can also cross-present HBsAg peptides. Indeed, preliminary data demonstrate that BDCA3^{hi} DCs are able to cross-present HBsAg (Figure 4). Further research is now required to determine whether BDCA3^{hi} DCs in CHB patients can still do so to warrant that BDCA3^{hi} DCs are feasible targets for immunotherapy to boost and/or induce HBV-specific cytotoxic T cell (CTL) responses. Cross-presentation of HBV antigens may occur *in vivo*, but since blood BDCA3^{hi} DCs of CHB patients do not have an activated phenotype (chapter 7), this may induce cross-tolerance in these patients or sub-optimal T cell skewing. Optimal cross-presentation and induction of effective CTL responses in CHB patients may thus not only require targeting of DCs with HBV-specific peptides, but may also require additional stimulatory signals.

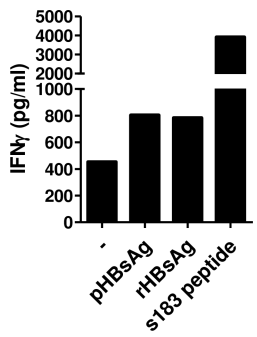

Figure 4. Cross-presentation of HBsAg by BDCA3^{hi} DCs.
Peripheral blood BDCA3^{hi} DCs of healthy controls (25.000 cells/150 μl) were incubated with 5 μg/ml patient-derived HBsAg (pHBsAg) or 5 μg/ml recombinant HBsAg (rHBsAg) in the presence of 20 μg/ml polyI:C. After 1 hour cells were washed and co-cultured with 50.000 T cells in 150 μl. After 20 hours supernatant was harvested and IFN-γ levels were determined by ELISA (n=1).

Regulation of IFN-λ production

The lack of an IFN-λ response in HBV patients (29, 30) and the diminished IFN-λ production in BDCA3^{hi} DCs by HBsAg (chapter 7) implies that in chronic HBV infection the IFN-λ response is dampened or even actively inhibited. To be able to understand how IFN-λ production can be suppressed, it is important to first scrutinize the regulatory mechanisms underlying production of IFN-λ in DCs.
In chapter 6 we therefore assessed the regulation of IFN-λ production in the major IFN-λ-producing cells, BDCA3^{hi} DCs, and demonstrated that the NFκB pathway and PI3K-PKB-mTOR pathway are involved in both polyI:C-induced IFN-λ1 as well as IFN-λ2 production. Whether these pathways are dysregulated in CHB, which may explain the diminished IFN-λ-producing capacity, is thus far unknown. We previously showed that PI3K-mTOR-mediated phosphorylation of S6 is disturbed in pDCs of HBeAg-positive CHB patients, and that also the IFN-α-producing capacity of these cells is diminished (31). As in chapter 7 only DCs from HBeAg-negative patients were used for functional characterization, it would be interesting to analyze the IFN-λ-producing capacity of DCs from HBeAg-positive patients as well. Further research is required to obtain more insight into the signaling pathways underlying IFN-λ production, which will contribute to discovery of targets for IFN-λ-manipulating therapy. The culture systems generating IFN-λ-producing CD34^{+}-derived DCs described above may be useful to perform this research.
Most studies on regulation of IFN-λ production have focused on IFN-λ1, -2 and -3. As IFN-λ4 was discovered more recently than the other IFN-λ subtypes, and is only produced in individuals with a certain genotype, this member of the IFN-λ family has been studied less well compared to the others. It will be interesting however, to assess whether the same regulatory mechanisms are involved in IFN-λ4 production.

Concluding remarks

DCs are pivotal players in the induction of antiviral immune responses. Although the function of BDCA1^{+} DCs and pDCs has been thoroughly studied, the function of BDCA3^{hi} DCs is still poorly understood due to the difficulty of creating knock-out mouse models and differences between murine and human DCs. The known functions of BDCA3^{hi} DCs point towards an important role in orchestrating antiviral immunity. However, assessment of human BDCA3^{hi} DCs is challenging because of low BDCA3^{hi} DC numbers *in vivo* and, as also demonstrated in this thesis, BDCA3 is co-expressed also on other DC subsets, which makes it even more complex to isolate these cells. The research described in this thesis is focused on the functional characteristics of BDCA3^{hi} DCs and their function in CHB infection. Our results provide insight into the effect of HBV infection on BDCA3^{hi} DCs, as well as the interaction and functional consequences of the viral envelope protein HBsAg on the function of BDCA3^{hi} DCs and BDCA1^{+} DCs *in vitro.* CHB has previously been shown to affect pDC and BDCA1^{+} DC function in a subset of patients. In addition to this, we demonstrate here that also the function of blood BDCA3^{hi} DCs, including IFN-λ-producing capacity, is impaired in CHB patients. As the signaling pathways underlying IFN-λ production, which may be dysregulated in HBV infection, are still incompletely understood, this requires further investigation. Development of models by which IFN-λ-producing BDCA3^{hi} DCs can be generated *in vitro* will contribute to this research, however, the currently available systems

are still not optimal. Other important questions that remain to be answered are whether BDCA3^{hi} DCs become activated during the course of an HBV infection and play a role in the induction of an HBV-specific immune response, as well as what mechanisms cause the impaired BDCA3^{hi} DC function we here observed. To answer these questions, assessment of intrahepatic DCs is essential. However, accessing liver material and isolation of intrahepatic DCs is even more challenging.

