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Hepatitis B Virus affects Dendritic cell activation and their cross-talk with Natural Killer cells

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Sommario

Abstract

The interplay between Natural killer (NK) cells and Dendritic cells (DCs) represents a first line of defence against viral infections. NK/DC interaction results in reciprocal cell activation and production of Interferon-γ (IFN-γ), a major antiviral cytokine. Chronic Hepatitis B (CHB) virus infection is characterized by a marked immune dysfunction that involves also NK cells, since IFN-γ production by NK cells is significantly reduced in both peripheral blood and liver of CHB infected patients.

Considering the crucial role of DCs in stimulating NK cell response, we sought to determine whether DCs could be responsible for NK cell impairment, as a consequence of HBV infection.

To this purpose, DCs were cultured in the presence of highly viremic sera from CHB patients and their phenotype and functions analysed.

In the presence of HBV serum, a drastic inhibition of DC maturation occurred, as demonstrated by the low expression of maturation markers, co-stimulatory molecules and IL-12, the latter representing a key cytokine for IFN-γ production by NK cells. Accordingly, DCs conditioned by the serum were significantly impaired in inducing both IFN-γ and TNF-α production by NK cells as well as in inducing NK cell proliferation. Similar data were obtained by adding HBV particles to DC cultures, suggesting that HBV has a direct role in the functional impairment of DCs.

In addition, the inhibitory role of HBV on DCs was apparently also exerted by a significant up-regulation of the tolerogenic enzyme Indoleamine 2 3-dioxygenase (IDO), which played a major role in the inhibition of NK cell proliferation induced by DCs.

Altogether, our data revealed that serum from chronic HBV patients significantly impairs DC functions and, in turn, both proliferation and IFN-γ production by NK cells upon DC/NK interactions. HBV is directly responsible for the phenotypic and functional alterations observed in DCs, suggesting that, during HBV chronic infection, abnormalities in the interactions between DCs and NK cells might occur, thus resulting in a reduced antiviral response.

Introduction

Hepatitis B virus (HBV) is a hepatotropic virus that causes variable degrees of liver disease in humans.. Despite the availability of a prophylactic vaccine, HBV is estimated to infect around 400 million people worldwide. Chronically infected patients are at risk of developing HBV-related diseases such as liver cirrhosis and hepatocellular carcinoma. However, HBV replication itself is not directly cytotoxic to cells, so to gain the name of stealth virus. Therefore, inadequate immune response toward HBV contributes to the establishment and progression of Chronic hepatitis B virus (CHB) infection (1).

Innate immunity is important in controlling infection immediately after contact with the pathogen, in order to limit the spread of the infection and to initiate an efficient development of adaptive immune response.

The immune system evolved to recognize specific structures on pathogens and eliminate foreign antigens exploiting specific receptor on cell surface. Among these, Toll like Receptors (TLR) play an important role in identification of viral antigens. Dendritic cells (DC), well equipped in TLRs, are extremely potent in initiating a primary immune response due to their ability to process foreign antigens in order to present them to effector cells. Additionally, DCs are able to activate natural killer cells (NK), orchestrating NK cell mediated innate immune responses. The outcome of NK/DC interaction during viral infection, is the activation of NK cell cytotoxicity and IFN-γ production, alike activated NK cells enhance DC maturation and IL-12 production. NK cells exert their non-cytolitic anti-viral function mainly by IFN-γ production (2).

Accumulating evidences suggested that an efficient induction of innate responses occurs during HBV acute infection by non-cytolytic mechanisms, advising that the innate immune response has the potential to contain the virus during early phases of HBV infection. However, studies indicate that NK cells from Chronic HBV infected patients are impaired in their non-cytolitic antiviral activity, as shown by the significant reduction of IFN- γ release. Still, NK cytotoxic function is maintained in peripheral blood and liver wondering that NK cells might also be implicated in disease pathogenesis.

The HBV-infected patients display large amounts of circulating HBV particles, which interact with DCs. Although, it has been demonstrated that HBV does not replicate into DCs, it has been proposed that HBV may cause alteration in DC functions probably interfering with signaling pathways involved in maturation and cytokine production (3)

Because DCs are potent activators of NK cells, remains to be clarified whether any DC dysfunction may affect NK functions. Therefore, in this study we wondered to investigated

whether the presence of HBV might alter dendritic cell activity, thus impairing NK/DCs cross-talk, resulting in a less efficient NK cell anti-viral response.

Innate Immunity in viral infection

Innate immunity have an important role in controlling infection immediately after contact with the pathogens, in order to limit the spread of the infection and to initiate an efficient development of adaptive immune response. Immune system is divided in two branches: Innate and Adaptive immunity, that work cooperatively to defend the host against infections. These two aspects of immunity differ with respect to how quickly it responds and for how long it responds to pathogens, for effectors cell types and for specificity to different classes of microbes.

Furthermore, the innate responses do not require prior exposure to the pathogen. On the contrary, adaptive immune responses are characterized by specificity and by the expansion of lymphocyte populations from a vast repertoire of lymphocytes bearing antigen-specific receptors that are generated through a mechanism generally known as gene rearrangement.

The innate immune response relies on recognition of evolutionarily conserved structures on pathogens, termed pathogen-associated molecular patterns (PAMPs), that are characterized by being invariant among entire classes of pathogens, essential for the survival of the pathogen. PAMPs are distinguishable from "self" through a limited number of germ line-encoded pattern recognition receptors (PRRs). PRRs upon recognition of pathogen structures trigger pro-inflammatory and antimicrobial responses by activating a multitude of intracellular signaling pathways, including adaptor molecules, kinases, and transcription factors. The signal transduction pathways ultimately result in the activation of gene expression and synthesis of a broad range of molecules, including cytokines, chemokines, cell adhesion molecules, and immuno-receptors, which together orchestrate the early host response to infection and at the same time represent an important link to the adaptive immune response.

Several and different pathogens, including viruses, bacteria, fungi, and protozoa, are recognized by PRRs that though are slightly different, display similar and overlapping mechanisms of action.

During viral infection, host PRRs detect viral components, such as genomic DNA, single-stranded (ss) RNA, double-stranded (ds) RNA, RNA with 5′-triphosphate ends and several viral proteins. Currently, different classes of PRRs have been shown to be involved in the recognition of virus-specific components in innate immune cells, among these Toll-like receptors (TLRs), that has been studied most extensively, together with retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) and NOD-like receptors (NLRs). Detection of viral components by RLRs and TLRs in immune cells activates intracellular signaling cascades, leading to the secretion of type I

IFNs, pro-inflammatory cytokines and chemokines. Type I IFNs activate intracellular signaling pathways via a type I IFN receptor, and regulate the expression of a set of genes. The IFN-inducible genes are involved in eliminating viral components from infected cells, inducing apoptosis of infected cells and conferring resistance to viral infection on uninfected cells. Type I IFNs are produced not only by professional innate immune cells, including dendritic cells (DCs) and macrophages, but also by non-professional cells, such as fibroblasts. Pro-inflammatory cytokines and chemokines are also critical for eliminating virus infection by provoking inflammation and recruiting innate and acquired immune cells. TLRs are transmembrane proteins suitable for detecting viral components outside of cells as well as in cytoplasmic vacuoles after phagocytosis or endocytosis. Among the at least ten known TLRs, present in mammals, TLR2, TLR3, TLR4, TLR7 and TLR9 are involved in the recognition of viral components. TLR2 and TLR4, present on plasma membrane, are involved in the recognition of viral envelope proteins on the cell surface, while TLR2 and TLR4 are critical for the recognition of bacterial components, lipoproteins and lipopolysaccharide, respectively. In contrast, TLR3, TLR7 and TLR9 are localized on cytoplasmic vesicles, such as endosomes and the endoplasmic reticulum, and recognize microbial nucleotides. TLR3 recognizes dsRNA, while TLR7 and TLR9 recognize ssRNA and DNA with CpG motifs, respectively. While TLR3 recognizes dsRNA in conventional DCs and possibly epithelial cells, TLR7 and TLR9 are highly expressed in plasmacytoid DCs (pDCs).

All TLRs, except TLR3, activate a common signaling pathway leading to the production of pro-inflammatory cytokines via MyD88 signaling pathway. Following the downstream of signaling transduction, finally NF-κB and MAP kinases are activated resulting in induction of genes involved in inflammatory responses.

Alternatively, in response to stimulation with dsRNA, TLR3 recruits another adaptor protein, TIR domain-containing adaptor inducing IFN-β (TRIF) that leads to TRAF6 activation and, finally, this latter is responsible for activating NF-κB leading to the expression of proinflammatory cytokines (4-7).

TLR3, as previously described, recognizes dsRNA, a universal viral molecular pattern, and thus, is involved in antiviral host immune responses (8). A synthetic ligand, Poly (I:C), structurally similar to double-stranded RNA, can also mediate responses through TLR3 triggering. The role of TLR3 was investigated in different types of viral infection and it was found to play a role in viral recognition and in promotion of various immune responses. For instance, it has been described a role for TLR-3 during Rhinoviruses infection, the major cause of the common cold, viral replication induces expression of TLR3 mRNA and its surface protein expression. TLR3 mediates protective antiviral activity in human bronchial epithelial cells infected with Rhinovirus

as confirmed by several impairment of antiviral response upon blocking of TLR-3, resulting in increased Rhinovirus replication (9).

Interestingly, TLR3 plays an important role in the pathogenesis of West Nile virus, a ssRNA flavivirus, mediating penetration of West Nile virus across the blood-brain barrier and induces neuronal injury. Another interesting feature of TLR3 is that it promotes cross-priming to virus-infected cells. To this regard, it has been proposed that TLR3 may have evolved to permit cross-priming of cytotoxic T cells against viruses that do not directly infect dendritic cells (10).

It has been described a role for TLR-3 also in course of Hepatitis B (HBV) infection. Wieland et al proposed that TLR3 ligand poly I:C induces intrahepatic IFN-β production , quite able to inhibits HBV replication by non-cytolytic mechanisms (11).

Additionally, Isogawa et al. (12) tested the ability of different TLR ligands to inhibit HBV replication in the HBV transgenic mouse model injectioning a single-dose of TLR3, TLR4, TLR5, TLR7, and TLR9 ligands able to suppressed HBV replication in the liver in an IFN-α/β-dependent manner. TLR3 activation by Poly I:C of hepatic Non-Parenchymal Cells (NPCs) such as Kupffer cells, could lead to release of IFN-β production and antiviral cytokines which are able to inhibit HBV replication in an *in vitro* co-culture mouse model (13).

Interestingly, several studies have suggested that HBV seems able to inhibit pattern recognition receptor (PRR) and IFN signaling. HBV surface and "e" antigen (HBsAg, HBeAg) and HBV particles could inhibit the activation of NPCs by TLR3 ligands by trigger IL10 production on hepatic cells and thereby attenuates the TLR3-mediated activation of NPCs. Additionally, at high amounts of HBV, even TNF-α and IL-6 expression in NPCs induced upon TLR triggering was suppressed, suggesting that mechanisms employed by HBV may counteract induction of an efficient innate response (14).

It also been reported that TLR3-mediated functions are impaired in patients with chronic HBV infection and may recover partially under successful antiviral treatment (15). In the woodchuck model of infection, peripheral blood mononuclear cells (PBMCs) from animals with chronic WHV infection show reduced responses to Poly I:C stimulation (16). All together these information confirming that the interaction of HBV or viral components of HBV with the innate immune system is complex, leading both to activation and inhibition of host innate responses.

As previously discussed, an effective immune response requires recognition of pathogen and consequent induction of innate and adaptive immune systems. The innate immune responses to infection are rapid and mostly dependent from *PRRs* that recognize PAMPs. Some of these receptors are present on the surface of professional phagocytic cells such as macrophages and

neutrophils, where they mediate the uptake of pathogens, and delivered them into the lysosome for degradation.

Additionally, other cells type take part to promote the implementation of innate response since, asserting innate immune system as crucial in the initiation and subsequent direction of adaptive immune responses. Natural Killer (NK) cells and Dendritic Cells (DCs) represent two central components of the innate immune system, and both play a key role in fighting early infection. DCs and NK cells interact each other through cell to cell contact and soluble factors in periphery or in secondary lymphoid tissues. The bidirectional crosstalk between NK cells and DCs results in maturation, activation, and cytokine production by both cells (17).

Dendritic cells: antigens recognition and immunological functions during microbial invasion

Dendritic cells (DCs) are professional antigen-presenting cells and essential mediators of immunity and tolerance. In 1973 Ralph Steinman discovered in the mouse spleen a rare population of cells characterized by stellate morphology and extended veils. He further studied what he named dendritic cells to provide evidences of the emerging functional specializations of these cell types. Over the following years, DCs was found to be critical in shaping innate and adaptive immunity, thanks to their ability in mounting immune responses to foreign antigens, and its contribution to the induction of tolerance to self-antigens. Consequently, subsequent studies focused on the potential therapeutic benefits of modulating DCs for vaccines or suppressive therapies against pathogens, tumors, and/or autoimmune diseases (18). Soon after the identification of DCs in lymphoid organs, and the discovery of epidermal Langerhans cells (LCs) led to the idea that more than one branch to the DC family might exist (19). Consequent studies had revealed the presence of cells with a similar phenotype in most non-lymphoid tissues that, upon antigen encounter, migrate through the lymph to lymphoid organs, where they localize in the T cell zone and present antigens to T lymphocytes. More recently, a further major division in the DC family occurs thanks to the identification of a population of cells that morphologically resemble plasma cells but, upon exposure to viral stimuli, produce enormous amounts of interferon IFN-α. Importantly, these cells also differentiate upon stimulation into immunogenic DCs that can prime T cells against viral antigens and were named plasmacytoid DCs (pDCs) (20). To distinguish pDCs from Steinman's DCs, the latter were renamed conventional DCs (cDCs), and remain so today.

DCs initiate an immune response by presenting the captured antigen, in the form of peptide–major histocompatibility complex (MHC) molecule complexes, to naive T cells in lymphoid

tissues (21). When compared with other APCs, such as macrophages, DCs are extremely efficient. In peripheral tissues, DCs capture antigens through several complementary mechanisms (22). Antigen-loaded DCs then migrate into the draining lymph nodes through the afferent lymphatics. Meanwhile, they process the proteins into peptides that bind to both MHC class I molecules and MHC class II molecules.

Immature non-activated DCs can also present self-antigens to T cells (23, 24), which leads to immune tolerance either through T cell deletion or through the differentiation of regulatory (T regulatory cells, Treg) or suppressor T cells. By contrast, mature activated and antigen-loaded DCs can start the differentiation of antigen-specific T cells into effector T cells with unique cytokine profiles and function.

Antigens can also directly reach lymph node-resident DCs through the lymph (25). Lymph node-resident DCs that acquired antigen directly from the lymph are the first to present peptides to naive $CD4^+$ T cells, which results in T cell priming. T cells primed by activated DCs produce high amount of interleukin-2 (IL-2), which in turn facilitates T cell proliferation and clonal expansion in LN. $CD4^+$ T cells and $CD8^+$ T cells upon interaction with DCs differentiate into antigen-specific effector T cells with different functions.

Additionally, DCs also have an important role in controlling humoral immunity but the mechanism by which DCs address antigens into compartment where they do not undergo degradation, that results in the presentation of unprocessed antigens to B cells is poorly characterized (26).

However, these DC properties allow the activation of both arms of the adaptive immune system. Following Ag encounter, DCs undergo maturation, that is associated with several events, such as the downregulation of antigen-capture activity, the increased expression of surface MHC class II molecules and co-stimulatory molecules, the increased ability to secrete cytokines, as well as the acquisition of CCR7, which allows migration of the DC into the draining lymph node (22).

Conventional DCs (cDCs) can be divided into at least two main subsets characterized by either CD8α and CD103 or CD11b expression Both subpopulations can be found in lymphoid tissue, including spleen, lymph node, and bone marrow (BM), as well as most non-lymphoid tissue. Heterogeneity within the DC population was first demonstrated by both the Shortman and Steinman groups, including the discovery of a CD8α-expressing DC subset in murine lymphoid organs (23). An equivalent population also exists in non-lymphoid tissues, although these cells do not express CD8 but are instead identified by the CD103 integrin marker (29, 30) and they also appear to be conserved through evolution. In both human and mice DC subset was discovered a potential common marker, the chemokine receptor XCR1, found by transcriptome profiling studies (32, 33). Development of $CD8\alpha^+$cDCs and the nonlymphoid tissue equivalent, the $CD103^+$ ($CD11b^-$) cDCs, is orchestrated by the same transcription factors: inhibitor of DNA

binding 2 (Id2), interferon regulatory factor 8 (IRF8), basic leucine zipper ATF-like 3 transcription factor (BATF3), and the nuclear factor interleukin 3 regulated (NFIL3). CD11b$^+$cDCs are the most abundant cDCs in lymphoid organs except for the thymus and can also be found in nonlymphoid tissue. In contrast to CD8α and CD103 DCs, the population currently defined as CD11b$^+$cDCs is heterogeneous and remains less well characterized.