Although the function of BDCA3^{hi} DCs in CHB patients requires further investigation, BDCA3^{hi} DCs seem a viable target to induce HBV-specific antibody and T cell responses for treatment of CHB. Answers to the above-mentioned questions will contribute to better understand the lack of HBV-specific immunity in CHB patients and development of effective (DC-targeted) therapy.

References

1. Poulin, L. F., M. Salio, E. Griessinger, F. Anjos-Afonso, L. Craciun, J. L. Chen, A. M. Keller, O. Joffre, S. Zelenay, E. Nye, A. Le Moine, F. Faure, V. Donckier, D. Sancho, V. Cerundolo, D. Bonnet, and C. Reis e Sousa. 2010. Characterization of human DNGR-1+ BDCA3+ leukocytes as putative equivalents of mouse CD8alpha+ dendritic cells. *J Exp Med* 207: 1261-1271.
2. Caux, C., B. Vanbervliet, C. Massacrier, C. Dezutter-Dambuyant, B. de Saint-Vis, C. Jacquet, K. Yoneda, S. Imamura, D. Schmitt, and J. Banchereau. 1996. CD34+ hematopoietic progenitors from human cord blood differentiate along two independent dendritic cell pathways in response to GM-CSF+TNF alpha. *J Exp Med* 184: 695-706.
3. Caux, C., C. Massacrier, B. Dubois, J. Valladeau, C. Dezutter-Dambuyant, I. Durand, D. Schmitt, and S. Saeland. 1999. Respective involvement of TGF-beta and IL-4 in the development of Langerhans cells and non-Langerhans dendritic cells from CD34+ progenitors. *J Leukoc Biol* 66: 781-791.
4. Thordardottir, S., B. N. Hangalapura, T. Hutten, M. Cossu, J. Spanholtz, N. Schaap, T. R. Radstake, R. van der Voort, and H. Dolstra. 2014. The aryl hydrocarbon receptor antagonist StemRegenin 1 promotes human plasmacytoid and myeloid dendritic cell development from CD34+ hematopoietic progenitor cells. *Stem Cells Dev* 23: 955-967.
5. Proietto, A. I., D. Mittag, A. W. Roberts, N. Sprigg, and L. Wu. 2012. The equivalents of human blood and spleen dendritic cell subtypes can be generated in vitro from human CD34(+) stem cells in the presence of fms-like tyrosine kinase 3 ligand and thrombopoietin. *Cell Mol Immunol* 9: 446-454.
6. Balan, S., V. Ollion, N. Colletti, R. Chelbi, F. Montanana-Sanchis, H. Liu, T. P. Vu Manh, C. Sanchez, J. Savoret, I. Perrot, A. C. Doffin, E. Fossum, D. Bechlian, C. Chabannon, B. Bogen, C. Asselin-Paturel, M. Shaw, T. Soos, C. Caux, J. Valladeau-Guilemond, and M. Dalod. 2014. Human XCR1+ dendritic cells derived in vitro from CD34+ progenitors closely resemble blood dendritic cells, including their adjuvant responsiveness, contrary to monocyte-derived dendritic cells. *J Immunol* 193: 1622-1635.
7. Balan, S., and M. Dalod. 2016. In Vitro Generation of Human XCR1(+) Dendritic Cells from CD34(+) Hematopoietic Progenitors. *Methods Mol Biol* 1423: 19-37.
8. Crozat, K., R. Guiton, M. Guilliams, S. Henri, T. Baranek, I. Schwartz-Cornil, B. Malissen, and M. Dalod. 2010. Comparative genomics as a tool to reveal functional equivalences between human and mouse dendritic cell subsets. *Immunol Rev* 234: 177-198.
9. Esashi, E., Y. H. Wang, O. Perng, X. F. Qin, Y. J. Liu, and S. S. Watowich. 2008. The signal transducer STAT5 inhibits plasmacytoid dendritic cell development by suppressing transcription factor IRF8. *Immunity* 28: 509-520.
10. Tamura, T., P. Tailor, K. Yamaoka, H. J. Kong, H. Tsujimura, J. J. O'Shea, H. Singh, and K. Ozato. 2005. IFN regulatory factor-4 and -8 govern dendritic cell subset development and their functional diversity. *J Immunol* 174: 2573-2581.
11. Mittag, D., A. I. Proietto, T. Loudovaris, S. I. Mannering, D. Vremec, K. Shortman, L. Wu, and L. C. Harrison. 2011. Human dendritic cell subsets from spleen and blood are similar in phenotype and function but modified by donor health status. *J Immunol* 186: 6207-6217.
12. Op den Brouw, M. L., R. S. Binda, T. B. Geijtenbeek, H. L. Janssen, and A. M. Woltman. 2009. The mannose receptor acts as hepatitis B virus surface antigen receptor mediating interaction with intrahepatic dendritic cells. *Virology* 393: 84-90.
13. Untergasser, A., U. Zedler, A. Langenkamp, M. Hosel, M. Quasdorff, K. Esser, H. P. Dienes, B. Tappertzhofen, W. Kolanus, and U. Protzer. 2006. Dendritic cells take up viral antigens but do not support the early steps of hepatitis B virus infection. *Hepatology* 43: 539-547.
14. Vanlandschoot, P., F. Van Houtte, A. Roobrouck, A. Farhoudi, F. Stelter, D. L. Peterson, J. Gomez-Gutierrez, F. Gavilanes, and G. Leroux-Roels. 2002. LPS-binding protein and CD14-dependent attachment of hepatitis B surface antigen to monocytes is determined by the phospholipid moiety of the particles. *J Gen Virol* 83: 2279-2289.
15. Boltjes, A., Z. M. Groothuismink, G. W. van Oord, H. L. Janssen, A. M. Woltman, and A.

Boonstra. 2014. Monocytes from chronic HBV patients react in vitro to HBsAg and TLR by producing cytokines irrespective of stage of disease. *Plos One* 9: e97006.

16. van der Molen, R. G., D. Sprengers, R. S. Binda, E. C. de Jong, H. G. Niesters, J. G. Kusters, J. Kwekkeboom, and H. L. Janssen. 2004. Functional impairment of myeloid and plasmacytoid dendritic cells of patients with chronic hepatitis B. *Hepatology* 40: 738-746.
17. Woltman, A. M., A. Boonstra, and H. L. Janssen. 2010. Dendritic cells in chronic viral hepatitis B and C: victims or guardian angels? *Gut* 59: 115-125.
18. Gehring, A. J., and J. Ann D'Angelo. 2014. Dissecting the dendritic cell controversy in chronic hepatitis B virus infection. *Cell Mol Immunol*.
19. Jongbloed, S. L., A. J. Kassianos, K. J. McDonald, G. J. Clark, X. Ju, C. E. Angel, C. J. Chen, P. R. Dunbar, R. B. Wadley, V. Jeet, A. J. Vulink, D. N. Hart, and K. J. Radford. 2010. Human CD141+ (BDCA-3)+ dendritic cells (DCs) represent a unique myeloid DC subset that cross-presents necrotic cell antigens. *J Exp Med* 207: 1247-1260.
20. Schreibelt, G., L. J. Klinkenberg, L. J. Cruz, P. J. Tacken, J. Tel, M. Kreutz, G. J. Adema, G. D. Brown, C. G. Figdor, and I. J. de Vries. 2012. The C-type lectin receptor CLEC9A mediates antigen uptake and (cross-)presentation by human blood BDCA3+ myeloid dendritic cells. *Blood* 119: 2284-2292.
21. Bertoletti, A., and C. Ferrari. 2012. Innate and adaptive immune responses in chronic hepatitis B virus infections: towards restoration of immune control of viral infection. *Gut* 61: 1754-1764.
22. Woltman, A. M., M. L. O. den Brouw, P. J. Biesta, C. C. Shi, and H. L. A. Janssen. 2011. Hepatitis B Virus Lacks Immune Activating Capacity, but Actively Inhibits Plasmacytoid Dendritic Cell Function. *Plos One* 6.
23. Shi, B., G. Ren, Y. Hu, S. Wang, Z. Zhang, and Z. Yuan. 2012. HBsAg inhibits IFN-alpha production in plasmacytoid dendritic cells through TNF-alpha and IL-10 induction in monocytes. *PLoS One* 7: e44900.
24. Maini, M. K., and A. Schurich. 2010. The molecular basis of the failed immune response in chronic HBV: therapeutic implications. *J Hepatol* 52: 616-619.
25. Sun, C., B. Fu, Y. Gao, X. Liao, R. Sun, Z. Tian, and H. Wei. 2012. TGF-beta1 down-regulation of NKG2D/DAP10 and 2B4/SAP expression on human NK cells contributes to HBV persistence. *PLoS Pathog* 8: e1002594.
26. van der Molen, R. G., D. Sprengers, P. J. Biesta, J. G. Kusters, and H. L. Janssen. 2006. Favorable effect of adefovir on the number and functionality of myeloid dendritic cells of patients with chronic HBV. *Hepatology* 44: 907-914.
27. Tjwa, E. T., G. W. van Oord, P. J. Biesta, A. Boonstra, H. L. Janssen, and A. M. Woltman. 2012. Restoration of TLR3-activated myeloid dendritic cell activity leads to improved natural killer cell function in chronic hepatitis B virus infection. *J Virol* 86: 4102-4109.
28. Kelly, A., R. Fahey, J. M. Fletcher, C. Keogh, A. G. Carroll, R. Siddachari, J. Geoghegan, J. E. Hegarty, E. J. Ryan, and C. O'Farrelly. 2014. CD141(+) myeloid dendritic cells are enriched in healthy human liver. *J Hepatol* 60: 135-142.
29. Duong, F. H., G. Trincucci, T. Boldanova, D. Calabrese, B. Campana, I. Krol, S. C. Durand, L. Heydmann, M. B. Zeisel, T. F. Baumert, and M. H. Heim. 2014. IFN-lambda receptor 1 expression is induced in chronic hepatitis C and correlates with the IFN-lambda3 genotype and with nonresponsiveness to IFN-alpha therapies. *J Exp Med* 211: 857-868.
30. de Groen, R. A., F. McPhee, J. Friborg, H. L. Janssen, and A. Boonstra. 2014. Endogenous IFNlambda in viral hepatitis patients. *J Interferon Cytokine Res* 34: 552-556.
31. van de Laar, L., A. van den Bosch, A. Boonstra, R. S. Binda, M. Buitenhuis, H. L. Janssen, P. J. Coffer, and A. M. Woltman. 2012. PI3K-PKB hyperactivation augments human plasmacytoid dendritic cell development and function. *Blood* 120: 4982-4991.
32. Cao, W., S. Manicassamy, H. Tang, S. P. Kasturi, A. Pirani, N. Murthy, and B. Pulendran. 2008. Toll-like receptor-mediated induction of type I interferon in plasmacytoid dendritic cells requires the rapamycin-sensitive PI(3)K-mTOR-p70S6K pathway. *Nat Immunol* 9: 1157-1164.