Splenic CD8α$^+$cDCs are functionally specialized in cross-presenting exogenous Ags on MHC-I molecules to CD8$^+$ T cells (38) and stimulation of TLR signaling on the CD8α$^+$ and CD103$^+$cDC lineage induces prominent secretion of "bioactive" IL12p70. In contrast in lymphoid organs, CD11b$^+$ DCs are not efficiency to cross-present and produce specific cytokines, such as IL-12. It has however been noted that CD11b$^+$cDCs are, as compared to CD8α$^+$cDCs, superior in the induction of CD4$^+$ T cell immunity, potentially because of their prominent expression of MHC-II presentation machinery (35, 36). This specialization of CD11b$^+$cDCs was recently attributed to their expression of the transcription factor IRF4 (37) CD11b$^+$ DCs can also be characterized by their cytokines production, such as IL-6 (38) and IL-23 (39). Furthermore, splenic CD11b$^+$cDCs were shown to be prominent producers of proinflammatory chemokines after TLR ligand exposure, such as CCL3, CCL4, and CCL5 (40).

cDCs generally display a short half-life of approximately 3–6 days and are constantly replenished from BM precursors in a strictly Flt3L-dependent manner. Myeloid and lymphoid branches of the immune system bifurcate early during hematopoiesis into common myeloid and lymphoid precursors (CMPs, CLPs). It has been proposed that CD8α-positive and -negative subsets, are of myeloid origin.

DCs thus initially share their origin with monocytes. Althought, a pre-DC population was subsequently identified in lymphoid tissues and in the blood. Pre-DCs were found to populate lymphoid organs via the circulation and give rise to cDCs in lymphoid and non-lymphoid tissues (41, 42, 43, 44)

DC tissue distribution and migratory properties

DC progenitors are not restricted to the BM but can be found in multiple locations, including the thymus, blood, lymph, and most visceral organs. DCs reside in lymphoid and selected non-lymphoid tissues. Lymphoid tissue–resident cDCs differentiate in lymphoid tissues, such as splenic compartment, where they spend their entire lives. Lymph Node also include non-lymphoid tissue migratory cDCs.

The spleen harbors different populations of resident cDCs: Among these, CD8α$^+$cDCs represent about 20%–30% of the total splenic DC compartment and are localized in the marginal zone where they sample lymph- and blood-borne Ags and pathogens. CD8α$^+$cDCs furthermore

efficiently uptake apoptotic or necrotic cells from peripheral blood (45) and are thus able to present exogenous tumor- or virus-derived Ags. Further functional properties of splenic CD8α$^+$cDCs are their highly specific expression of the double-stranded RNA sensor

In Lymph node (LN), cDCs can be subdivided into CD8α$^-$ and CD8α$^+$ subsets (46) but harbor in addition migratory DCs that entered via the afferent lymphatics from associated non-lymphoid tissues. The latter probably import pathogen antigens for T cell stimulation.

In contrast to tissue-migratory cDCs that arrive in the LNs in a mature state, lymphoid tissue CD8$^+$cDCs are phenotypically immature in the steady state (47). Activation to a phenotypically mature state occurs upon stimulation with microbial products or when cDCs are isolated from the lymphoid tissue and cultured in vitro. In LN, CD11b$^+$cDC represents another subset that predominates the lymphoid-resident cDC population; they proliferate in situ in response to Flt3L and are characterized by the lacking of CD8 marker. CD11b$^+$cDCs crucial feature is the high production the CD4$^+$ T cell attractant chemokines CCL17 and CCL22.

Like lymphoid organs, most non-lymphoid tissues contain at least two major subsets of cDCs that often share the αE integrin CD103 marker but can be distinguished according to CD11b expression. Non-lymphoid tissue DCs are in contact with body surfaces, such as the skin, lung, and intestine and have different specializations. For istance, Langherans Cells (LC) residing in the skin epidermis represent the principal skin DC population and play a critical role in the defense against external threats. Indeed, LCs efficiently phagocytose pathogens after epidermal cell injury and switch their chemokine expression pattern from CCR6 to CCR7, which allow them to migrate toward cutaneous LNs. In contrast to most DCs, LCs develop independently of Flt3 (48). Compared with dermal cDCs, LCs are characterized phenotypically by lower MHC-II levels, intermediate CD11c levels, and very high levels of the C-type lectin langerin, CD207, which is responsible for the generation of Birbeck's granules, the ultra-structural hallmark formed by LCs. LCs that are transiting through the dermis or that have migrated to skin-draining LN can be identified based on expression of Langerin, EpCam and CD11b and the absence of CD8 and CD103 expression.

In contrast, dermal DCs are discriminate from Langherans Cells and most often identified in the dermis and LN based on expression or the absence of Langerin, CD103 and the absence of CD11b, CD8 and EpCam expression. Importantly, careful examination of subset ontogeny has clearly identified that LCs and Langerin$^+$dDCs are distinct DC subsets (49).

Additionally, other markers help to identified DCs: among these HLA-DR and DC-SIGN (DC specific intercellular adhesion molecule-3 ICAM-3), a c-type lectin receptor, that recognize high-mannose-containing glycoproteins on viral envelopes acting as receptor for several viruses such as HIV and Hepatitis C. Moreover, it is used as a marker for immature DCs.

Recently, the human DC subset-specific markers termed: blood DC Ag-1 BDCA-1 (CD1c), BDCA-2 (CD303), BDCA-3 (CD141), and BDCA-4 (CD304) have been also identified (50).

DCs are sparsely distributed through the liver, and immunohistochemical studies of patient liver biopsies indicate that they are primarily found in the portal regions and occasionally in the parenchyma.

The DC population in the liver can be further divided into two major functionally and phenotypically distinct subsets. In mice, the hepatic $CD11c^{hi}MHC\text{-}II^{hi}$ DC population contains a more prevalent $CD103^{-}CD11b^{hi}$ population and a rarer $CD103^{+}CD11b^{low}$ population. CD11b expression on the $CD103^{-}$ DC population tends to be heterogeneous, and the $CD103^{-}CD11b^{low}$ subset may represent a less mature population. Corresponding counterparts to the $CD11b^{hi}$ and $CD103^{+}$ DC populations can be further identified in human livers by the markers CD1c (BDCA1) and CD141 (BDCA3) (51)

Recent studies suggest that $CD141^{+}$ DC subset may play a specialized role during hepatic viral infections. The frequency of $CD141^{+}$ DCs is higher in the liver than in the peripheral blood and has been found to further increase in the setting of hepatitis C virus infection along with high levels expression of TLR3 (52). Remarkably, DC population present in human liver, produces higher levels of IL-10, compared to DCs from the blood, spleen and skin, which accounts for their reduced allogeneic stimulatory capacity. In normal liver, DCs reside as "immature" expressing low levels of surface MHC and costimulatory molecules (CD40, CD80, CD86) necessary for T cell activation. However, these immature DCs are extremely well-equipped for Ag capture, processing, and loading onto MHC class II molecules for export to the cell surface.

Circulating DCs and their precursors circulate the blood and traffic in response to tissue-specific recruitment signals that are expressed on vascular epithelial cells. These signals include pro-inflammatory chemokines, and chemotactic cytokines, that are produced from sites of inflammation or from normal tissues, able to recruit DC precursors. DCs express specific adhesion molecules and maturation-dependent chemoattractant receptors that allow them to respond to a variety of ligands (53). Immature DCs enter to non-lymphoid peripheral tissues and travel within them, by utilizing specific chemokine receptor-ligand pathways, such as CCR2-CCL2 (54, 55), CCR5-CCL5 (56), and CCR6-CCL20 (57). When DCs encounter Ags becoming mature and downregulate their responsiveness to these inflammatory chemokine pathways and traffic to the draining LNs by upregulating CCR7, which responds to two ligands, CCL19 and CCL21 (48, 53). These chemokines are expressed by peripheral lymphatic endothelial cells as well as LN stroma cells and guide DCs to downstream LNs. Leukocyte extravasation occurs in a series of distinct steps including tethering, rolling, activation by a chemoattractant, firm adhesion, and diapedesis. Mainly for circulating DCs, tethering and rolling are primarily mediated by one

or more of the three members of the selectin family: integrins, in particular LFA-1 (αLβ2), VLA-4 (α4β1), Mac-1 (αMβ2) and α4β7 mediate arrest of the rolling cells by binding to members of the immunoglobulin superfamily, including ICAM-1 (ligand for LFA-1 and Mac-1), ICAM-2 (ligand for LFA-1), VCAM-1 (ligand for VLA-4 and weakly for α4β7) and MAdCAM-1 (ligand for α4β7) (58). A large variety of pro-inflammatory stimuli induce DCs to migrate from peripheral tissues to LNs, such as chemical (e.g. contact sensitizers and irritants), physical (e.g. UV radiation or trauma) or biological stimuli (e.g. microbial or tissue necrosis). At the site of injury, DC antigen-uptake occurs, followed, within hours, by maturation process whereby DCs regain their motility, re-arrange their chemokine receptor repertoire, upregulate their Ag presentation machinery and eventually migrate to LNs (59).

DC pathogen recognition and activation of immune responses

DCs process and present antigen to activate both $CD4^+$ and $CD8^+$ T cells. This appears to be the most prominent role for DCs, since only DCs are capable of activating naïve T cells. Dendritic cells, present throughout peripheral tissues, constitutively sample the environment for the presence of pathogens that upon recognition are internalized and processed into peptides. Peptides can be generated either by lysosomal proteases in the endocytic pathway, or by proteasomes. Thus, generated peptides may associate intracellularly with either MHC class I or MHC class II molecules, and in that context can be transferred and displayed at the plasma membrane. DCs migrate to lymphoid tissues, thus MHC–peptide complexes can be recognized by T cells. Antigen specific $CD8^+$ cytotoxic T cells are activated by recognition of MHC-I on DCs that load Ag, helping in elimination of infected and malignant cells. In contrast to MHC-II, MHC-I is expressed by nearly all cell types, and in non-professional antigen-presenting cells is exclusively loaded with peptides that are generated from cytosolic proteins by the ubiquitin-proteasome system. Cytosolic peptides, upon processing, can be translocated into the lumen of the endoplasmic reticulum for loading onto MHC-I. Peptide-loaded MHCI is then transported out of the ER via the Golgi apparatus to the plasma membrane, where it is stably exposed. Infected cells that display pathogen-derived peptides on MHC-I can be killed by cytotoxic T cells that specifically recognize relevant MHC-I/peptide complexes (60).

In contrast, MHC class II molecules are normally found only on antigen-presenting cells such as dendritic cells, mononuclear phagocytes, some endothelial cells, thymic epithelial cells, and B cells.

The antigens presented by class II peptides are derived from extracellular proteins. Loading of a MHC class II molecule are loaded with peptides upon phagocytosis; extracellular proteins are endocytosed, digested in lysosomes, and the resulting peptide fragments are loaded onto MHC class II molecules. Peptide-loaded MHC II is thus presented to $CD4^+$ T cells.

DCs have evolved specific surface molecules to uptake pathogens, in addition to expressing a number of specific receptors for certain viruses such as CD4 and CXCR4 for HIV, the previously named DC-SIGN and its close relative DC-SIGNR, or L-SIGN found on liver endothelium, are some of the most intensively studied pathogen receptors (61). Additionally, DCs sense the environment through both surface and intracellular receptors, which comprise several families, including cell surface C-type lectins (CLRs), surface and intracellular TLRs, and intracellular helicases. The helicases are a very large family of molecules, including retinoic acid-inducible gene I (RIGI), which recognize nucleic acids.

The different signals that are provided by different microbes either directly or through the surrounding immune cells induce DCs to acquire distinct phenotypes. DC maturation varies according to different microbes because microbes express PAMPs that trigger distinct DC molecular receptors. Strikingly, although most microbes activate DCs, same of these can block DC maturation. As previously mentioned, viral products, pro-inflammatory cytokines, bacterial or double-stranded RNA trigger the maturation of immature DCs and in this context cytokines milieu play a critical role.

Concerning DC cytokines profiles it is dependent from signals occurs upon pathogens encounter. It is now clear that the interaction between TLRs and PAMPs plays a key role in enhancing the release of cytokines, such as interleukin-10 (IL-10), IL-12 and type I interferons. Production of IL-12 and IL-10 by mature murine DCs can be elicited by many pathogens or their products (62-64) In human system, Gram-negative bacteria, but not Gram-positive bacteria, prime DCs to produce the IL-12 (65). In contrast, *Mycobacterium tuberculosis* blocks DC maturation and induces IL-10 release by targeting DC-SIGN (66).

Considering DC role in directing the development of T-cell responses, the pattern of cytokines that they release upon their activation play an important role in determining the T-cell response. Importantly, DCs are also able to produce type I interferon in response to viral infection or following interaction with T cells. It is now well established that IL-12-secreated DC drive T helper type 1 (Th1) responses whereas IL-10 inhibits them, promote Th2-type responses. Also, Type I interferons have been shown to regulate T-cell differentiation, but their role has not yet been clearly resolved.

The major events in DC maturation are probably the up-regulation of MHC and costimulatory molecules on their surface. Pattern of costimulatory molecules is well known. A variety of inflammatory or pathogen-derived molecules rapidly up regulate expression of very early costimulatory signals. Members of B7 family,such as CD80/CD86 are expressed on DCs and constitutes the most important costimulatory pathway in T cell activation since through binding of CD28 on T cells is promoted the production of IL-2, a factor that supports expansion and survival of T cells. Importantly, CD80/CD86 by binding with CD28, also strongly interferes with tolerogenic properties of immature DC. Interestingly, the same costimulatory molecules are also

responsible for shutting down T cell activation exploiting the upregulation of an inhibitory molecule, CTLA-4, on T cell surface. Briefly, CTLA-4 binds with higher affinity to CD80/CD86 than CD28 and thereby competes for interaction with both costimulatory molecules provides a very simple negative feedback loop (67). Human immature DCs constitutively express intermediate amounts of CD86 and lack CD80. Hence, for characterization of human DC maturation, CD80 is considerably more reliable, as it is exclusively induced on mature DC while CD86 is already present on immature DC and further up-regulated upon stimulation (68).

The ligation of the co-stimulatory receptor CD40 (also known as TNFRSF5) is an essential signal for the differentiation of immature DCs into fully mature DCs.

CD40 ligation on DCs, indeed, increases expression of costimulatory, adhesion and MHC molecules and promotes the production of T cell stimulatory cytokines such as IL-12. Recombinant CD40 ligand therefore is often used to induce DC maturation (69).

It is important to note that in the absence or low levels of costimulation signal, including IL-1, IL-12, TNF-α, CD40, CD80, CD86, the cross-linking between T cell receptors and MHC-bound peptides expressed on the surface of DCs leads to anergy or apoptosis of antigen-specific T cells. The functional properties of DCs are thus mainly dependent on their status of maturation and activation. Accordingly, subsets of immature DCS induce and maintain peripheral T cell tolerance whereas differentiated mature DCs efficiently induce the development of effector T cells. To this regard, it is well stated that a crucial role for DCs in maintaining immuno-tolerance also occurs. Several studies have shown that DCs in the steady state are able to have negative effect on T cell survival. Certain subpopulations of DCs in the periphery were found to induce $CD4^+CD25^+$ Treg cells, a subsets well known to display regulatory functions in vitro and in vivo among these subsets liver-derived DC, IL-10-producing DC or lymphoid-derived DC. In the absence of inflammation thus in homeostatic conditions, circulating immature DC have the primary function to migrate in Lymph-node and induce the differentiation of naive or resting T cells into Treg cells.

A key factor that alters the maturation process of DC is IL-10. The immunosuppressive properties of IL-10 on DC result in a reduced expression of MHC class II molecules as well as co-stimulatory and adhesion molecules. Steinbrink et al (70) demonstrated that human DC from the peripheral blood, matured in the presence of IL-10, induce anergic T cells, inhibiting IL-2 production and T cell proliferation. Therefore, the presence of IL-10 during DC-maturation inhibits the development of immunostimulatory DCs.

Upon pathogens encounter, DCs undergo maturation process, resulting in DC maturation by phenotypical and functional change that include the up-regulation of MHC I and co-stimulatory molecules and progressive variation in Ag-processing machinery (APM) component expression. The proteasome is a central element of APM and can be expressed in two forms, constitutive and

inducible also called immunoproteasome, endowed with different protein cleavage specificities. The switch of constitutive proteasome in the immunoproteasome form occurs in sites of infection and suggests that, during the peak phase of viral or bacterial elimination, this last form is more efficient. Immunoproteasome adopts a distinct manner to cleave proteins thus generating more peptides capable to better bind to MHC class I molecules.

Immunoproteasome proteins were significantly upregulated also in response to the major immunomodulatory cytokine, interferon-gamma (IFN-γ).

The constitutive proteasome is a four-ring structure; the outer rings contain seven non-catalytic α-type subunits, whereas the inner rings contain seven β-type subunits, three of which have catalytic properties (delta/β1, Z/β2, and X/β5). The immunoproteasome, inducible by TNF-α and IFN-γ, contains alternative forms of the catalytic subunits (LMP2, MECL1, and LMP7), that replace the corresponding constitutive homologs β1, β2, and β5. LMP2 and LMP7 were located within the major histocompatibility complex (MHC) class II region where they are clustered with the TAP-1 and TAP-2 genes, while MECL-1 is encoded outside the MHC class II region. After proteolysis of a ubiquitinated proteins in the immunoproteasome core, peptides are further cut by a battery of aminopeptidases, and bind to TAP-1 and TAP-2 for transport into the ER where the N-terminus of peptides were further cleaved and loaded into the MHC class I complex. The rising MHC- I complex is formed chaperoned by calnexin (CNX), tapasin (TPN), and calreticulin (CRT). Peptides generated by either partial or complete degradation of proteins can be loaded into MHC class I molecules for recognition by CD8 T cells as part of immune surveillance process (71).