Nederlandse samenvatting

Chapter 10

Achtergrondinformatie

Hepatitis B is een ontsteking van de lever die veroorzaakt wordt door HBV. HBV kan worden overgedragen van moeder op kind bij de geboorte, door bloed-bloed contact of via onbeschermd seksueel contact, en infecteert vervolgens specifiek de lever. Wereldwijd zijn ongeveer 250 miljoen mensen chronisch geïnfecteerd met HBV, wat kan leiden tot een chronische ontsteking van de lever. Deze chronische ontsteking kan resulteren in de vorming van littekenweefsel in de lever (leverfibrose en levercirrose), leverfalen en leverkanker. In 90% van de mensen die op volwassen leeftijd geïnfecteerd raakt, is het immuunsysteem in staat om het virus zelf op te ruimen en immuniteit tegen het virus te ontwikkelen. In de overige 10% is dat niet het geval, waardoor een chronische infectie ontstaat. Echter, van de pasgeborenen en kleine kinderen die besmet zijn geraakt met HBV, ontwikkelt maar liefst 90% een chronische infectie. Het is op dit moment onbekend welke factoren bepalen of een infectie onder controle komt van het immuunsysteem of in een chronische infectie resulteert.

Voor de afweer van virussen is een goed werkend immuunsysteem van belang. Het immuunsysteem kan onderverdeeld worden in twee delen; het aangeboren (*innate*) afweersysteem dat een ziekteverwekker herkent, geactiveerd raakt en vervolgens snel op een vrij a-specifieke manier virusreplicatie en infectie remt, en het verworven (*adaptive*) immuunsysteem dat door het aangeboren afweersysteem op een ziekteverwekker-specifieke manier wordt geactiveerd en gericht kan aanvallen/opruimen en vervolgens zorgt voor een langdurige bescherming. Het aangeboren immuunsysteem bevat verschillende cellen, waaronder DC. DC zijn in bloed, lymfe en weefsels aanwezig en scannen hun omgeving op ziekteverwekkers. Deze cellen zijn van groot belang voor de antivirale immuunrespons aangezien ze virussen niet alleen opruimen door ze op te nemen en te vernietigen, maar ook de unieke functie hebben om activatie te initiëren van cellen van het verworven immuunsysteem die virusgeïnfecteerde cellen doden en langdurige bescherming tegen het virus bieden.

Wanneer DC een virus herkennen raken ze geactiveerd, wat gepaard gaat met het verschijnen van activatiemarkers op het oppervlak van de cel en secretie van oplosbare eiwitten, cytokinen. Deze activatiemarkers en cytokinen zijn nodig om andere immuuncellen te activeren. Daarnaast worden kleine delen van het opgenomen virus op het celoppervlak gepresenteerd. Deze kleine delen worden antigenen genoemd, en het presenteren ervan antigeenpresentatie. DC kunnen de gepresenteerde antigenen op verschillende manieren verkrijgen, namelijk via infectie van de DC zelf, via opname van het virus uit de omgeving, en via interactie met of opname van virusgeïnfecteerde cellen. Antigeenpresentatie via de laatstgenoemde route wordt kruispresentatie genoemd. De gepresenteerde antigenen kunnen worden herkend door cellen van het verworven immuunsysteem, waaronder $CD8^+$ T cellen, die vervolgens mede door de activatiemarkers en cytokinen geactiveerd raken en virusgeïnfecteerde cellen kunnen doden.

Naast het activeren van andere immuuncellen hebben veel cytokinen die geproduceerd worden door DC ook een directe antivirale werking. Belangrijke antivirale cytokinen zijn interferonen (IFN). Deze IFN remmen de productie van virus, beperken de infectie van ongeïnfecteerde naburige cellen, en reguleren de werking van andere immuuncellen. De IFN worden verdeeld in 3 typen; type I IFN (IFN-α/β), type II IFN (IFN-γ) en type III IFN (IFN-λ). Vanwege de bovengenoemde functies spelen DC een belangrijke rol in het aanzetten van afweerreacties tegen virussen. De familie van DC bestaat uit verschillende subtypen, waaronder plasmacytoide DC, $BDCA1^+$ myeloide DC en $BDCA3^+$ myeloide DC. Deze subtypen

kennen veel overeenkomsten, zoals de unieke capaciteit om T-cellen te activeren, maar hebben ook elk hun specifieke functies, zoals het produceren van verschillende cytokinen. De BDCA3$^+$ DC subset is relatief recent gekarakteriseerd en kenmerkt zich door een zeer hoge expressie van BDCA3 op het celoppervlak. BDCA3$^+$ DC hebben excellente functionele capaciteiten wat betreft kruis-presentatie van antigenen aan CD8$^+$ T-cellen en het produceren van IFN-λ. Hoewel er over de rol van BDCA3$^+$ DC in antivirale immuunresponses nog relatief weinig bekend is, wijzen deze eigenschappen op een belangrijke rol voor BDCA3$^+$ DC in het induceren van antivirale immuniteit.