The location of the LMP2 and LMP7 immunoproteasome subunits in the MHC region, and their production in response to proinflammatory cytokines, suggested their role in antigen processing. Study with KO mice for one or both of these subunits help to investigate subunit-specific properties. It was found that mice deficient in LMP7 exhibited a modest reduction in surface expression of MHC class I, and resulted in mice with a reduced response to antigens. Deficiency in LMP2 was found to result in a reduced of CD8 T cell activation. Additionally, LMP2-deficient cells were less able to activate NF-κB pathway (72). Findings derived from the use of these KO mice, have demonstrated and confirmed that iproteasome subunits have a roles in antigen-specific interactions with microbial challenges. Importantly, van Helden and colleagues (73) reported that MECL-1 and LMP7 deficiency resulting in a reduced MHC class I expression, made those cells susceptible to natural killer cell-mediated killing in mice whose immune system had been activated by a viral infection.

Natural Killer cells: antiviral response and regulation of cytolitic activity

Natural killer (NK) cells interact with DCs to reciprocally activate and influence subsequent effector functions. The intricate cross-talk between NK cells and DCs serves to modulate the anti-viral immune responses. Although specific NK cell responses depend on viral context, they require other cells to coordinate an effective antiviral response.

NK cells are the prototypic innate lymphoid cell (ILC) and are considered crucial components of innate immune system given their pivotal role in the first line of defense.

NK cells are characterized by a wide spectrum of effector functions such as killing and controlling of target cells, mainly tumor and virally-infected cells, the ability to influence various steps of the immune response (74-76).

NK cells comprise 5–10% of human peripheral blood lymphocytes, however, this proportion can vary with age. The traditional cell surface phenotype defining human NK cells; analyzing the lymphocyte gate by flow cytometry NK cells are characterized by the absence of CD3 and expression of CD56, the 140-kDa isoform of neural cell adhesion molecule (NCAM) found on NK cells and a minority of T cells.

Human NK cells can be subdivided into different populations based on the relative expression of the markers CD16 (or FcγRIII, low-affinity receptor for the Fc portion of immunoglobulin G) and CD56: $CD56^{bright}$ $CD16^{-}$ (50–70% of $CD56^{bright}$), $CD56^{bright}$ $CD16^{dim}$ (30–50% of $CD56^{bright}$), $CD56^{dim}$ $CD16^{-}$, and $CD56^{dim}$ $CD16^{bright}$ subset (77).

The two major subsets are $CD56^{bright}$ $CD16^{dim/-}$ and $CD56^{dim}$ $CD16^{bright}$, respectively. The $CD56^{dim}$ $CD16^{bright}$ NK cells represent at least 90% of all peripheral blood NK cells and are therefore the major circulating subset (78, 79). A maximum of 10% are $CD56^{bright}$ NK cells.

NK Distibution and trafficking

One of the challenges in identifying tissue NK cells is the discrimination of these cells from other ILCs, since they share several markers. NK cells are present in healthy skin and gut, in the liver, in the lungs, and are abundant in uterus during pregnancy. In addition, human NK cells were investigated also in other tissues such as the kidney (80), joints (81), and breast under pathophysiological conditions. In the normal intestinal mucosae, NK cells (82) are found predominantly as intraepithelial lymphocytes and within the lamina propria, but are rarely associated to lymphoid aggregates, although they can be found in the parafollicular region of cecal lymphoid patches, Peyer's patches, and mesenteric lymph nodes.

NK cells in healthy human liver strikingly account for almost 20–30% of all human hepatic lymphocytes and are found among the non-parenchymal cells that populate this organ (83).

However, in steady-state, NK cells are preferentially located in the hepatic sinusoids, often adhering to the endothelial cells (84). A particular subset of NK cells is found in the placenta, where it regulates specific developmental processes at the fetal–maternal interface. During the first trimester of pregnancy, NK cells represent a subpopulation with unique phenotypic and functional properties, representing about 50–90% of the lymphoid cells infiltrating in this tissue. Carrega et al. (85) showed that NK cells expressing NKp46marker populate the normal lung, counting for ~10% of lymphocytes present in this tissue. In addition, it has been described that the majority (~80%) of lung-NK cells belong to the $CD56^{dim}CD16^{+}$ subset. The localization of NK cells in different tissues indicate that they could migrate to various organs then reside there, where they acquire peculiar activities. Although, different theories argue the possibility that NK cells could re-circulate constantly through the tissues.

At date, NK cell subsets, mostly represented by $CD56^{bright}$ populate gut, liver, lung, and other different human solid tissues thus indicating that specific homing signals are important to drive the localization of NK cells to the different tissues. Importantly, it has been described that NK cells are present in in human afferent lymph draining peripheral tissues suggesting that NK cells might even exit the organ and traffic through tissues in normal conditions (86). Concerning NK trafficking, it has been demonstrated that the expression of CCR7 and L-selectin (CD62L), drive NK cell trafficking.

NK cells continuously traffic toward tissue through a combination of stimuli able to promote their mobilization, such as chemokines. Chemokines bind with high efficiency to physiologic L-selectin ligands on peripheral LN high endothelial venules (HEVs) thus promoting a conformational change in the receptor, that trigger intracellular signals, to finally drive cell polarization, migration, and adhesion, thus resulting in the induction of leukocyte trafficking and homing. NK cell subsets display a differential pattern of chemokine receptor expression. $CD56^{bright}$ NK cells are targeted to lymph nodes via CCR7, preferentially express CXCR3 and have higher CXCR4 expression levels as compared with $CD56^{dim}$ cells. $CD56^{dim}$ NK cells, in contrast, uniquely express CXCR1, and CX_3CR1. CXCR3 ligands are expressed at low levels in homeostatic conditions, but their expression can be upregulated (87). It has been described that in multiple myeloma patients with active disease, to an up-regulation of CXCR3 ligand, CXCL10, corresponded to marked down-regulation of CXCR3 expression levels by BM NK cells, an event that was linked to reduction of NK cell localization in the BM in multiple myeloma-bearing mice (88). CXCL10/CXCR3 axis is also involved in hepatic trafficking of NK cells, in which a truncated form of ligand can bind to CXCR3 without signaling, thus preventing NK cells to migrate into infected liver but they instead accumulate in the peripheral circulation (89).

The *in vivo* NK cell developmental pathway has remained somewhat of a mystery in contrast to the pathways for B cell and T cell development, it was generally accepted that NK cells develop

exclusively within the bone marrow similar to most other leukocyte populations. BM ablation results in NK cell deficiency in mouse models, and human NK cells may be derived *in vitro* from BM-derived $CD34^+$ hematopoietic precursor cells (HPC) (90). Moreover NK cell commitment requires the expression of transcription factors such as nuclear factor IL-3 regulated (NFIL3) and thymocyte selection-associated HMG box factor (TOX). Interestingly, NK cell precursors are normally detected in the circulation, and recent data indicate that specific $CD34^+$ NK cell precursors are selectively enriched in extramedullary tissues where unique subsets of mature NK cells reside, suggesting that the latter may derive locally *in situ* (91).

Scientific consensus at date, suggest that NK cells derived from $CD34^+$ hematopoietic progenitor cells (HPCs). However, the site of maturation and details of the process are only now beginning to emerge. Human T cells develop in the thymus and human B cells develop in the bone marrow, and the intermediate populations can be isolated in situ from their respective maturational sites. In contrast, the characterization of the full NK cell developmental pathway from $CD34^+$ HPCs within the bone marrow or in the thymus needs to be further clarified. A first clue that NK development might not occur wholly in the bone marrow came from the observation that $CD56^{bright}$ could be isolated from lymph nodes and tonsils or secondary lymphoid tissue (SLT). $CD56^{bright}$ NK cells are relatively dominant in SLT compared with their more abundant $CD56^{dim}$ NK counterpart found in bone marrow, blood, and spleen (92).

Further studies have suggested that the $CD56^{dim}$ NK-cell subset is derived directly from the $CD56^{bright}$ NK subset. Interestingly, one of these recent studies also revealed an important role for the CD56 molecule itself in promoting this terminal maturational step. Using an in vitro co-culture system consisting of purified $CD56^{bright}$ NK cells and human fibroblasts, Studies have demonstrated that antibody blockade of the interaction between CD56 and fibroblast growth factor receptor-1 significantly inhibited the generation of $CD56^{dim}$ NK cell (93). Supporting these data, it was found that the $CD56^{bright}$ NK subset is the major NK cell population that is derived early *in vitro* when $CD34^+$ HPCs are cultured in NK development supportive conditions, whereas $CD56^{dim}$ NK cells develop later over time. Additionally, $CD56^{bright}$appear to accumulate earlier in the blood following bone marrow or stem cell transplantation, display longer telomeres compared to $CD56^{dim}$ NK cells.

Additionally, within the SLT relative to blood or bone marrow was found a selective enrichment of both $CD34^+CD45RA^+$ pre-NK cells and $CD56^{bright}$ NK cells in close contact with an abundance of dendritic cells (DCs) and other antigen presenting cells (APCs) that express membrane-bound IL-15. Il-15 is required for NK-cell maturation, suggesting that SLT may be a site, although not preferentially, for NK-cell development *in vivo*.

Among multiple growth factors likely facilitate the development of NK cells, IL-15 is considered the most important NK cell homeostatic cytokine. IL-15 promotes the proliferation and survival

of mature NK cells and is also capable of inducing the differentiation and maturation of $CD34^+$ HPC into $CD56^{bright}$ NK cells in stroma-free medium (94, 95). IL-15 mostly produced by dendritic cells, is typically provided in soluble form *in vitro*, yet *in vivo* it is presented *in trans* as a membrane bound ligand in association with the IL-15 receptor alpha chain (IL-15Rα) (96, 97). In order to respond to IL-15, NK cells must express the common gamma chain (CD132) as well as the IL-2Rα (CD122) (98). As such, human NK cell precursors have been traditionally defined as $CD34^+CD122^+$ cells (IL-2Rα).

Caligiuri et al, described five putative stages of human SLT NK cell development according to the differential expression of CD34, CD117, CD94, and CD16. Stage 1 cells (Lin−CD34+CD117−CD94−CD16−) lack expression of the common IL-2/IL-15 receptor beta chain (IL-2/15Rβ, CD122) and are thus not responsive to exogenous soluble IL-2 or IL-15 ex vivo. However, they can generate NK cells when cultured in IL-15 plus other cytokines, such as Flt3 ligand and c-Kit ligand (KL) that likely induce CD122 expression and hence IL-15 responsiveness. In contrast, stage 2 cells (Lin−CD34+CD117+CD94−CD16−) constitutively express CD122 and a functional high affinity IL-2 receptor, including the IL-2Rα subunit (CD25), can generate functionally mature NK cells in vitro in the presence of exogenous soluble IL-15 in media without other cytokines or support cells. Stage 3 cells (Lin−CD34−CD117+CD94−CD16−) lacked T cell and DC developmental potential and were proposed to represent committed NK cell precursors (99, 100).

The sequential acquisition of receptors and functional capabilities occurs during NK cell differentiation. Immature, not yet cytolytic NK cells acquire activating receptors such as NKp46, NKG2D, and DNAM-1 and the complex CD94/NKG2A, first inhibitory receptor to be expressed. Concomitantly to CD94 acquisition, immature NK cells down-regulate CD117. These events mark the achievement of a mature phenotype reminiscent of peripheral blood $CD56^{bright}$ NK cells.

In peripheral blood, $CD56^{dim}$ NK cells, thought to represent the most mature NK cell population in humans, are capable of robust natural cytotoxicity and target-induced cytokine production. In contrast, a small population of NK cells in the blood shows bright CD56 expression and a relatively higher capacity for *ex vivo* proliferation and cytokine production but relatively lower capacity for natural cytotoxicity in comparison to the $CD56^{dim}$ NK subset. A recent study also shows that peripheral $CD56^{dim}$ NK cells produce low levels of IFN-γ earlier than $CD56^{bright}$ NK cells (91)

Activator and inhibitory Receptors

The regulation of NK cell responses is managed by a balance between signals derived from activating and inhibitory receptors. It is important to note that NK cells respond rapidly to activation signals and, through perforin and granzymes, thus they can directly exploit their

cytolytic activity without the requirement for transcription or cell proliferation. However, an inappropriate NK cell activation may present a danger to healthy thus, it is important that the process of NK cell activation must be tightly regulated.

Many inhibiting NK cell receptors interact with major histocompatibility complex (MHC) class I proteins, which are ubiquitously expressed on the surface of all nucleated cells. Because of the abundant expression of MHC-I, NK cells remain non-responsive to healthy tissue. But when cells have a decreased expression of MHC-I, which can occur during certain viral infections or in tumors, they can become target for NK cell killing. The process by which NK cells detect cells with aberrant MHC-I expression has been termed by Kärre et al. as "missing-self" detection (101).

However, further studies have indicated that NK cell activation may be determined, not only by lack of MHC class I expression, but also the expression of ligands for NK cell-activating receptors. The 'induced self' model of NK cell activation describes the recognition of cellular stress ligands, induced upon malignant transformation or viral invasion.

Additionally, for a correct development of functional NK cells in the bone marrow, interactions between inhibiting receptors and MHC-I are necessary (102). This process is called NK cell "education" and determines the threshold for activation in mature NK cells. Depending on the strength of the inhibitory signals received during development, NK cells balance their activation threshold (103).

Inhibiting NK cell receptors is characterized by the presence of immunoreceptor tyrosine-based inhibitory motifs (ITIM) in their cytoplasmic tail that can decrease the state of activation (104) Commonly, Src homology 2 domain containing phosphatases (SHP1 or 2) are recruited after phosphorylation of a tyrosine residue. Dephosphorylation and specific phosphorylation of intracellular components are thought to be the mechanism by which Inhibitory receptors interfere with activating signaling. The interference in activating signaling can prevent not only NK cell-mediated cytotoxicity, but also interfere with adhesion of NK cells to target cells. In contrast, activating receptors lack ITIMs, but contain a positively charged amino acid (arginine or lysine) in their transmembrane region, and are associated with signaling adaptor molecules containing immuno-receptor tyrosine-based activating motifs (ITAM) (105). After phosphorylation, the Src homology 2 domain containing kinases (Syk or ZAP70) are recruited, leading to a signal cascade, which results in degranulation and transcription of cytokine and chemokine genes. Some activating receptors are associated with adaptive molecules DAP10 or DAP12 which results in different signaling events: DAP-12 signaling results in cytokine secretion and cytotoxicity and DAP-10 signaling results only in cytotoxicity.

Among the activating receptors is a specialized group of receptors called natural cytotoxicity receptors NCRs, which play a key role in recognition and killing of tumor and virally infected cells. Comprising the NCRs are the NKp44, NKp30, and NKp46 receptor. Binding of one or more of these receptors with a specific ligand induces strong NK cell activation and cytotoxicity. NCRs belong to the Ig-superfamily. In humans, NCRs NKp46, NKp80 and NKp30 are expressed on activated and resting NK cells, but NKp44 is upregulated upon IL-2 stimulation of some NK cells. The NCRs were originally believed to be strictly activating NK cell receptors. However, NKp44 and NKp30 have recently been shown to exhibit both inhibitory and activating functions.

Nkp44 (106): is restricted to activated NK cells, its expression is responsible for a dramatic increase in NK cell killing activity, actually the cross linking the receptor results in the release of cytotoxic granules, IFN-γ, and TNF-α. Reported ligands for NKp44 and also forNKp46, include viral hemagglutinins.

Interestingly, the cytoplasmic tail of NKp44 contains a tyrosine sequence resembling an ITIM, the latter is functional and inhibits the release of cytotoxic agents and IFN-γ, thus conferring to this receptor both activating and inhibitory functions. NKp44 surface expression is dependent on its association with the ITAM containing DAP-12 accessory protein. Upon recognition of activating ligands, signaling transduced through the ITAMs in Dap-12 result in release of cytotoxic agents, tumor necrosis factor-α, and IFN-γ (28-40).

While only found on activated NK cells in circulation, NKp44 is constitutively expressed by a specialized subset of NK cells in the decidua, implicating a role for NKp44 during placentation. Decidual NK cells (dNK) make up 50–90% of lymphocytes in the uterine mucosa during pregnancy and constitutively express NKp44 (107). Trophoblast cells and maternal stromal cells of the decidua both express unidentified NKp44 ligands. As an inhibitory ligand for NKp44 that can inhibit NK cell effector function, the extracellular proliferating cell nuclear antigen, PCNA, over expressed in trophoblast cells during the first trimester expression on trophoblast cells could be a candidate, thus explaining the diminished ability of dNK cells to lyse trophoblasts despite low levels of classical HLA I expression (108).

NKp44 is implicated in recognition and killing of numerous types of cancer: neuralblastoma, choriocarcinoma, pancreatic, breast, lung adenocarcionma, colon, cervix, hepatocellular carcinoma, Burkitt lymphoma, diffuse B cell lymphoma, prostate. While NK cells utilize NKp44 to recognize and kill targets, tumors may also exploit NKp44 to escape NK cell recognition. By engaging NKp44, as well as the other NCRs, tumors can induce NK cell death via up regulation of Fas Ligand on the NK cell, inducing Fas-mediated apoptosis. Tumors may also down-regulate NKp44 surface expression by shedding soluble MHC Class I chain-related molecules or by releasing indoleamine 2, 3-dioxygenase (IDO) and prostaglandin E2 (109).