Vraagstelling

Gezien het belang van DC in het aanzetten van antivirale immuunresponsen, zou het virus voor zijn overleving baat hebben bij een sub-optimale functie van DC in HBV-geïnfecteerde patiënten waardoor een chronische infectie kan ontstaan en voortbestaan. De sleutelrol van DC impliceert dat het gericht activeren en instrueren van DC in HBV-geïnfecteerde patiënten ook gebruikt zou kunnen worden als therapie om responsen van het verworven immuunsysteem op te wekken die het virus specifiek kunnen opruimen. Om het ontstaan van een chronische infectie, in het bijzonder hepatitis B, beter te kunnen begrijpen en therapieën die gericht zijn op DC te kunnen ontwikkelen, is het belangrijk te begrijpen hoe antivirale functies van DC gereguleerd worden, hoe DC reageren op blootstelling aan HBV, of en waar DC in HBV-geïnfecteerde patiënten met (chronische) hepatitis B aanwezig zijn, en wat hun functionele capaciteit is. Het onderzoek in dit proefschrift is daarom gericht op eigenschappen en functies van DC die van belang zijn bij de afweer tegen virale infecties in het algemeen, de interactie met en het effect van het HBV envelop eiwit HBsAg op DC, en de functionele staat van DC bij chronische HBV infectie. Aangezien de functie van BDCA3$^+$ DC in HBV-geïnfecteerde patiënten nog geheel onbekend is, is het bovengenoemde onderzoek met name gericht op deze DC subset.

Resultaten

In hoofdstuk 1 wordt een algemene introductie gegeven van onder andere het verloop van een HBV infectie en de algemene antivirale immuunrespons en de rol van DC hierin. Vervolgens worden de huidige inzichten in de rol van DC en interferonen in HBV infectie uiteengezet, en een overzicht gegeven van de mogelijkheden voor hepatitis B therapie. Ten slotte zijn de doelen en hoofdlijnen van dit proefschrift beschreven. In aanvulling hierop toont hoofdstuk 2 een overzicht van de verschillende mechanismen die DC gebruiken voor (kruis-) presentatie van virale antigenen aan T-cellen. Uit dit overzicht blijkt dat kruis-presentatie een zeer efficiënt mechanisme is voor de inductie van virus-specifieke immuniteit.
In vergelijking tot andere DC subsets hebben BDCA3$^+$ DC de beste capaciteit voor kruis-presentatie van antigenen afkomstig van cellen, wat zou kunnen wijzen op een belangrijke rol voor deze cellen in het induceren van antivirale immuunresponsen. In hoofdstuk 3 wordt een overzicht gegeven van de eigenschappen van BDCA3$^+$ DC met betrekking tot hun uiterlijk, functie en ontwikkeling. Een belangrijk en specifiek karakteristiek van BDCA3$^+$ DC is dat ze grote hoeveelheden van het antivirale cytokine IFN-λ kunnen produceren. Daarnaast hebben de BDCA3$^+$ DC specifieke moleculen op hun celoppervlak, zoals XCR1 en CLEC9A. CLEC9A is een receptor die dode cellen kan herkennen en bijdraagt aan een extra goede

opname van dode cellen en daaropvolgende kruis-presentatie van antigenen aan T-cellen door BDCA3$^+$ DC. Deze excellente IFN-λ productie en kruis-presentatie van antigenen van dode cellen duiden erop dat BDCA3$^+$ DC een belangrijke rol spelen in het aanzetten van antivirale immuunresponsen, maar aanvullend onderzoek is noodzakelijk om dit te bewijzen. Onderzoek naar de BDCA3$^+$ DC wordt sterk bemoeilijkt door het lage aantal van deze cellen in het lichaam en het ontbreken van een homoloog in de muis. In hoofdstuk 4 is daarom getracht om BDCA3$^+$ DC uit een specifiek soort stamcellen te kweken, CD34$^+$ stamcellen, zodat grotere hoeveelheden cellen verkregen kunnen worden voor onderzoek. In het ontwikkelde kweeksysteem konden BDCA3$^+$ cellen gedetecteerd worden, maar gedetailleerde analyse van deze cellen toonde aan dat ze niet volledig hetzelfde waren als BDCA3$^+$ DC in het lichaam. Dit systeem kan dus niet gebruikt worden voor verder onderzoek naar BDCA3$^+$ DC, maar aangezien de cellen in het systeem wel in staat zijn om grote hoeveelheden IFN-λ te produceren, is het een waardevol model om IFN-λ productie te bestuderen.
Hoewel BDCA3$^+$ DC hun naam ontlenen aan de expressie van BDCA3 op hun celoppervlak, blijkt dus dat cellen met BDCA3 expressie niet perse tot de specifieke BDCA3$^+$ DC subset die in het lichaam aanwezig is behoren. Daarnaast is bekend dat BDCA3 ook tot expressie kan worden gebracht door een deel van de BDCA1$^+$ DC populatie. Het is echter niet bekend of er een verschil is tussen de BDCA3$^+$ en BDCA3$^-$ cellen binnen de BDCA1$^+$ DC populatie en of de BDCA3$^+$BDCA1$^+$ DC meer op BDCA3$^+$ DC lijken dan BDCA3$^-$BDCA1$^+$ DC. In hoofdstuk 5 zijn daarom de overeenkomsten en verschillen in genexpressie en functie tussen zowel BDCA3$^+$BDCA1$^+$ DC en BDCA3$^-$BDCA1$^+$ DC, als BDCA3$^+$BDCA1$^+$ DC en BDCA3$^+$ DC onderzocht. De resultaten tonen aan dat BDCA1$^+$ DC een apart subtype is van BDCA3$^+$ DC, ongeacht de expressie van BDCA3 op de BDCA1$^+$ DC. De functie van BDCA3 op DC is nog onbekend, maar BDCA3$^+$BDCA1$^+$ DC lijken meer geactiveerd te zijn en hogere hoeveelheden cytokinen te produceren dan BDCA3$^-$BDCA1$^+$ DC.
Een DC produceert niet zomaar cytokinen, zoals IFN. Een signaal van buitenaf, zoals bijvoorbeeld een virus dat bindt aan bepaalde receptoren op de cel, zorgt voor het aan- of uitzetten van verschillende functies van cellen. Deze signalen worden in de cel vertaald via signaalroutes, waarlangs het signaal wordt doorgegeven tot het uiteindelijk tot productie van een molecuul leidt. Hoe de productie van type I IFN en type II IFN in een cel aangezet wordt en welke processen daar bij betrokken zijn is tot in detail beschreven. Voor het recent ontdekte type III IFN, IFN-λ, is dat echter niet het geval. In hoofdstuk 6 is onderzocht welke processen in BDCA3$^+$ DC betrokken zijn bij de productie van IFN-λ. In dit hoofdstuk wordt beschreven dat twee signaalroutes, namelijk de PI3K-PKB-mTOR route en NFκB route, betrokken zijn bij de productie van IFN-λ door BDCA3$^+$ DC. Deze kennis is van belang om bijvoorbeeld te begrijpen hoe virussen de productie van IFN-λ in een cel kunnen verhinderen door deze signaalroutes te onderbreken, en om bijvoorbeeld therapie te ontwikkelen die de productie van IFN-λ manipuleert.
Een verlaagde IFN-λ producerende capaciteit van BDCA3$^+$ DC in HBV-geïnfecteerde patiënten met chronische hepatitis B (CHB) wordt getoond in hoofdstuk 7. Dit hoofdstuk beschrijft zowel de frequentie en functie van BDCA3$^+$ DC in lever en bloed van CHB patiënten, als het effect van het envelopeiwit van HBV, HBsAg, op de functie van BDCA3$^+$ DC *in vitro*. Het aantal BDCA3$^+$ DC in HBV-geïnfecteerde levers is verhoogd vergeleken met levers van ongeïnfecteerde personen. De functie van BDCA3$^+$ DC in bloed van CHB patiënten is echter verminderd. Deze BDCA3$^+$ DC raken minder goed geactiveerd na stimulatie en de capaciteit om IFN-λ te produceren is verlaagd. Deze verminderde functie zou veroorzaakt kunnen zijn door de chronische leverontsteking in de patiënten of door het virus zelf. Wanneer