Recognition of tumor cells is partially mediated through charged-based binding of NKp44 with heparan sulfate proteoglycans (HSPGs) on the surface of tumor cells. Of note, recognition of HSPG only evokes IFN-γ release by NK cells, not cellular cytotoxicity Truncated isoform of mixed-lineage leukemia-5 (MLL5) is an activating cellular ligand for NKp44. This MLL5 isoform contains a specific exon encoding a C-terminus, which interacts with NKp44. Typically located only in the nucleus, MLL5, at date, is considered a possible NKp44 ligand; it is a lysine methyltransferase implicated in hematopoietic differentiation and control of the cell cycle. Contrary to normal MLL5, the isoform recognized by NKp44 is not found in the nucleus but in the cytoplasm and endoplasmic reticulum, destined to be expressed at the cell surface. While MLL5 is expressed in normal tissue, the isoform recognized by NKp44 is only present on tumor and transformed cells. NKP44 recognizes the C terminus of the MLL5 ligand. Due to the dual nature of NKp44 signaling, it will be of interest to determine whether the modulation of NK cell activity via the NCRs, in particular NKp44 could depend on the recognition of the DAMPs molecules, either PCNA or MLL5 (110).

NKP30: The NKp30 activating receptor has emerged as a promising therapeutic target in multiple tumors. Downregulation is observed in patients with cervical cancer and high-grade squamous intraepithelial lesions. In lymphoma and leukemia models, ligation of NKp30 has been shown to activate human NK cells, trigger degranulation, and increase cytotoxicity. In patients with gastrointestinal sarcoma, the NKp30 isoform predicts the clinical outcome; patients with the immunostimulatory NKp30a and NKp30b isoforms have increased survival relative to patients with the immunosuppressive NKp30c isoform. The ligand for NKp30, B7-H6, was highly expressed in neuroblasts, and the serum soluble form of B7-H6 correlated with tumor load and disease dissemination. Concerning NK and DC interaction it is important to note that NKp30 has been reported to have a unique role in determining the fate of immature DCs (iDCs) during their interactions with NK cells. Under certain conditions, NK cells can kill autologous iDCs via ligation of NKp30 (111). Alternately, instead of inducing iDC killing, NK cells can also mediate the maturation of iDCs via engagement of NKp30 and the release of TNF-α and IFN-γ (20). The mechanism controlling the dual roles of NKp30 is not clear, although the ratio of NK cells to DCs is thought to be important (112).

Nkp46: It represent an important regulator of NK cell function and was found recently expressed on some ILCs and a small subset of T cells. Engagement of the CD335 receptor on NK cells results in increased cellular activation, in terms of increased cytokine production and release of cytolytic granules. Although the identity of an endogenous ligand for CD335 is not yet known, the receptor can confer tumor cell recognition activity NK cells and can specifically bind viral hemagglutinins, supporting roles for NK cell CD335 expression in antitumor and antiviral immunity. Normal NK cells in humans and in rodents uniformly express CD335, which is

upregulated during NK cell maturation following commitment to the NK cell lineage. Sivori et all found that NKp46 triggering strongly induces the NK cell-mediated cytolytic activity (113).

As a tumor immunosuppressive mechanism, the surface expression of NKp46 on NK cells can be down-modulated by exposure to l-kynurenine, a m catabolism product generated by IDO enzyme in tumor microenvironments (114). Even though NKp46 is associated with ITAM-bearing subunits, stimulation of primary resting NK cells with NKp46 Abs was not sufficient to activate degranulation (115). However, when combined with signals from any one of the receptors 2B4, DNAM-1, NKG2D or CD2, NKp46 induced degranulation. This stands in contrast to signaling by CD16, which is sufficient to activate degranulation.

NKp80, the most recent discovered receptor, was expressed at the cell surface as a dimer of approximately 80 kDa (NKp80). In polyclonal NK cells, mAb-mediated cross-linking of NKp80 resulted in induction of cytolytic activity and $Ca2^{+}$ mobilization (116). Recently, Freud et al demonstrated that NKp80 expression is closely correlates with NK cell functional maturity in SLTs suggests that NKp80 may fulfill an important regulatory role during the maturation process in SLTs, potentially related to the acquisition of cytotoxic and cytokine-production. NKp80, that seems have activating properties, stimulates Sykphosphorylation, but the signals downstream of Syk are yet not known.

Among inhibitory receptors the leukocyte immunoglobulin-like receptors (also known as LIR, ILT or CD85) are expressed on NK cells and bind MHC class I molecules. The function of LIR in the regulation of NK cell activation is unclear, as leukocyte immunoglobulin-like receptor receptors are able to inhibit NK cell activation, although inhibitory KIR and CD94–NKG2 receptors are thought to be more dominant. KIRs have evolved from the Ig-superfamily and consist of type 1 transmembrane glycoproteins with two or three Ig-like domains and possess either a short or long cytoplasmic tail. The overall KIR repertoire is determined by KIR genotype. The repertoire of *KIR* genes expressed within one individual forms a KIR haplotype (117).

KIR are characterized by two (KIR2D) or three (KIR3D) extracellular immunoglobulin domains. In addition, they have either short (S) or long (L) intracytoplasmic tails which transduce activating or inhibitory signals, respectively. The known ligands for inhibitory KIRs are all represented by MHC class I molecules: HLA-C is recognized by KIR2DL1, KIR2DL2 and KIR2DL3; HLA-B by KIR3DL1 and HLA-A by KIR3DL2, while HLA-G is recognized by KIR2DL4. The HLA determinants that bind to inhibitory KIRs are known as KIR epitopes. In particular, HLA-C allotypes have either the C1 epitope, recognized by KIR2DL2/3, or the C2 epitope that are the ligands for KIR2DL1. Similarly, all HLA-B allotypes have either the Bw4 or Bw6 epitope, but only the Bw4 epitope is a ligand for KIR, its cognate inhibitory receptor being KIR3DL1. Concerning the role of KIR in viral infection, several studies reported association

between some viral infection and diverse KIR aplotypes. An association study performed by Lu et al. demonstrated a lower frequency of A haplotype for KIRs and higher presence of the B haplotype in patients exposed to hepatitis B virus compared to healthy controls (117).

In addition to KIRs, CD94-NKG2A (lectin-like) inhibitory receptor recognizes MHC molecules; this complex is specific for HLA-E and peptides derived from the leader sequences of many, but not all, HLA-A, HLA-B and HLA-C polypeptides. Importantly, individual NK cells express only one or few inhibitory KIRs, while some NK cells do not express KIR but express CD94-NKG2A or inhibitory KIR and CD94-NKG2A receptors may be co-expressed by subsets of NK cells. Loss of HLA molecules is a common event in cells undergoing tumour transformation or infection with some viruses. When NK cells expressing KIR with specificity for a particular allotypic determinant interact with target cells that have lost these allele, they are not inhibited and kill the potentially harmful target cells (117).

In mouse and human, several NKG2 molecules have been identified and shown to dimerize with CD94. These include NKG2A and its splice variant NKG2B, which form inhibitory receptors, as well as NKG2C and NKG2E, which form activating receptors. CD94/NKG2 receptors are expressed on NK cells during ontogeny and are maintained into adulthood. Depending on the NKG2 molecules that dimerize with CD94, either an inhibitory or an activating receptor is formed.

Receptors considered costimulatory include members of the signaling lymphocytic activation molecule (SLAM) family, such as 2B4 (CD244), as well as unrelated receptors such as DNAM-1 (CD226). Among these, 2B4 receptor is present on all human and mouse NK cells, expressed as one of two isoforms, of which only one has been shown to activate NK cell cytotoxicity;

NK cells express a long list of other receptors with activation potential. DNAM-1 receptor (also known as CD226) is a member of the Ig-superfamily, and is constitutively expressed upon approximately 50% of NK cells. The ligands for this co-stimulatory activating receptor are CD155 (also referred to as Polio virus receptor, PVR or Necl-5) and CD112 (Nectin-2), and these ligands can be upregulated on some tumor cells, implicating DNAM-1 in some NK cell-mediated anti-tumor responses. Furthermore, DNAM-1 participates, together with NKp30, in the killing of dendritic cells (118). Additionally, another NK receptor with important function is NKG2D that binds to a number of ligands that are induced on cells that are under stress due to infection, transformation or DNA damage. These include MICA and MICB and the family of ULBP molecules in humans, and H60 and Mult1 and the family of RAE-1 molecules in mice (119). Therefore, it has an important function in targeting NK cell responses towards abnormal cells. Expression of cellular stress ligands MIC-A and MIC-B has been reported to be induced upon malignant transformation, reportedly as a result of the DNA damage response pathway.

NK-DC cooperation during viral infection

Several events occur upon microbial and non-microbial invasion. These represent the first events that promote NK-DC interaction. In response to different stimuli, cells were recruited in the site of injury, where chemokines are released thus promoting NK-DC encounter. The interaction and consequent activation of these two innate immune components are crucial to ensure an appropriate anti-viral response.

Immature DCs release CCL3, CXCL8. Also, macrophages and endothelial cells produce CC22, CX3CL1 during inflammation enhancing immune cell recruitment. On the other hand, NK cells express receptors that are specific for these factors, such as CXCR1, CX3CR1.

NK cells are readily recruited and activated at the sites of viral infections where they can directly killing infected cells. However, it is now established that cytokines such as IFN-γ and TNF-α, produced by NK cells, contribute to the control of multiple human viral infections. Additionally, the NK cell effector functions are stimulated through direct contact with activated DCs. Altogether these findings have paved the way to further investigation about the cell interaction between these two cell type. Several studies have demonstrated that DCs have a major effect on resting NK cells from human blood. DCs were able to induce proliferation, cytolytic function and also promote the release of cytokines by NK cells, mainly TNF-α and IFN-γ, this latter well known for controlling viral replication. DC-mediated NK cell activation occurs mainly through the release of soluble factors. In vitro studies have demonstrated a central role for DC-derived IL-12 in the induction of IFN-γ production by NK. IL-18 can synergize with IL-12 by inducing the expression of IL-12R on NK cells. Moreover, IL-18 and IL-12 synergize to enhance NK cell cytotoxic activity (120).

Importantly, NK cells can also be activated independently of IL-12 or IL-18. Indeed, Granucci et al. (121) found that following stimulation with TLR4 ligands such as LPS, NK were efficient in producing IFN-γ. Another relevant cytokine for NK cell development and functions is IL-15, which is mostly produced by DCs. This cytokine can be presented by DCs via its binding to IL15R alpha or as trans-membrane protein; it can stimulate NK cell proliferation, survival, and priming of protective NK cell response. It has been described by Ferlazzo et al (122) that IL-15 production by DCs could be particularly important to trigger NK-cell proliferation; study reported that IL-15 production by human monocyte-derived DCs or spleen DCs induces NK cells to proliferate. Optimal NK-cell activation by DCs also requires direct cell-to-cell contacts

Recently, Borg et al (123) found that NK cells and DCs form stimulatory synapses involving cytoskeleton rearrangement and lipid raft mobilization in DCs. Synapse formation allows the polarized secretion of preassembled stores of IL-12 by DCs toward the NK-cell. This IL-12 secretion leads to IFN-γ production (Imagine 1). Similar findings have been reported for IL-18.

Also, the interaction of CXC3CL1 expressed on DCs with CX3CR1 on NK cells was found to result in IFN-γ release by NK cells (48). Interestingly, it has been shown that influenza virus-infected DCs can support IFN-γ production by triggering the activating receptors NKp46 and NKG2D. Collectively, these studies indicate that in vitro NK cell activation induced by DCs requires the synergistic action of several cytokines and a direct contact between DCs and NK cells.

Among human NK cell subsets, $CD56^{bright}$ NK cells were found to be particularly responsive to activation by DCs. Interestingly, NK cells are enriched in SLO and in most solid tissues and their presence in afferent lymph also suggests that they may re-circulate from peripheral solid tissues to SLO; thus, it is conceivable that, *in vivo*, NK–DC cross-talk may occur either in peripheral tissues or in lymph nodes, where, in both cases, NK cells can encounter distinct myeloid DC subsets.

The activation of NK cells upon interaction with DCs has important consequences not only for the lysis of virus-infected cells, but it can also boost ongoing adaptive responses by the release of IFN-γ, which promotes type 1 polarization of T cells. It is important to note that NK/DC cross-talk during viral invasion is bi-directional; therefore, DCs, upon recognition of viral antigens and, are able, through cytokine release and cell-to cell contact, to stimulate NK cell functions, as well as NK are responsible for DC maturation (120).

To this regard, during the early phase of an anti-viral immune response, the microenvironment is influenced by a peculiar cytokine milieu, which includes cytokines directly released following NK cell activation. NK cells release large amounts of TNF-α and IFN-γ, which are known, as well as the engagement of surface receptors, to affect DC maturation. TNF-α enhances the expression of costimulatory molecules on DCs and, synergizing with IFN-γ, thus contributing to DC production of IL-12 (120).

Additionally, it has been reported that cell to cell contact is also crucial in NK/DC interaction and to this regard NKP30 play a major role. It has been reported a balance between activating and inhibitory signals exploit by NK cell to mediate DC maturation. NK cell mediated monocyte-derived DC maturation depends on the triggering of NKp30 on NK cells and is counter regulated by KIRs and NKG2A inhibitory receptors signalling (111).

The interaction occurring between DCs and NK cells lead to the direct production of IFN-γ. However, some reports suggest a role for NK cells in promoting antigen cross-presentation by DCs. It has been shown that DCs can take up dying cells killed by NK cells and present them on MHC class I molecules. Obviously, NK cell ability to lyse virally infected or tumor cells could help uptake and cross-presentation of antigens by DCs but whether NK cells also play a direct role in favoring DC cross-presentation is still not clear. Although, studies show that the capability of mono-derived DCs, generated in the presence of IFN-α, to prime $CD8^{+}$T cells against human

tumor antigens is dependent on NK cells; NK cell removal indeed leads to generation of IFN-DCs with no priming activity of tumor Ag-specific T cells (124). It is conceivable that NK cell killing of tumor cells could provide antigens subsequently taken up, processed, and cross-presented by DCs; at the same time, activation of NK cells is associated to the secretion of cytokines, such as TNF-α or IFN-γ, potentially able to help cross-priming of specific CTLs.

As previously described, *in vitro*, crosstalk between NK cells and matured DCs result in activation and cytokine production by both cell types.

NK–DC interactions were shown to be important for optimal immune cell expansion and activation during viral infection *in vivo* (125, 126). Subsequently, several human studies have investigated the range of interactions between NK cells and DCs. NK/DC interaction was found to occur in vivo. Decidual antigen-presenting cells were observed in situ to have intimate contact with decidual NK cells, during human pregnancy. Interaction between these two cell types was found, also in skin lesions or in some chronic inflamed tissue.

As previously mentioned, NCRs triggering on NK cells lead to the destruction of virus-infected cells or tumors.Once NK cells have reached the site of infection, direct contact between NK cells and iDCs can occur. The interaction of NK cells with myeloid DCs takes place during the early phases of inflammation when DC-derived cytokines such as IL-12 and IL-15 are produced in response to triggering by pathogen-associated products and provoke NK cell maturation. Subsequently, cytotolytic function and release of cytokines such as TNF-alpha and IFN-gamma NK cells are up-regulated, in turn, these latter promote the maturation of DCs. In that phase NK cells acquire the capability to kill autologous iDC, an event known as Editing. Additional studies revealed that only a fraction of NK cells is capable of killing autologous iDC. This subset is able to discriminate immature and mature DCs by their level expression of MHC-I. iDCs are characterized by a severe low level of HLA class I expression and are killed by NK cells, while the up-regulation of HLA class I by DCs undergoing maturation allows these cells to resist to NK cell-mediated killing. Furthermore, the recognition of immature DCs by NK cells was found to rely almost entirely on the NKp30 activating receptors. Although, also mature DCs are able to trigger the same receptor, they seem to use an independent mechanism to actively stimulate resting NK cells. Importantly, Editing by NK cells serves as important mechanism to check the quality of DC undergoing maturation before their migration to lymph nodes (111, 112).

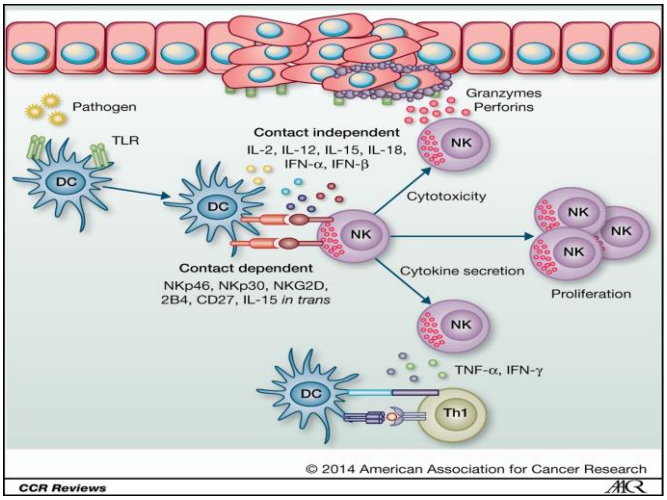

Image 1: DC-induced NK cell activation. DCs can affect NK cell function by augmentation of cytotoxicity, cytokine secretion (IFN-γ and TNF-α), and proliferation. Van Elssen et al, Clinical Cancer Research, 2014.

Hepatitis B virus: Role of innate immunity in controlling HBV infection and its involvement in viral pathogenesis

Hepatitis B virus (HBV) infects more than 300 million people worldwide and is a common cause of liver disease and liver cancer despite the availability, from 1982, of safe and effective vaccines that have as a major goal the prevention of the spread of this virus. To date, vaccination programs have focused on eliminating mainly perinatal transmission to neonates, where the risk of developing chronic infection is ranking from 70% to 95%, and to young infants, but sexual transmission and injection drug use remain the major risk factors for acquiring infection. The prevention of morbidity and mortality associated with chronic HBV infection are considered the main hot spots. Efforts are focused mainly to achieve a risk reduction of progressive liver disease.

HBV, a member of the *Hepadnaviridae* family, is a small DNA virus with unusual features similar to retroviruses. Related viruses are found in woodchucks, ground squirrels, tree squirrels, that shared 70% sequence homology to human virus, thus are used as model to better understand disease pathology. Based on sequence comparison, HBV is classified into eight genotypes, A to H. Each genotype has a distinct geographic distribution: genotype A prevails in north-western Europe and in the United States, genotypes B and C in Asia, genotype D in the Mediterranean

basin, the Middle East, and India, genotype E in Western Africa, genotype F in South and Central America, genotype G in the United States and France, genotype H in Northern Latin America, genotype I in Laos, Vietnam, Eastern India and North-Western China and genotype J in Japan (1).

HBV consists of an outer lipid envelope and an icosahedral nucleocapsid core composed of several proteins. The nucleocapsid encloses the viral DNA and a DNA-polymerase,able to act also as a reverse transcriptase. HBV has a double-stranded DNA genome of approximately 3200 base pairs organized in four overlapping open reading frames (ORFs: *S*, *C*, *P*, and *X*). The *S* ORF encodes the viral surface envelope protein, the HBsAg, and can be structurally and functionally divided into the pre-S1, pre-S2, and S regions. The core or *C* gene has the precore and core coding regions; the *C* ORF encodes either the viral nucleocapsid HBcAg or hepatitis B e antigen (HBeAg). The polymerase (pol) is a large protein of about 800 amino acids, encoded by the *P* ORF and is functionally divided into three domains: the terminal protein domain, which is involved in encapsidation and initiation of minus-strand synthesis; the reverse transcriptase (RT) domain, which catalyzes genome synthesis; and the ribonuclease H domain, which degrades pregenomic RNA and facilitates replication. The HBV *X* ORF encodes a 16.5-kd protein HBxAg, whose specific role is still to be clarified, but it has been described to be involved in multiple functions, including signal transduction, transcriptional activation, DNA repair, and inhibition of protein degradation(1).

Although, the genome of HBV is made of circular DNA, it is unusual since DNA is not fully double-stranded. The partially double-stranded DNA is rendered fully double-stranded by completion of the (+) sense strand and removal of sequence from the (−) sense strand and a short sequence of RNA from the (+) sense strand. The initial phase of HBV infection involves the attachment of mature virions to host cell membranes. After entry of the viral genome into the nucleus, the single-stranded gap region in the viral genome is repaired by the viral pol protein, and the viral DNA is circularized to the covalently closed circular form (cccDNA). Persistent viral infections require that the viral genome be present in the infected cell in a stable form that is not lost during cell division, and which therefore can be used for the continuous production of progeny genomes. However, it has been reported that HBV DNA is able to integrate into host chromosomes only in tissues of Hepatocellular Carcinoma (HCC) patients or in cell lines derived from HCC. It was hypothesized that HBV could act as an insertional mutagen causing the activation of proto-oncogenes. In a woodchuck model, viral DNA was found to integrate close to members of the Myc proto-oncogene family and the resulting oncogene deregulation appeared to be a major cause of liver cancer. In humans, HBV integration is random and at date, it is difficult to predict possible insertion points as well as preferential sites. Conversely, it is clear that HBV integration plays a direct role in carcinogenesis, although in only a few cases, where the viral

DNA is inserted next to important growth regulatory genes. Viral DNA integration may also promote the general instability of chromosomal DNA in the infected cells, which may be exacerbated by the chronic inflammation in the infected liver (127). However, the cccDNA form allows HBV to establish persistent infection. Additionally, this form of HBV DNA serves as the template for transcription of several species of genomic and sub-genomic RNAs. The transcripts from the cccDNA consists primary of the pregenomic and the precore RNAs. The latter directs the translation of the precore gene products, while the pregenomic RNA (pgRNA) serves as the template for reverse transcription and as messenger-RNA for core and polymerase. Furthermore, pgRNA is the template for generation of new DNA genomes by reverse transcription. The next crucial step in HBV replication is the specific packaging of pgRNA, where a crucial role is played by encapsidation signal ε, which binds P protein together with pgRNA. Once pgRNA and P protein are being encapsidated, reverse transcription starts its action. The polymerase begins to reverse transcribe the pgRNA template for three to four bases. The polymerase protein is actually covalently attached onto the growing (-) DNA strand. Reverse transcription of the pgRNA generates the (-) DNA strand.

As (-) DNA occurs, the newly copied pgRNA template is degraded by RNase H activity of the polymerase protein. However, the 15 to 18 capped oligoribonucleotides at the 5' end of the pgRNA remain undegraded once the (-) DNA strand is completed. This oligoribonucleotide cap serves as the primer for (+) DNA strand synthesis. Among viral particles, the HBV Reverse Transcription needs to have a special note; HBV RT is a multifunctional protein that consists of four domains: terminal protein (TP), reverse transcriptase, RNase H, and a non-conserved spacer domain between the TP and RT domains. Initiation of reverse transcription and nucleocapside assembly in HBV is carried out by RT. The unique TP domain is used as a protein primer to initiate reverse transcription catalyzed by the RT domain.

Concerning regulation of HBV transcription, Pollicino et al (128) reported that HBV replication seems to be regulated by the acetylation status of histones H3 and H4 bound to cccDNA minichromosome. These results suggested that HBV transcription can be modulated by epigenetic changes to the cccDNA and provide new insight into how cellular host factors might regulate HBV replication.

Viral particles are visualized in serum from infected-patients by electron microscopy. It was observed spheres and filaments of about 22 nmol/L, that was found to be composed of hepatitis B surface antigen (HBsAg) and host-derived lipids without viral nucleic acids and are therefore considered noninfectious. HBsAg is an envelope glycoprotein that is currently the primary element for diagnosis and target of immunoprophylaxis of HBV infection. Several mutations in the S region have been described and those most frequently reported in the literature. The majority of mutations were located in the S region, but some mutations were also identified in the

pre-S1 or pre-S2 regions. HBV envelope contains the embedded proteins HBsAg, pre-S1 and pre-S2 involved in the viral binding and entry into susceptible cells. The infectious HBV virion (Dane particle) has a spherical, double-shelled structure 42 nm in diameter, consisting of a lipid envelope containing HBsAg that surrounds an inner nucleocapsid composed of hepatitis B core antigen (HBcAg) complexed with virally encoded polymerase and the viral DNA genome.

Hepatocytes are the only confirmed site of replication for HBV. The infection of hepatocytes follows a multiple stepwise process. Subsequently, viruses interact with their specific receptor(s) with high affinity, triggering the early entry process, although the precise mechanism for triggering viral internalization has not yet been clarified. However, sodium taurocholate cotransporting polypeptide NTCP was finally revealed to be a specific entry receptor of HBV. NTCP is distributed almost exclusively in the liver in humans, thus explaining the specific viral tropism.

Experiments demonstrated that both tupaia sodium taurocholate cotransporting polypeptide (tsNTCP) and human sodium taurocholate cotransporting polypeptide (hNTCP) bind to the preS1 peptide; this indings were confirmed by knockdown experiments of endogenous hNTCP or tsNTCP in HepaRG, PTH, and primary human hepatocytes (PHH) that have resulted in the reduction of HBV and HDV infections. Confirming these data, ectopic expression of hNTCP confers viral susceptibility to HepG2 and Huh-7 cell line, which otherwise do not support HBV or HDV infections. These data indicate that NTCP is essential for HBV and HDV infection. Expression of NTCP correlates well with the susceptibility to viral infections; it is abundantly expressed in susceptible PHH and differentiated HepaRG cells, whereas little or no NTCP is detected in resistant cell lines, such as HepG2, Huh-7, FLC4, and HeLa cells (129).

For reasons that need to be further investigated, ectopic expression of hNTCP confers susceptibility to only a fraction of human hepatocyte cell lines, such as Huh-7, HepG2, and undifferentiated HepaRG cells, So far, HepG2 cells show the highest infection efficiency following expression of hNTCP; however, <10% of the cells were infected with HBV according to the original studies. These data indicate that susceptibility to HBV infection is governed by factors other than simple expression levels of NTCP. Such factors include post-translational modification of NTCP, cell cycle status, and the presence of other unknown factors essential for HBV infection.

HBV infection leads to a wide spectrum of liver disease ranging from acute hepatitis (including fulminant hepatic failure) to chronic hepatitis, cirrhosis, and hepatocellular carcinoma.

HBV infection can be presented in Acute or Chronic form. Patients with Acute infection have a mild, asymptomatic and subclinical illness that usually remains undetected. Laboratory diagnosis is essential to monitoring the progression or the phases of HBV infection. The incubation period

is followed by a short pre-icteric or prodromal period of constitutional symptoms such as fever, fatigue, anorexia, nausea, and body aches. During this phase, serum ALT levels rise and high levels of HBsAg and HBV DNA are detectable. Additionally, IgM Abs specific for the HBcAg are present, and patients are also seropositive for HBeAg that represents usually a marker of high levels of replication of the virus. The presence of HBeAg indicates that the blood and body fluids of the infected individual are highly infectious. During convalescence, jaundice is resolved along with the clearance of HbsAg, followed by the disappearance of detectable HBV DNA level from serum. In acute resolving infections, the response of the innate and adaptive immune system to HBV is efficient and timely. Viral clearance involves the induction of a robust adaptive T cell reaction inducing both a cytolytic dependent and independent antiviral effect via the expression of antiviral cytokines.(1).

Chronic hepatitis B (CHB) has a variable and dynamic course. Early during infection, HBeAg, HBsAg, and HBV DNA are usually present in high titers. Chronic HBV infection progresses through distinct disease phases that are strongly associated with age reflecting the interaction between HBV replication and the host immune response. Chronic infection begins when the immune response that normally clears the infection fails to take place or is too weak to be effective in viral clearance. The prognosis for many chronic carriers of HBV who were infected with HBV, mainly adults, is poor. The immune-mediated pathological processes were found to ultimately lead to cirrhosis and hepatocellular carcinoma in about 25% of patients. Concerning HBV treatment, there are currently two approved therapeutic agents for chronic HBV, alpha-interferon and nucleoside analogues (1).

Both control of infection and liver cell injury are strictly dependent by protective immune responses. Resolution of acute hepatitis B is associated with functional and efficient antiviral T-cell responses and by long-lasting protective memory. While Chronic infection persistence is characterized by a lack of protective T-cell memory maturation and by an exhaustion of HBV specific T-cell responses. Thus, a key role in HBV control and liver cell injury is played by cell-mediated immune responses.

HBV infection acquired in adult life is that HBV remains quiescent for some weeks before starting an active and exponential phase of replication.

Thus, the role of innate responses in the initial phase of HBV infection is found to play a crucial role in the control of viral replication, although HBV has developed specific strategies to evade recognition by the innate immunity. In particular, the use of a transcriptional template, cccDNA, that is sequestered within the nucleus of infected cells in its replication cycle may allow HBV to escape detection by the innate DNA sensing cellular machinery. In addition to escaping innate immunity, HBV seems also to be able to actively inhibit innate responses, for example, affecting

production and blocking antiviral activity of type I IFN by interference with IFN signaling by its non-structural protein. .In addition, secretory HBV proteins (HBsAg, HBeAg) have been reported to suppress TLR expression and to abrogate TLR-induced responses.

Because of the poor induction of innate intracellular immunity, innate immunity contributes to the early non-cytolytic control of infection. The contribution of innate effectors is likely to be of particular relevance, mainly in the liver, where their frequencies are greatly enriched. Different key components of innate immunity are able to respond to HBV or contribute to its control or, eventually, to HBV pathogenesis. Among innate immune cell type, NK cells are able to efficiently respond to Acute HBV infection in the liver. In the healthy liver, the percentage of NK is typically increased, and their enrichment is maintained in the inflammatory infiltrate characteristic of HBV infection. Resident healthy intrahepatic NK cells display functional characteristics that are distinct from their circulating counterparts. The majority have a $CD56^{bright}$ phenotype and a more activated phenotype

The antiviral potential of NK cells has been demonstrated in animal models of HBV infection, in which NK cells efficiently inhibiting HBV replication in transgenic mice and making a contribution to viral clearance in the hydrodynamic injection model of Acute HBV.

Moreover, in acute HBV infection it has been observed an increase in the number of circulating NK cells (130) but their activation and effector function was suppressed as viral load increased and only peaked once viremia had resolved (131) However, the delayed in NK cell activation observed during acute infections, results temporally associated with an induction of IL-10, again raising the possibility that HBV can actively evade immune responses.

Data observed in acute infection suggest that NK cells may make their major contribution to HBV control in the earliest phase of infection. However contradicting information about the role of NK cells during the early stages of infection have been reported. During acute infection NK cells were promptly activated before peak viremia occurred, as indicated by the early increase of NK cells expressing the activation markers CD69 and NKG2D.

In contrast, in peripheral blood of patients with chronic HBV infection with high-level viremia, NK numbers is reported to be maintained or to reduced; additionally, their activation and anti-viral cytokine production, such as IFN-γ and TNF-α are significantly impaired (132).

Indeed, several studies have demonstrated that NK cells, in both liver and peripheral blood of chronic HBV infected patients are defective in the production of cytokines like IFN-γ, thus their non-cytolyic antiviral capacity results impaired among with their ability in promoting T cell responses. It has been proposed that this selective defect in NK cell function may be attributable

to the influence of IL-10 and TGF-β in the liver, since it was restored following *in vitro* blockade of these immunosuppressive cytokines (133, 134).

Additionally, it has been proposed that activated intrahepatic NK cells could exacerbateHBV-related liver damage through lysis of infected hepatocytes through granzyme- perforin or death receptor pathways, suggesting that their cytotoxic ability remain intact (135, 136). However some studies suggest also that hepatocytes may be relatively resistant to perforin/granzyme-mediated cytotoxicity and mouse models have supported a role for death ligands from the TNF superfamily in mediating liver damage through the induction of hepatocyte apoptosis. Human NK cells were reported to express minimal amounts of TRAIL in both the circulation and liver of healthy individuals, but NK cells, mainly $CD56^{brigh}$t subset could upregulate this ligand in the setting of HBV-related liver inflammation (134, 135).

Additionally, The Fas pathway was found implicated in hepatocyte apoptosis; evidence show that DC-activated NK cells are capable of inducing HBV-infected hepatocyte degeneration in a humanized mouse model through the Fas/FasL system However, a direct role for NK cells mediating liver damage via the Fas pathway has not been fully established.

The regulation of activatory and inhibitory NK cell receptors by cytokines milieu or viral particles might play an important role in involvement of NK cells in HBV infection control. In patients with CHB, in which NK cells was found impaired in their anti-viral activity, it has been reported a partial recovery of IFN-γ production by NK cellsupon antiviral therapy; These observations were linked to the downregulation of NKG2A (132). While, Micco et al (137) described an enhancement of NK cell effector function during IFN-α treatment associated with an induction of NKp46 expression.

Peppa et al observed that activated HBV-specific T cells in the intrahepatic compartment, upregulate death receptors that render them susceptible to elimination by NK cells suggesting that in CHB infection NK cell might play a role in disarming antiviral T cell response, rending ineffective viral control.

Impaired functionality of NK cells in CHB has also been attributed to the capacity of TGF-β to downregulate the NKG2D/DAP10 and 2B4/SAP pathways In a recent study, HBV modulation of plasmacytoid Dendritic cells pDC–NK cell crosstalk was found to contribute to impaired IFN-γ production by NK cells, highlighting the importance of reciprocal interactions with other innate cells (138).

To this regard, myeloid dendritic cells (mDC), of patients with chronic HBV was found impaired in their capacity to mature compared to mDC of healthy controls, as well as to stimulate T cells (139).

The different steps of binding, uptake and subsequent replication of HBV might all compromise DC functions. Contradictory results have been reported about the possible presence and active replication of HBV in *in vitro*-generated monocyte-derived DC of patients with chronic HBV. Recent studies showed that myeloid DC actively internalize HBsAg and that the presence of either purified HBsAg or intact viral particles during mDC maturation gives rise to mDC with some functional changes such as significant less immunogenic phenotype compared to DC of healthy controls, suggesting that HBV might have evolved to interfere with the function of DC to escape the host immune response. However, this DC impairment was recently challenged by a study of Tavakoli (140).

The reduction of co-stimulatory molecules on DC has been suggested as a possible explanation for the decreased capacity in T cell stimulation.