immuuncellen uit bloed blootgesteld worden aan het envelopeiwit HBsAg wordt de IFN-λ productie door BDCA3$^+$ DC verminderd. Dit geeft aan dat de verminderde IFN-λ productie in patiënten (deels) veroorzaakt zou kunnen zijn door dit eiwit, dat onderdeel is van het virus, maar ook los in het bloed van patiënten voorkomt.
De vermindering van IFN-λ productie door BDCA3$^+$ DC na blootstelling aan HBsAg zoals getoond in hoofdstuk 7, vindt alleen plaats indien er andere immuuncellen aanwezig zijn. Dit impliceert dat HBsAg een directe functionele interactie aangaat met andere immuuncellen. Eén van de typen immuuncellen waarvan de functie direct door HBsAg beïnvloed wordt zijn BDCA1$^+$ DC. De receptoren voor HBsAg op BDCA1$^+$ DC die een effect hebben op de functie van deze cellen zijn echter nog niet bekend. In hoofdstuk 8 is daarom de interactie tussen HBsAg en BDCA1$^+$ DC en het effect hiervan op BDCA1$^+$ DC functie bestudeerd. Blootstelling van BDCA1$^+$ DC aan HBsAg *in vitro* resulteert in opname van HBsAg, verhoogde expressie van activatiemarkers, productie van cytokinen en verhoogde activatie van T-cellen door BDCA1$^+$ DC. Daarnaast wordt in dit hoofdstuk aangetoond dat CD14, dat ook als oplosbaar eiwit in bloed aanwezig is, en TLR4 de receptoren zijn die HBsAg herkennen en betrokken zijn bij de activatie van BDCA1$^+$ DC. Kortom, HBsAg kan worden herkend door BDCA1$^+$ DC en deze cellen activeren, wat een belangrijke stap zou kunnen zijn in de initiatie van HBV-specifieke immuunresponsen en van belang kan zijn voor de ontwikkeling van immunotherapie tegen hepatitis B.
Een samenvatting en discussie van bovengenoemde resultaten is beschreven in hoofdstuk 9.