To this regard, it has been reported that the restoration of T cell stimulatory capacity of mDC was observed upon treatment with nucleoside analogue adefovir, suggesting that the reacquired ability of DC to prime T cell response, might be the result of viral load reduction.

Conflicting data have been reported about DC ability to increased proportion of regulatory T cells in HBV infection setting. It has been reported that T regulatory $CD25^{+}$ cells are increased in peripheral blood of CHB patients. However, recent studies have demonstrated that either HBV and HBsAg-treated DC did not significantly induce regulatory T cells in an allogeneic MLR.

Aim of the study

The activation of an efficient innate immune reaction, mainly in the early phase of HBV infection, is crucial to ensure an appropriate antiviral response. To this regard, the interaction between the two main innate components, NK and DCs play a pivotal role in counteracting viral replication. It has been reported that NK cells from both peripheral and liver compartment display defective function, mainly in the production of IFN- γ, known for their role in controlling viral replication Since DCs are potent activator of NK cells, the aim of this study was to investigate whether the presence of HBV might affect dendritic cell functions, thus impairing NK/DCs cross-talk, resulting in a less efficient NK cell anti-viral response.

Materials and Methods

Cell isolation and generation of human monocyte-derived dendritic cells

Peripheral blood mononuclear cells (PBMCs) were obtained from healthy donors by Ficoll-Isopaque gradient centrifugation (Euroclone). A written informed consent was obtained from each donors as appropriate. This research study was approved, as request, by our Institute.

NK cells were isolated from PBMCs either by magnetic cell-sorting technique (Miltenyi Biotec) or by FACS Aria cell sorter (BD) as $CD3^-$, $CD56^+$ cells. Purity of NK cells were consistently >95%.

$BDCA1^+$ DCs were isolated from healthy donors or Chronic Hepatitis B virus (CHB) patients by FACS sorting using the following antibodies and strategy: LIN- (CD3,CD19,CD14), CD1c+.

Dendritic Cells were generated as previously described (Sallusto and Lanzavecchia, J. Exp. Med, 1994). Briefly, monocyte-derived DCs were obtained from plastic adherent PBMCs cultured in the presence of IL-4 (MiltenyiBiotec) and GM-CSF (Sargramostim), at 20 ng/mL and 25 ng/mL respectively, in RPMI 1640 supplemented with 2% of sera collected from Chronic Hepatitis B virus (CHB serum) infected patients (HBV DNA levels ranging from 106 to 108 IU/mL) or 2% of AB control serum. Alternatively, monocytes were isolated from PBMCs by sorting as $CD14^+$ and cultured as previously described.

After 6 days of culture, cells were characterized by the $CD14^-CD11c^+CD83^-$ phenotype corresponding to immature DCs. CHB and AB sera were heat inactivated at 56° C for 1h and filtrated with 0,22 µm filters (BD) before use.

Dendritic cell *in vitro* stimulation and analysis

Immature (iDCs) were stimulated to undergo maturation with Poly I:C (Sigma-Alrich) at 25ug/mL in the presence of 2% of CHB or AB serum for 24 h or at different time points and viral load to perform time and dose-dependent experiments.

Alternatively, iDCs were stimulated with Poly I:C with or without HBV particles, purified as described below.

Cells were harvested, washed twice with PBS and analysed by FACS Canto II for the expression of the most relevant maturation and activation markers: CD80, CD83, HLA-DR, CD86, CD25, CD40, HLA-I.

DCs were analysed for intracellular IDO expression. Briefly, DCs were treated with/without HBV particles used at MOI 100, fixed with 1% paraformaldehyde for 10 minutes on ice and in the dark, then permeabilized with Saponin Buffer (0.5 x of BSA and 0.1% of Saponin) and stained with IDO purified mAb.

DCs were analysed for the expression of Ag presentation machinery subunit: the mAb anti–low-molecular-mass polypeptide (LMP)-2 (SY-1), anti–LMP-7 (HB-2), anti–LMP-10 (TO-7), anti–

TAP-1 (NOB-1), anti–TAP-2 (NOB-2), anti-calnexin (TO-5), and anti-Tapasin (TO-3) were developed and kindly provided by Soldano. Ferrone. Cells were washed with PBS containing 1% BSA, fixed in 2% paraformaldehyde at room temperature for 20 min. Following three washings, cells were treated in a microwave oven at 200 W for 45 s. At the end of treatment, cells were chilled on ice for 10 min. The cell membrane was permeabilized by incubating cells for 30 min at room temperature in PBS containing 1% BSA and 0.1% saponin. DCs were finally incubated for 30 min at room temperature with an appropriate amount of primary mAb or isotype control. Following three washings with PBS containing 1% BSA and 0.1% saponin, cells were incubated for 30 min at room temperature in the dark with an optimal amount of IgG goat Abs specific to mouse IgG Fc fragments.

Hepatitis B Virus particles purification

HBV-transfected HepG2 cells were grown until confluence in Williams'E medium for 7 days. HBV virions were concentrated from the culture supernatant by sucrose gradient-centrifugation Briefly (at 40000 rpm, 4° C, overnight) and quantified (amount of HBV DNA: 107 IU/mL) before use. Unless otherwise stated, purified HBV particles were used at multiplicity of infection of 100 (MOI 100): DC.

Cytokine Measurement

To evaluate NK cytokine production following DC stimulation, NK cells were cultured with DCs, previously maturated with Poly I:C in the presence or not of CHB serum, or alternatively in the presence of HBV purified particles, as described above.

NK cells were plated together with DCs for 24h at 37° C and a blocking mix (2 μM Monensin, Sigma-Aldrich, 2 mg/mL of Brefeldin) were added for the last 12h.

Cells were harvested, wash twice and fixed with 1% paraformaldehyde for 10 minutes on ice and in the dark, then permeabilized with Saponin Buffer (0.5 x of BSA and 0.1% of Saponin). The permeabilized cells were subsequently stained with mAbs anti-IFN-γ (Biolegend), and anti-TNF-alpha (BD Pharmigen) for 45 minutes at room temperature in the dark. Samples were washed twice with PBS before acquisition by Flow Cytometer.

The amount of IL-12 secreted by DCs were measured from cell culture supernatant upon 48h of DC stimulation with Poly I:C in the presence of CHB serum or AB control serum by Flowcytomix Multiplex Immunoassay (Abcam) following the manufacture's instructions.

Cytotoxicity Assay

NK cell cytotoxic ability upon DC stimulation was evaluated against target cells. Briefly, freshly NK cells were cultured alone or with DCs for 12h at 37° C at 2:1 ratio. Dendritic cells were previously stimulated with Poly I:C in the presence or not HBV particles, used at MOI 100, for

24h: washed twice with PBS before co-culture with NK cells. Target cells (K562 or HepG2) were added to NK/DC co-culture at ratio 2:1 (Effector : Target) for a 6h of killing assay.

To evaluate NK cytolytic activity against DCs, NK cells were activated with PHA (1ug/ml) and IL-2 (500 UI/ml) for 24h, washed twice and cultured with autologous immature or mature DCs, that were previously conditioned by the presence or not of HBV particles, washed and used as target cells in a 6h killing assay. Blocking experiments were performed in the presence of HLA-I blocking mAb (A632). NK cell cytotoxic ability was assessed as percentage of $CD107a^+$ NK, used as a marker of NK cell degranulation. The anti-CD107a mAb was present in the cultures throughout the test period. The experiments were performed in triplicates, and the data were shown as mean ± sd from one of three experiments

NK cell Proliferation Assay

To evaluate DC-mediated NK cell proliferation, freshly NK cells were cultured together with DCs, previously stimulated as described above, and washed before co-culture.

Where indicated, IDO specific inhibitor, 1-Methyl-D Tryptophan (Sigma- Aldrich) were added into co-culture at the final concentration of 0.125mM. After 5 days, NK cell proliferation was measured evaluating the percentage of $Ki67^+$ NK cells by intranuclear staining, following manufacturer's instructions (Intranuclear Buffer Set-Miltenyi). Briefly, cells were resuspended in cold and freshly preparated Permeabilization/Fixation Solution for 30 minutes at 4° C, then, washed twice with Permeabilization Buffer and stained with anti-Ki-67 mAb for 30 minutes at 4° C in the dark. Cells were then washed and cell pellets were resuspended in a suitable amount of volume for Flow Cytometry analysis.

RT-PCR

The relative amount of IDO transcribed was assessed using the TaqMan™ real-time PCR technology. Briefly, freshly $BDCA1^+$ dendritic cells from CHB infected patients or healthy controls, were isolated as previously described, and total mRNA was extracted using the RNeasy mini kit (Qiagen) and treated with RNase-free DNase I to remove contaminating genomic DNA; the cDNA was synthesized using a first-strand cDNA synthesis kit from Quiagen (QuantiTac Rev Transcription Kit).

Real-time PCR was performed with automated fluorimeter (Applied Biosystems) performing the PCR cycles as follow: 2 min at 60 ° C followed by 40 cycles of 10 min at 95° C, 30 sec at 98° C, and 2 min at 60° C. Relative quantification of gene expression was determined by comparison of threshold values. All results were normalized to β-actine.

Flow Cytometry mAb

The following mouse anti-human mAbs were used in this study: CD56, IFN-γ, CD14, CD11c, CD80, CD83, CD25, CD40, CD19, C107a, CD3, CD1c, TNF-α and Ki67 all from BD Pharmigen. For Ag presentation machinery subunit analysis: the mAb anti–low-molecular-mass polypeptide (LMP)-2 (SY-1), anti–LMP-7 (HB-2), anti–LMP-10 (TO-7), anti–TAP-1 (NOB-1), anti–TAP-2 (NOB-2), anti-calnexin (TO-5), and anti-Tapasin (TO-3) were developed and kindly provided by S. Ferrone (Harvard Medical School, Massachusetts General Hospital). Anti-HLA-I W632 and blocking-HLA-I A636 were kindly provided by Prof. Alessandro Moretta, Genoa, Italy. Recombinant Human anti-IDO purified antibody (Adipogene) and IgG goat anti-rabbit (Invitrogen).

Statistical Analysis

Statistical analyses were performed using Prism Version 4 (GraphPad Software). Data are expressed as means ± SEM of independent experiments. Student's matched pair t-test was used to compare means and P values lower than 0.05 were considered statistically significant.

Results

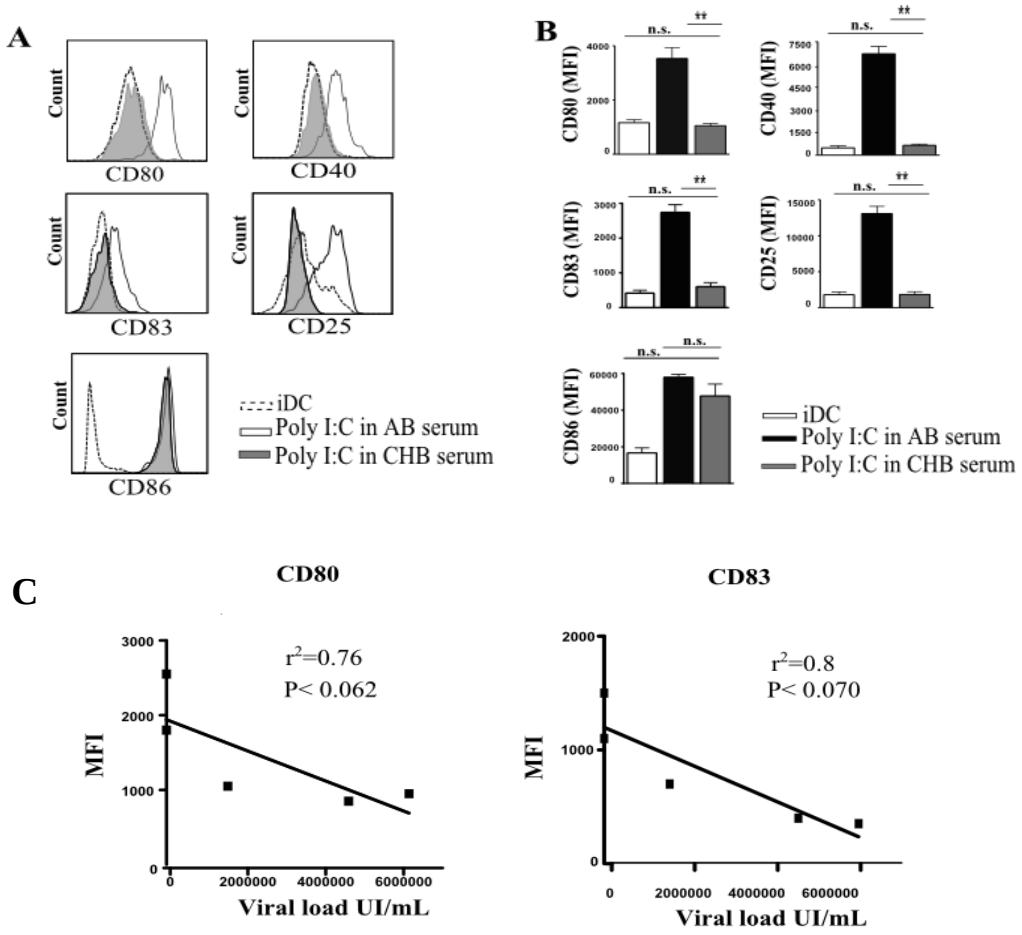

Figure 1. DC maturation is significantly impaired in the presence of serum from Chronic Hepatitis B virus (CHB) patients

Expression of DC maturation markers CD80, CD86, CD40, CD83, CD25 were assessed by Flow Cytometry.

A. Representative histograms showing level expression of each maturation marker for immature DCs (dotted line), or DCs stimulated for 24h with Poly I:C (25ug/mL) in the presence of CHB serum (grey histogram) or AB serum (black line) as control. A representative experiment is shown.

B. Mean fluorescence intensity (MFI) recorded for each membrane markers for immature DCs (white bar), and DCs stimulated with Poly I:C in the presence of CHB serum (grey bar) or AB serum control (black bar). The mean ± standard error of six independent experiments is shown. The statistical significance of differences between conditions was $P< 0.05$.

C. Correlation between level expression of DC maturation CD80 and CD83 markers and CHB sera viral load is shown. DCs were stimulated with Poly I:C in the presence of CHB sera with different viral load expressed as UI/mL and DC phenotype was analysed upon 24h.

Result

DC maturation is drastically affected in the presence of serum from Chronic Hepatitis B infected patients.

We wondered to investigate whether serum collected from Chronic Hepatitis B virus (CHB) might influence DC differentiation and maturation. To this purpose, human peripheral blood monocytes, obtained by adhesion or sorted as $CD14^+$ cells from healthy donors were cultured in the presence of IL-4 and GM-CSF, used at 20ng/mL and 25ng/mL respectively and CHB serum, or AB serum as control, were added to DC culture at concentration of 2% in the medium, either at the start of DC differentiation on day 0 and along with the addition of maturation stimuli.

After 5 days of culture, the cells exhibited morphologic and phenotypic features characteristic of immature DCs (iDC), including the down-regulation of the monocyte/macrophage marker CD14 and the up-regulation of CD11c, confirming that the differentiation process occurs in both conditions (data non shown).

We then investigated the effect of CHB serum on DC phenotype undergoing maturation *in vitro*.

iDCs were stimulated with Poly I:C, a potent type I IFN-inducing TLR3 agonist, used at 25ug/mL for 24h.

We observed that DCs in the presence of CHB serum show an impaired phenotype, as demonstrated by the low level expression of costimulatory molecules and maturation markers analysed, such as CD80, CD40, CD25,CD83 (Figure 1 A-B) suggesting that DC maturation was seriously affected by the presence of CHB serum.

Considering the different viral load measured in used CHB sera we wondered to investigate whether increased viral load could further aggravate DC functional impairment.

Hence, we assessed the effect of 4 CHB sera with increased viral load, from lower up to higher, on DC maturation, performing 24h of stimulation with Poly I:C. Analysis revealed a significant correlation between increasing viral load and the inhibition rates of DC maturation, here expressed as MFI of CD80 and CD83 MFI (Figure 1-C). Therefore, our data indicate that increased viral load may further stifle dendritic cell activity.

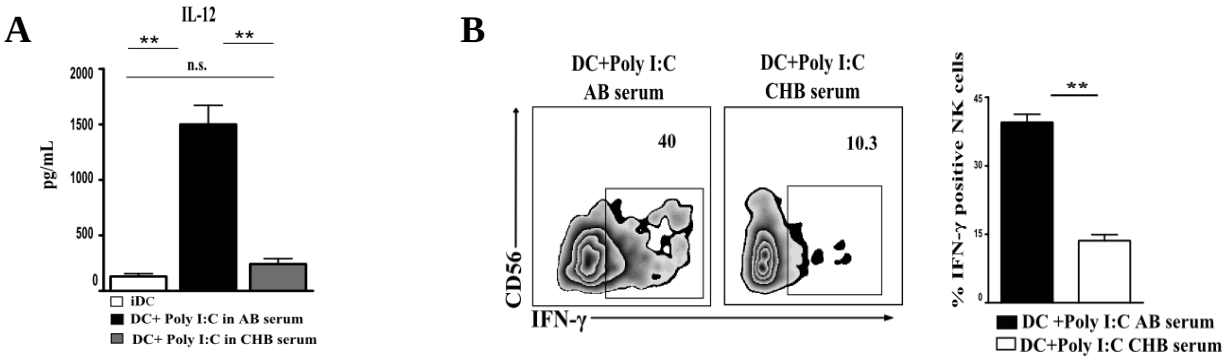

Figure 2. CHB serum drastically impairs IL-12 secretion by DCs, affecting in turn IFN-γ production by NK cells following DC stimulation

A. IL-12 production by DCs was measured in the supernatant of immature DCs (white bar) or DCs stimulated with Poly I:C in CHB serum (grey bar) or AB control serum (back bar) upon 24h of culture using FlowCytomix Assay. IL-12 concentration was expressed in pg/mL. The statistical significance of differences between conditions was $P < 0.05$.

B. The percentage of IFN-γ positive NK cells following DC stimulation, was analysed by intracellular staining. Resting NK cells were cultured with DCs, previously stimulated for 24h with Poly I:C in the presence of CHB serum (white bar) or AB serum (black bar) and washed before co-culture with NK cells. After 12h of co-culture a Golgi stop were added for additional 6h. Cells were fixed, permeabilized and stained with IFN-γ mAb. Representative dot plot and relative statistical analysis of four different experiments are shown. The statistical significance of differences between conditions was $P < 0.05$.

Result

IL-12 production by DCs is drastically impaired in the presence of CBH serum, affecting in turn IFN-γ secretion by NK cells following DC stimulation.

As DCs that differentiated and matured in the presence of CHB serum displayed several impaired characteristics in the context of maturation phenotype, it was important to determine whether CHB serum could affect also DC cytokine production.

Hence, functional analysis was performed to examine DC ability in secreting IL-12. To this purpose supernatant of DCs stimulated with Poly I:C for 24 h in the presence or not of CHB serum were collected to measured IL-12 production by Flow Cytomix Assay.

Analysis revealed that, while Poly I:C-stimulated DCs produced large amount of IL- 12, in contrast, in the presence of CHB serum, the ability of DCs to produce IL-12 was greatly reduced (Figure 2-A).

Considering the key role of IL-12 during NK/DC interaction, for its role in inducing IFN-γ production by NK cells (122), we wanted to evaluated whether IFN-γ production by NK cells, following DC stimulation, could suffer for the less amount of IL-12 secreted by DC-conditioned with CHB serum.

According with our hypothesis, DCs fail in stimulating IFN-γ production by NK cells upon 18h of co-culture, as demonstrated by the significant reduction of percentage of IFN-γ positive NK cells compared as control (Figure 2-B).

Our data could suggest that the less amount of IFN-γ by NK cells observed in CHB patients could result from the reduction of IL-12 release by DCs, questioning that a defecting NK/DC cross-talk could occurs also *in vivo*, where it may not providing enough IFN-γ to counteract viral replication.

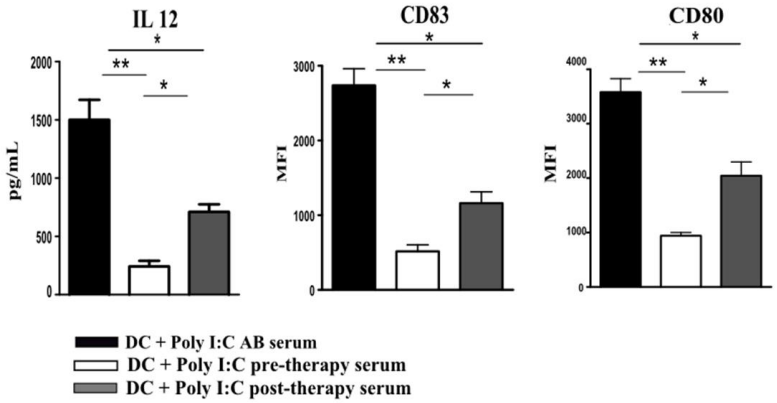

Figure 3. DC activity is partially recovered upon anti-viral therapy

Concentration of IL-12 expressed in pg/mL measured in DC supernatant and representative level expression of CD80 and CD83 expressed as MFI were showed. DCs were stimulated with Poly I:C in the presence of CHB serum collected from patients before (white bar) or upon (grey bar) undergoing treatment with analogue nucleosides, or AB serum as control (black bar). After 48h, DCs were stained for maturation makers surface expression, while supernatant were collected to evaluate IL-12 concentration by Flow Cytomix Assay, following manufacture's instruction. The statistical significance of differences between conditions was $P < 0.05$.

Result

Functions and IL-12 production by DCs are partially recovered in the presence of post-therapy CHB serum

It has been stated that the antiviral therapy with nucleoside analogues restores IFN-γ production by NK cells in CHB affected patients (132). Therefore, we wanted to investigate whether the

recovered ability of NK cells in producing IFN-γ, described in this patient, could be, at least in part, the consequence of the restoration of DC activity. To address this question, DCs were cultured in the presence of CHB sera collected from the same infected patients before and after treatment and stimulated for 24h with Poly I:C. Supernatants were collected to analyse IL-12 production and phenotypical analysis was assessed on harvested DCs.

Flow Cytomix assay revealed that DCs in the presence of post-therapy CHB serum significantly increase IL-12 production compare to pre-therapy condition although it not achieve control level (Figure 3).

In line with this finding, also the level expression of maturation markers was up-regulated compare to pre-therapy condition (Figure 3-B).

As know, administration of antiviral treatment, to date one of the main stay of treatment, can significantly have a favourable impact on the reduction of viral load. Our data might suggest that the partial restoration of DC functions, observed in the presence of post-treatment CHB sera, could be related to the diminished viral count indicating that the decreasing of viral load could abruptly reverse the dysfunction of DCs.

In addition, this data suggests that one of the possible mechanisms of IFN-γ production recovery, observed in patients undergoing anti-viral therapy, might be the result of re-established IL-12 release by DCs.

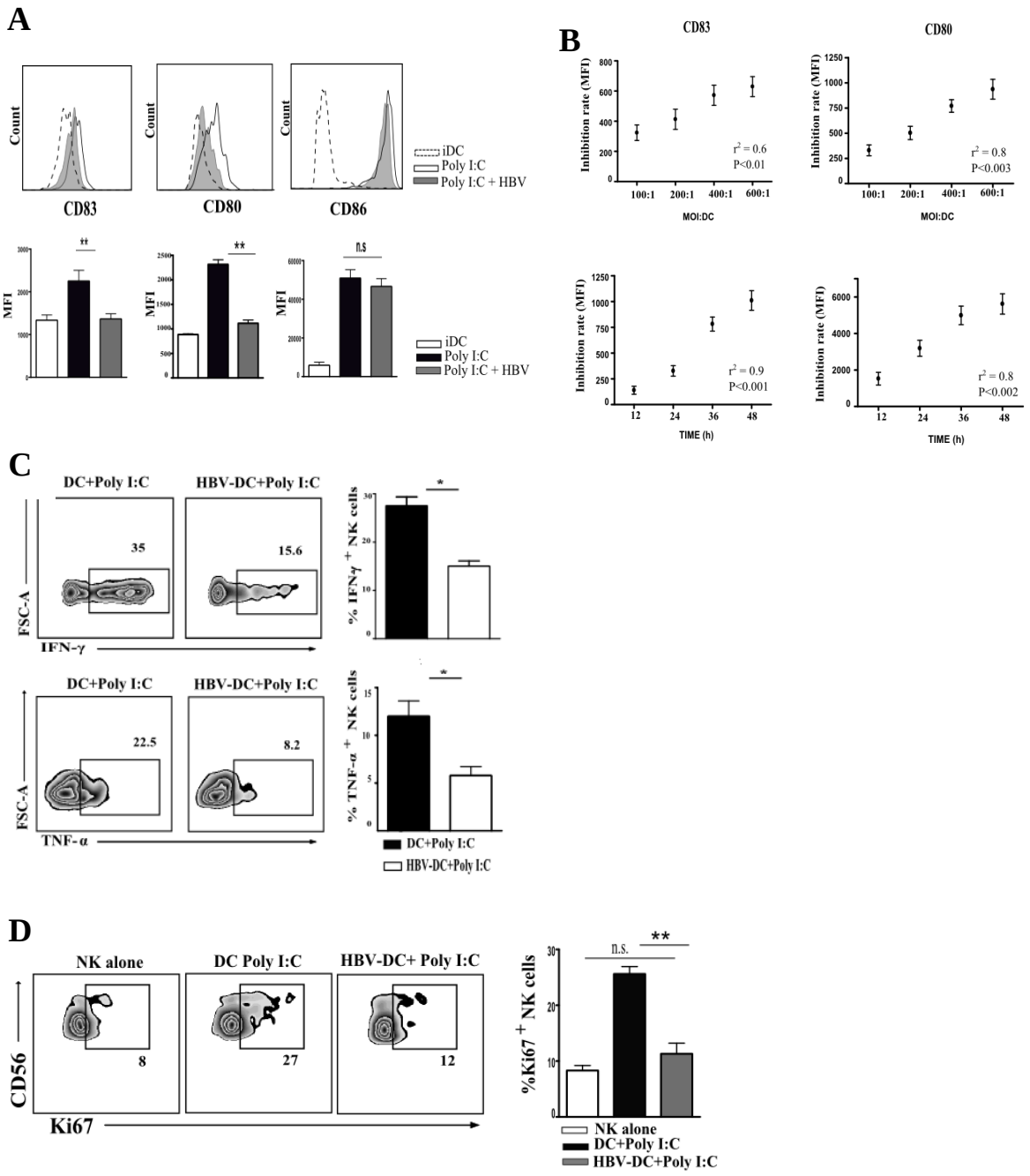

Figure 4. Purified HBV is directly involved in DC impairment in a time and dose-dependent manner, resulting in DCs with less ability to stimulate proliferation and cytokine production by NK cells.

Expression level of DC maturation markers CD80, CD83, CD86, was assessed by Flow Cytometry.

A. Representative histograms and relative statistical analysis showing level expression of the indicated maturation markers for immature DCs (dotted line), or DCs stimulated with Poly I:C in the presence (grey histogram) or not (black line) of purified HBV particles, used at MOI 100.

Mean fluorescence intensity (MFI) was recorded for the indicated surface markers for immature DCs (white bar), and DCs stimulated with Poly I:C in the presence(grey bar) or not (black bar) of HBV particles. The mean ± standard error of five independent experiments is shown. The statistical significance of differences between conditions was $P< 0.05$.

B. Correlation between time (lower panel) or dose (upper panel) of exposition of DCs to purified HBV particles was shown. For time course experiments, iDCs were stimulated in the presence of HBV particles at MOI 100 and their phenotype was assessed at different time points up to 48 h. Alternatively, in dose-dependent experiments, iDCs were exposed for 24h to increasing MOI, from 100 up to 800. Cells were harvested and stained for the indicated maturation markers. Inhibition rate of DC maturation markers was calculate as delta of CD80 or CD83 MFI value between HBV- conditioned DCs and control.

C. Representative Flow cytometry dot plots and relative statistical analysis showing intracellular staining for IFN-γ (upper) and TNF-α (lower) production by NK cells following co-culture with DCs. DCs were previously stimulated with Poly I:C in the presence or not of HBV particles and washed before overnight culture with NK cells. A Golgi stop was added for further 6h. Cell were fixed, permeabilized and stained with mAb for IFN-γ and TNF-α.

D. Representative dot plots and statistical analysis show NK cell proliferation following DC stimulation, assessed by intranuclear staining for KI67 marker. NK cells were cultured alone (white bar) or with DCs maturated with Poly I:C in the presence (grey bar) or not (black bar) of HBV particles, added at MOI 100. DCs were washed before co-colture with NK cells. After 5 days of culture, cells were harvested and stained with mAB for Ki67. The percentage of $Ki67^+$ NK cells was shown. The mean ± standard error of five independent experiments is shown. The statistical significance of differences between conditions was $P< 0.05$.

Result

HBV particles direct affect DC function in a time and dose-dependent manner, impairing in turn proliferation and cytokine production by NK cells

According with our results, the viral load have a significant impact in disrupting DC functions, thus we hypothesized that HBV particles could be the direct responsible for DC impairment. To address this question HBV particles were purified starting from HepG2-transfected cell line by ultracentrifugation as described and their effect on DC activities were examined. As show in Figure 4-A, the level expression of the most important DC maturation markers are drastically reduced upon only 24h of stimulation along with HBV particles, used at Multiplicity of Infection MOI of 100:1 with DCs. These data are consistent with these obtained culturing DCs in the presence of CHB serum, underlying a direct involvement of HBV in DC impairment.

In addition, we found that higher dose of HBV particles or increasing time of exposure to viral particles significantly aggravates DC functional damaging. Indeed a positive correlation between

increasing dose of HBV particles in DC culture, ranking from MOI 100 up to 800, or increasing time of stimulation, from 12h up to 48h, and inhibition rate of DC maturation markers, occurs (Figure 4-B). These data confirm that HBV negatively affects DC functions in a time and dose-dependent manner.

As expected, DCs cultured in the presence of HBV particles are less able to stimulate optimal IFN-γ and TNF-α production by NK cells. In Figure 4-C is showed the percentage of IFN-γ and TNF-α positive NK cells following stimulation overnight with DC conditioned by HBV particles.

As a matter of fact, DCs are able to promote NK cell activation and proliferation (111) thus we wondered to investigate whether HBV-conditioned DCs had this potential.

Hence, DCs, stimulated with Poly I:C along with HBV, were used in culture with resting purified NK cells. Upon 5 days cells were harvested and proliferation of NK cells were analysed evaluating Ki67, an intranuclear proliferative marker. Analysis revealed that DC-conditioned by HBV are less able to promote NK cell proliferation compare to control, as demonstrated by the reduction of percentage of Ki67 positive NK cells (Figure 4-D). Concerning NK cell number in HBV infected patients, at date, there are conflicting data. It has been reported no change or an increase in NK cell absolute number. However, some study have reported significant reduction in NK cell numbers in HBV-infected patients, especially in the late stage of Hepatocellular Carcinoma HCC (144); considering our data may suggest that the reduction of NK cell numbers observed in this subjects could be the results of an improper DC stimulation.

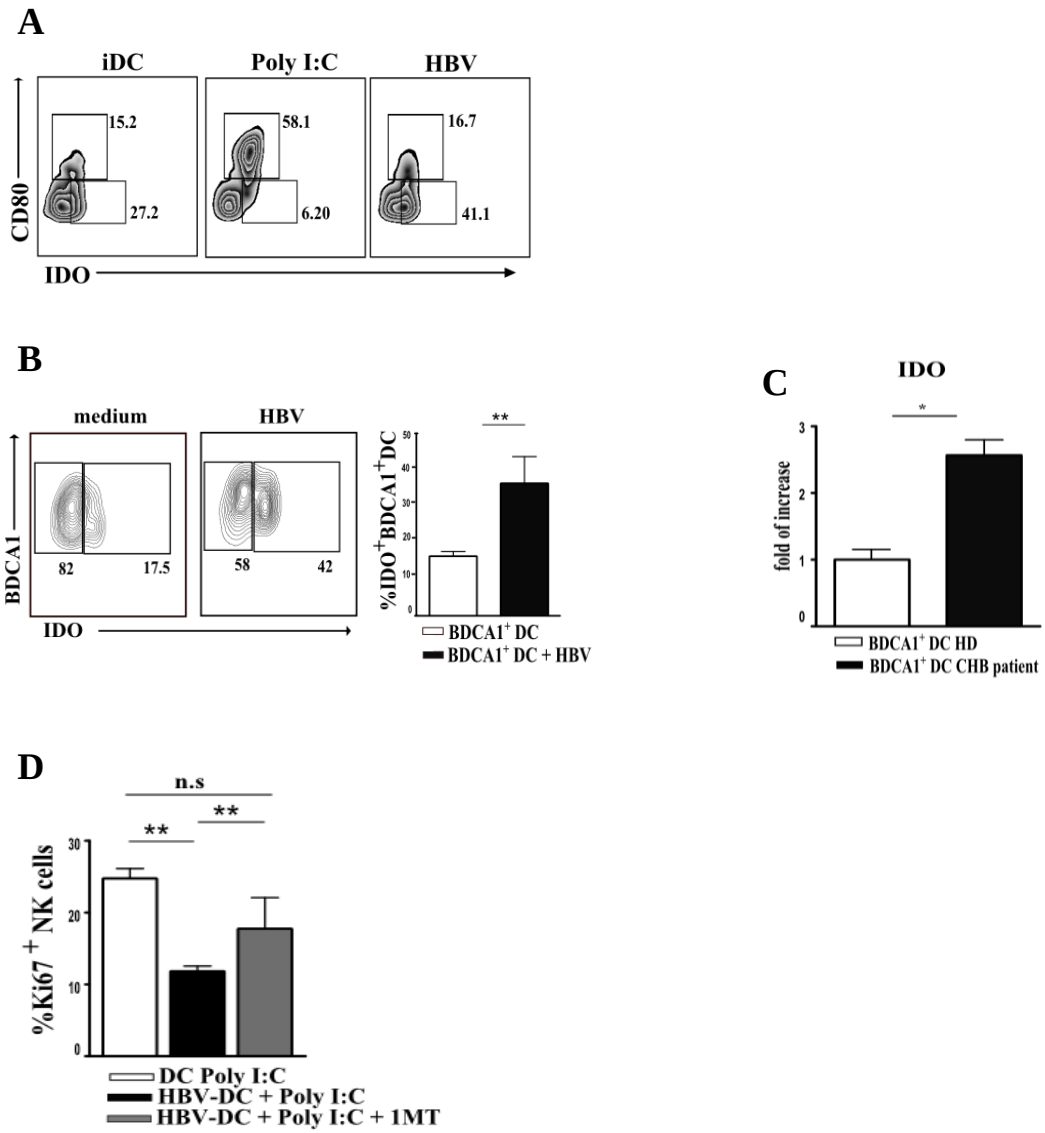

Figure 5. IDO expression by DCs, significantly up-regulates by HBV, is involved in the inhibition of DC-mediated NK cell proliferation.