Conclusie

DC zijn van groot belang in het induceren van antivirale immuunresponsen. Vergeleken met andere DC subtypen is over de functie van BDCA3$^+$ DC echter nog relatief weinig bekend. De functies van BDCA3$^+$ DC die beschreven zijn duiden op een belangrijke rol in het tot stand brengen van antivirale immuniteit. Het bestuderen van BDCA3$^+$ DC is gecompliceerd doordat de aantallen van deze cellen in het lichaam zeer laag zijn en doordat BDCA3 ook op andere cellen voorkomt, waardoor het nog complexer is om deze cellen uit het lichaam te isoleren. Het onderzoek beschreven in dit proefschrift is gericht op de eigenschappen van BDCA3$^+$ DC en hun functie in CHB. De resultaten bieden inzicht in zowel het effect van HBV infectie op BDCA3$^+$ DC *in vivo*, als de consequenties van de interactie van BDCA3$^+$ DC en BDCA1$^+$ DC met het virale envelop eiwit HBsAg *in vitro*. In het verleden is aangetoond dat in een deel van de CHB patiënten de functie van BDCA1$^+$ DC en plasmacytoide DC is aangetast. In toevoeging hierop wordt in dit proefschrift aangetoond dat ook de functie van BDCA3$^+$ DC, waaronder IFN-λ productie, verminderd is in CHB patiënten. De signaalroutes die betrokken zijn bij IFN-λ productie zijn nog niet volledig ontrafeld. Om onder andere de verminderde IFN-λ productie door BDCA3$^+$ DC te doorgronden is verder onderzoek naar deze signaalroutes van belang. De ontwikkeling van kweeksystemen waarmee IFN-λ producerende BDCA3$^+$ DC gegenereerd kunnen worden *in vitro*, zal bijdragen aan dit onderzoek, al zijn de momenteel beschikbare systemen voor de generatie van BDCA3$^+$ DC *in vitro* nog niet optimaal. Overige vragen die nog beantwoord dienen te worden zijn of BDCA3$^+$ DC geactiveerd raken tijdens het verloop van een HBV infectie, of deze cellen een rol spelen in het induceren van een HBV-specifieke immuunrespons, en welke mechanismen de hier aangetoonde verminderde BDCA3$^+$ DC functie veroorzaken. Om deze vragen te beantwoorden is het bestuderen van intrahepatische DC in de lever essentieel. Het verkrijgen van levermateriaal en het isoleren van intrahepatische DC is echter gecompliceerd. Al is meer onderzoek naar de functie

van BDCA3$^+$ DC in CHB patienten vereist, BDCA3$^+$ DC lijken een geschikt doelwit voor het induceren van HBV-specifieke immuunresponsen ter behandeling van CHB. Antwoorden op bovengenoemde vragen zal bijdragen aan het doorgronden van het ontbreken van HBV-specifieke immuniteit en ontwikkeling van effectieve (DC-gerichte) therapie.

Appendix

Dankwoord
List of publications
PhD portfolio
Curriculum Vitae

Chapter 11

Dankwoord

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List of publications

van der Aa E, Buschow SI, Biesta PJ, Janssen HL, Woltman AM. The Effect of Chronic Hepatitis B Virus Infection on BDCA3+ Dendritic Cell Frequency and Function. PLoS One. 2016 Aug 16;11(8).

van Montfoort N, **van der Aa E**, van den Bosch A, Brouwers H, Vanwolleghem T, Janssen HL, Javanbakht H, Buschow SI, Woltman AM. Hepatitis B Virus Surface Antigen Activates Myeloid Dendritic Cells via a Soluble CD14-Dependent Mechanism. J Virol. 2016 Jun 24;90(14):6187-99.

van der Aa E, van de Laar L, Janssen HL, van Montfoort N, Woltman AM. BDCA3 expression is associated with high IFN-λ production by CD34(+)-derived dendritic cells generated in the presence of GM-CSF, IL-4, and/or TGF-β. Eur J Immunol. 2015 May;45(5):1471-81.

van der Aa E, van Montfoort N, Woltman AM. BDCA3(+)CLEC9A(+) human dendritic cell function and development. Semin Cell Dev Biol. 2015 May;41:39-48.

van Montfoort N, **van der Aa E**, Woltman AM. Understanding MHC class I presentation of viral antigens by human dendritic cells as a basis for rational design of therapeutic vaccines. Front Immunol. 2014 Apr 23;5:182.

PhD portfolio

Name of PhD student:	Evelyn van der Aa
Department:	Gastroenterology and Hepatology, Erasmus MC University Medical Center, Rotterdam
PhD period:	September 2011 – December 2015
Promotor:	Prof.dr. Harry L.A. Janssen
Co-promotor:	Dr. Andrea M. Woltman

General academic and research skills: courses

2014	Course Biostatistical Methods, Netherlands Institute for Health Sciences (NIHES), Rotterdam
2013	Course Integrity in Research, Dept. of Medical Ethics and Philosophy, Erasmus MC University Medical Center, Rotterdam
2013	Course Scientific English Writing, Netherlands Institute for Health Sciences (NIHES), Rotterdam
2013	Course Molecular Immunology, Erasmus Postgraduate school Molecular Medicine (MolMed), Erasmus University, Rotterdam
2012	Course qPCR, Life Technologies, Leiden
2012	Course Presenting Skills, Erasmus Postgraduate school Molecular Medicine (MolMed), Erasmus University, Rotterdam

Oral presentations

2015 T cell consortium meeting, Erasmus MC

2015 MolMed day, Erasmus Postgraduate school Molecular Medicine, Erasmus University, Rotterdam
Chronic HBV infection affects BDCA3$^+$ dendritic cell frequency and IFN-l production.

2014 Dutch Society for Immunology (NVVI) annual meeting, Kaatsheuvel.
IFNl is mainly produced by BDCA3-expressing myeloid dendritic cells and regulated by the NFkB and PI3K-PKB-mTOR pathway.
Chronic HBV infection affects BDCA3$^+$ dendritic cell frequency and IFNl production.

2013 Dutch Society of Hepatology (NVH), Dutch Liver Retreat, Spier
Characterization and quantification of intrahepatic dendritic cells in healthy and diseased livers.

Poster presentations

2015 Dutch Society for Immunology (NVVI) annual meeting, Noordwijkerhout.
Chronic hepatitis B virus infection affects BDCA3$^+$ dendritic cell frequency and IFN-l production

2015 International Meeting on Molecular Biology of Hepatitis B Viruses, Bad-Nauheim, Germany.
Chronic hepatitis B virus infection affects BDCA3$^+$ dendritic cell frequency and IFN-l production.

2015 Dutch Experimental Gastroenterology and Hepatology (DEGH) meeting, Veldhoven
Interaction of BDCA3$^+$ dendritic cells with HBsAg specifically inhibits IFN-λ production.

2014 Dutch Society for Immunology (NVVI) annual meeting, Kaatsheuvel.
Interaction of BDCA3$^+$ dendritic cells with HBsAg specifically inhibits IFN-λ production.

2014 International Meeting on Molecular Biology of Hepatitis B Viruses, Los Angeles, CA, USA
Interaction of BDCA3$^+$ dendritic cells with HBsAg specifically inhibits IFN-λ production.

2014 Dutch Experimental Gastroenterology and Hepatology (DEGH) meeting, Veldhoven
Characterization and quantification of intrahepatic dendritic cells in healthy and diseased livers.

2014 MolMed day, Erasmus Postgraduate school Molecular Medicine, Erasmus University, Rotterdam
Characterization and quantification of intrahepatic dendritic cells in healthy and diseased livers.

2013 Keystone, Understanding Dendritic Cell Biology to Advance Disease Therapies
In vitro generation of CD34$^+$-derived IFN-λ-producing BDCA3$^+$ dendritic cells, resembling the BDCA3$^+$ dermal dendritic cell subset.

2013 MolMed day, Erasmus Postgraduate school Molecular Medicine, Erasmus University, Rotterdam
In vitro generation of CD34$^+$-derived IFN-λ-producing BDCA3$^+$ dendritic cells, resembling the BDCA3$^+$ dermal dendritic cell subset.

2013 Dutch Society for Immunology (NVVI) annual meeting, Noordwijkerhout.
Intrahepatic BDCA1$^+$ and BDCA3$^+$ dendritic cell subsets differ in their location and response to chronic viral infection.

2012 Dutch Society for Immunology (NVVI) annual meeting, Noordwijkerhout.
In vitro generation of CD34$^+$-derived IFN-λ-producing BDCA3$^+$ dendritic cells, resembling the BDCA3$^+$ dermal dendritic cell subset.

Additional conferences attended

2013 Dutch Society for Immunology (NVVI) Symposium, Lunteren.
APCs revisited: the function of antigen presenting cells in health and disease.

2012 European Association for the Study of the Liver (EASL) Monothematic Conference: Immune Mediated Liver Injury, Stratford upon Avon, UK.

2011 Dutch Society for Immunology (NVVI) annual meeting, Noordwijkerhout.

Grants and awards

2015 Travel grant, Dutch Society for Hepatology

2015 Poster prize, Dutch Experimental Gastroenterology and Hepatology (DEGH) meeting, Veldhoven

2014 Travel grant, Dutch Society for Hepatology

2013 Travel grant, Erasmus Trustfonds

2013 Research award, Dutch Society for Hepatology

2013 Travel grant, Dutch Society for Immunology

2013 Travel grant, Erasmus Trustfonds

Teaching activities

April 2014 – March 2015	Que Zhu, MSc thesis, Wageningen University
October 2013 – December 2013	Yoga Vignesh Chancra Mohan, MSc thesis, University of Kaiserslautern, Germany
November 2012 – July 2013	Fadime Ayhan, MSc thesis, UVA
October 2014, 2013, 2011	Organization of labrotations for MSc students

Curriculum vitae

Evelyn van der Aa werd op 5 augustus 1988 geboren te Sliedrecht. In 2006 behaalde zij haar VWO diploma aan De Lagewaard in Papendrecht. Vervolgens startte zij met haar studie Life Science and Technology aan de Universiteit Leiden en Technische Universiteit Delft. Haar bachelor sloot zij af met een onderzoeksstage op de afdeling Humane Genetica van het Leids Universitair Medisch Centrum (LUMC) onder begeleiding van Dr. Yvonne Krom en Prof.dr. Silvère van der Maarel. Tijdens deze stage werd de spierfunctie in een muismodel voor facioscapulohumorale spierdystrofie (FSHD) gekarakteriseerd. In 2009 begon zij aan de master Research in Life Science and Technology aan de Universteit Leiden. In het eerste jaar van haar master deed zij ervaring op in het bedrijfsleven door middel van een stage bij Crucell onder begeleiding van Dr. Pieter Rijken en Els van Deventer. Deze stage stond in het teken van de ontwikkeling van een high-throughput assay voor de bepaling van de oplosbaarheid van antilichamen. Het tweede jaar van haar master startte zij met een onderzoeksstage aan de afdeling Maag-, Darm- en Leverziekten van het Erasmus MC in de onderzoeksgroep van Dr. Andrea M. Woltman. Tijdens deze stage, waarbij zij begeleid werd door Dr. Lianne van de Laar en Dr. Nadine van Montfoort, optimaliseerde zij een kweeksysteem voor de in vitro ontwikkeling van BDCA3^{+} dendritische cellen. Na het behalen van haar mastertitel in 2011 kwam zij terug bij de Maag-, Darm-, Leverziekten afdeling van het Erasmus MC en startte zij haar promotieonderzoek, waarvan de resultaten in dit proefschrift beschreven staan. Het promotieonderzoek vond plaats onder leiding van co-promotor Dr. Andrea M. Woltman en promotor Prof.dr. Harry L.A. Janssen. Sinds april 2016 is zij werkzaam bij Janssen Vaccines and Prevention in Leiden als scientist op het gebied van bacterial vaccines.