A. IDO expression by DCs was assessed by Flow Cytometry analysis. CD80 surface expression and IDO intracellular staining was performed on immature DCs or DCs stimulated for 24h with Poly I:C or HBV at MOI 100.

B. Representative dot plots and relative statistical analysis for IDO expression on *ex-vivo* $BDCA1^+$ DCs in the presence (black bar) or not (white bar) of HBV particles at MOI 100 upon 24h of culture, were shown. The statistical significance of differences between conditions was $P< 0.05$.

C. IDO gene expression was analyzed using qRT-PCR on BDCA1$^+$ DCs isolated from CHB infected patients or healthy donors by cell sorting. β2-actin was used as an internal control. Data are presented as fold of increase of three separate experiments.

D. Statistical analysis shows percentage of Ki67$^+$ NK cells assessed by intranuclear staining in the presence of IDO specific inhibitor. NK cells were cultured with DCs previously stimulated with Poly I:C together with or without HBV particles (MOI 100). Upon 5 days of NK/DC co-culture in the presence (grey bar) or not (black bar) of IDO inhibitor 1-Methyl- Tryptophan, or control DCs (white bar) the percentage of Ki67$^+$NK cells was evaluated by Flow cytometry. The mean ± standard error of four independent experiments is shown. The statistical significance of differences between conditions was $P< 0.05$.

Result

HBV significantly up-regulates IDO enzyme on *in vitro* and *ex-vivo* DCs, and blocking experiments confirm IDO involvement in inhibition of DC-mediated NK cell proliferation

In an attempt to identify the mechanism involved in DC impairment, we found that DCs conditioned by HBV display significant high level expression of IDO, known to have a role in tolerance induction. As known, in immune cells, IDO expression is finely regulated and mainly induced by cytokines and TLR-ligands. In our vitro experiments we found that the percentage of IDO positive DCs is significantly enhanced when cultured with HBV particles. Moreover, it is important to note how, in parallel with IDO upregulation, the level expression of CD80 on HBV-DCs considerably decrease (Figure 5-A). Our data could suggest that HBV might address DCs to a tolerance state. Interestingly, BDCA1$^+$ DCs display a significant increase of IDO expression when treated *in vitro* with HBV particles (Figure 5-B). Furthermore, ex vivo BDCA1$^+$ DCs, purified from CHB infected patients show higher IDO gene expression, compare to healthy DCs, performing a qRT-PCR, thus confirming our *in vitro* observations and suggesting that, in CHB infected patients, DCs might circulate with tolerogenic features (Figure 5-C).

It has been described that IDO activity in cells of the immune system results in the degradation of essential aminoacid tryptophan and the latter has been reported be the main mechanism of IDO-induced T cell suppression. Thus, we wondered to investigate whether the upregulation of IDO on HBV-conditioned DCs may have critically altered DC- mediated NK cell proliferation.

To address IDO involvement, we performed NK/DC co-culture to trigger resting NK cell proliferation adding the specific IDO inhibitor, 1-Methyl Tryptophan, that was present in the culture throughout the assay period. As shown in Figure 5-D, the inhibition of IDO activity significantly restores NK cell proliferation, thus confirming, at least in part, the role of IDO in the impairment of NK expansion following HBV-conditioned DC stimulation.

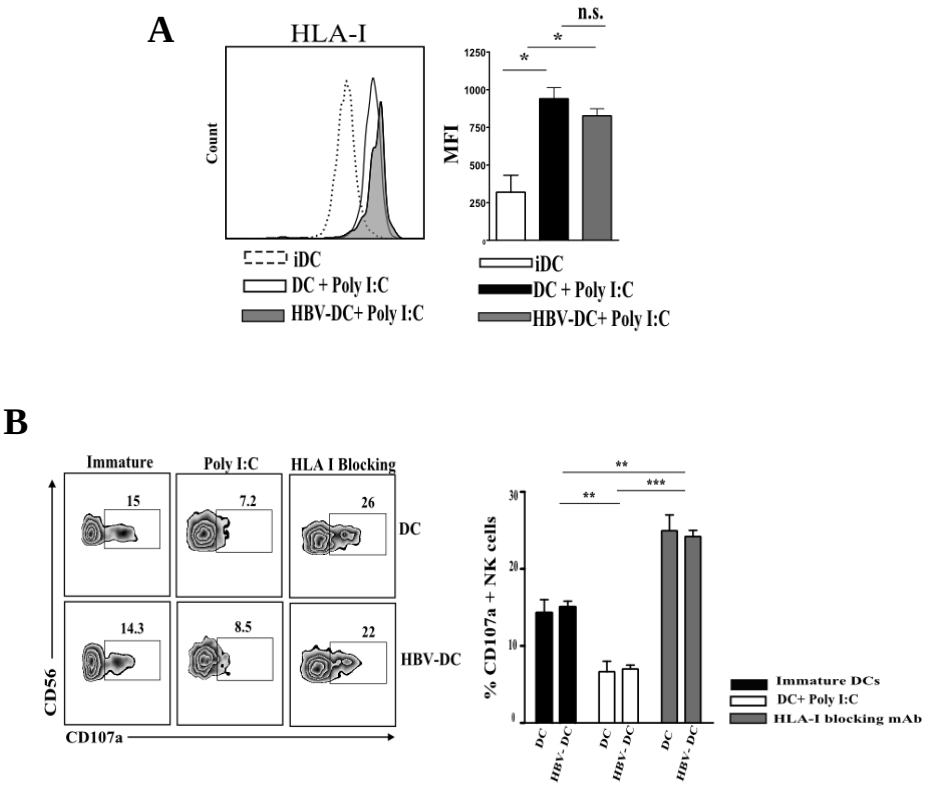

Figure 6. High level of HLA-I in HBV-conditioned DCs protects them from NK-mediated lysis.

A: Representative histogram of HLA-I expression level of immature DC (dotted line) and DCs stimulated in the presence (grey line) or not (black line) of purified HBV particles, assessed by Flow Cytometry.

B. Representative dot plots and relative statistical analysis of NK cell-mediated lysis of DCs. Immature DCs (black bar) conditioned or not with HBV particles or DCs stimulated with Poly I:C (white bar) in the presence or not of HBV, were used as targets in a 6 h degranulation assay based on CD107a expression on NK cell surface. HLA-I blocking mAb (grey bars) were added in blocking experiments. The percentage of $CD107a^+$ NK cells were evaluated by Flow Cytometry. The mean ± standard error of three independent experiments is shown. The statistical significance of differences between conditions was $P < 0.05$.

Result

HBV does not affect HLA-I expression on DCs protecting them from NK cell -mediated killing

Despite the impairment of DC maturation, that we previously observed by the evaluation of maturation markers and cytokine production, we found that HBV particles does not alter HLA-I level expression, that reaches comparable level expression to control (Figure 6-A).

HLA class I level regulates Editing, a physiological process operated by NK cells. It is well established that NK cells are able to discriminate between mature and immature DCs and to kill only immature, potentially tolerogenic, DCs based on their low amount of surface human leukocyte antigen (HLA) class I molecules. By Editing, NK cells play an important regulatory role by removing inappropriate DCs, especially during the course of immune responses (141).

Considering the high level expression of HLA-I on HBV-conditioned DCs, we performed a NK cell-mediated killing assay to evaluate whether HBV-conditioned DCs are killed by autologous NK cells.

To this purpose, DCs, previously stimulated in the presence of HBV, were used as targets in a 6h NK/DC co-culture, in which the ability of NK cells to recognize immature and mature DCs were evaluated by monitoring surface expression of CD107a, used as a marker for NK cell degranulation.

Our analysis revealed that, HBV-conditioned DCs are sheltered from NK mediated lysis because of the high amount of HLA-I on their surface, as confirmed by HLA-I blocking experiment (Figure 6-B).

Our data suggest that HBV, not affecting HLA-I expression, might allow DCs to escape from NK cell killing. Likewise, DCs with tolerogenic features might circulate in an immature state in CHB patients as a consequence of a failed killing by NK cells.

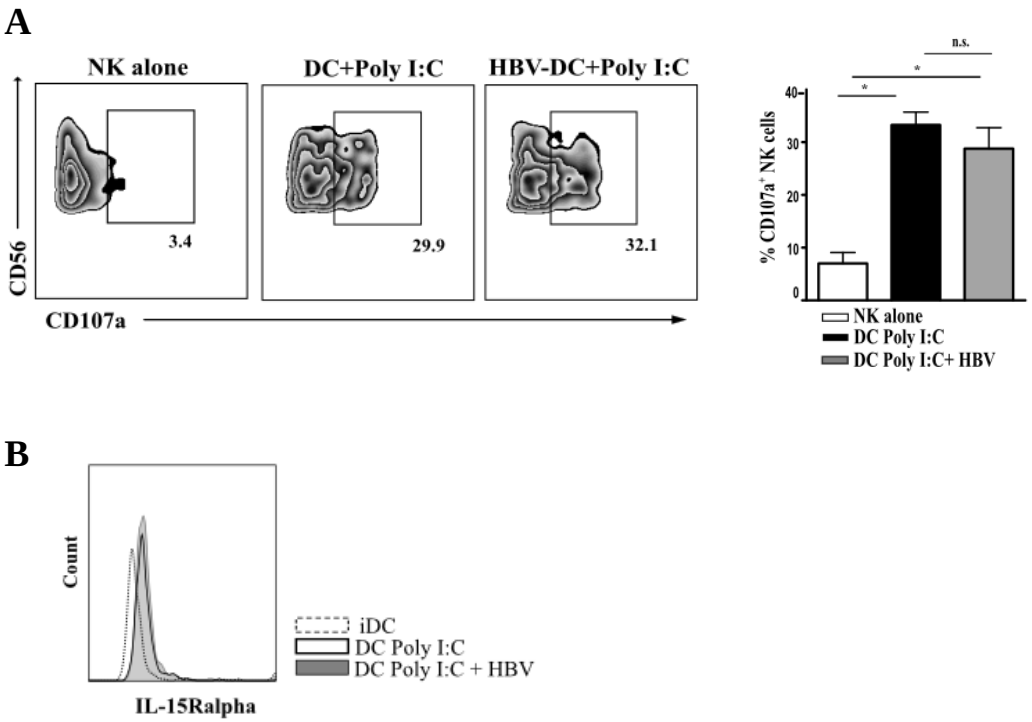

Figure 7. NK cells, following HBV-DC stimulation, display considerable cytotoxicity towards Hepatocellular carcinoma cell line

A. Representative dot plots and relative statistical analysis of NK cell cytotoxic ability toward HepG2 cell line are shown. NK cell were cultured, alone (white bar) or with DCs, previously activated with Poly I:C in the presence (grey bar) or not (black bar) of purified HBV particles, used at MOI 100. DCs were washed before co-culture at ratio 1:1 with NK cells for 24 h. HepG2 cell line were added at ratio 1:1 (Effector:Target) to the NK/DC culture for additional 6h of degranulation assay based on CD107a expression on NK cell surface. CD107a was present troughout the assay. The percentage of $CD107a^+$ NK cells were evaluated by Flow Cytometry analysis. The mean ± standard error of three independent experiments is shown. The statistical significance of differences between conditions was $P < 0.05$.

B. Representative histogram of IL15-Rα expression by iDCs (dotted line) or DCs stimulated with Poly I:C in the presence (grey line) or not (white line) of HBV particles.

Result

HBV-conditioned DCs are able to stimulate NK cell cytolitic activity against hepatocarcinoma cell line

In order to determine whether HBV-conditioned DCs, that display defective functions, are capable of modulating NK cell cytotoxic function, resting NK cells were tested for their cytotoxic ability against tumor cell targets, HepG2 cell line, following a 24h stimulation with or without HBV-DCs or in medium alone.

As shown in Figure 7-A, NK cells displayed considerable cytotoxicity towards HepG2 tumor cell line, suggesting that HBV-DCs, despite their noteworthy defects in maturation, were able to activate and stimulate NK cytotoxic ability. These data are in line with literature observations reporting that NK cells of chronic HBV infected patients have a reduced capacity to produce IFN-γ, compared with healthy controls but, conversely, NK cell cytotoxicity is maintained, hypotizing that the preserved cytotoxicity might contribute to liver inflammation (134).

It has been stated that several cytokines modulate NK/DC interaction, among these, IL-15, of which DCs are the major producer, has been found to play an important role in enhancing various NK functions, including activation and cytotoxicity. IL-15 is not secreted as free protein but it is associated with its high affinity receptor, IL-15Rα, expressed on DC surface: through this mechanism, IL-15 can be trans-presented to NK cells to contribute to their optimal activation.

Considering the comparable level expression of IL-15Rα that we observed on HBV-conditioned DCs (Figure 7-B) we speculate that in CHB infected patients, liver inflammation, that several study has ascribed to NK cytotoxic ability, might be perpetuate by IL-15-produced DCs.

This hypothesis may provide a possible mechanism through which liver inflammation may spread.

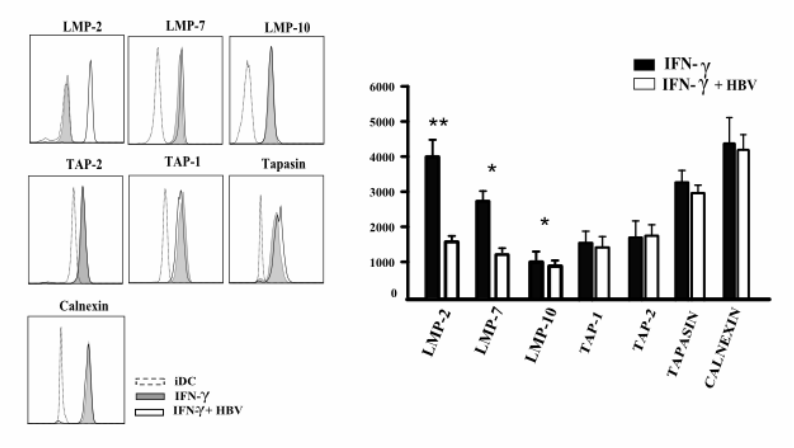

Figure 8. Immunoproteasome machinery subunits are significantly impaired in HBV conditioned DCs

Representative hystograms and relative statistical analysis show intracellular staining with mAbs specific for the indicated components of the Ag-processing machinery. Analysis was performed on DCs stimulated with IFN-γ (1000 UI/mL) in the presence (white bar) or not (black bar) of HBV particles. The mean ± standard error of three independent experiments is shown. The statistical significance of differences between conditions was $P < 0.05$

Result

The expression of specific immunoproteasome subunits is significantly impaired on HBV-conditioned DCs

An important feature of DCs is the ability to acquire exogenous, potentially pathogenic, antigen in peripheral tissues, processed them and present them to T cell in SLO. In order to process antigen, DCs exert immunoproteasome. Iproteasome is formed by several catalytic subunits able to influence the pool of peptides available for presentation by MHC-I molecules. It has been described that expression of IFN-inducible proteasome catalytic subunits correlates with the IFN-γ-mediated noncytopathic inhibition of HBV in transgenic mice and hepatocytes, as well as with clearance of the virus in acutely infected chimpanzees. Hence, the ability of the IFN-induced proteasome catalytic subunits might influence the progression of infection. Thus, analysing Iproteasome subunits on DCs we found some alteration in the endogenous Ag-processing and presentation machinery between HBV-conditioned DCs. DCs were stimulated with IFN-γ in the presence of HBV particle. We found that HBV conditioned DCs, following IFN-γ stimulation in the presence of HBV, display defective expression of specific iproteasome subunits, in particular LMP-2 expression is drastically reduced as well as LMP-7 and Tapasin although at lesser extent. These data might suggest that in the presence of HBV, DCs might display defective ability to shape adaptive response.

Conclusion and Discussion

The pathogenesis and the clinical manifestation of hepatitis B virus (HBV) infection is driven by complicated interplays between the virus and the host immune system.

HBV has various effects on innate immune responses and antiviral signaling pathways. To this regard, it has been reported that HBV exerts different and active mechanisms aimed at inactivating various components of the innate immune system. These mechanisms may represent important key steps for viral replication and the establishment of infection.

Innate immune components, such as NK and DCs are major contributor during viral invasion.

NK/DC bi-directional activation during infection is crucial in controlling viral replication, acting at the early phase of infection and is linked to a favourable clinical outcome and subsequent robust adaptive immune responses.

It has been reported that during Chronic Hepatitis B infection, NK cells isolated from both peripheral and hepatic compartment, display decreased production of anti-viral cytokine IFN-γ. Considering that IFN-γ represents the non-cytolytic mechanism for HBV clearance (142), through which it is possible to control viral replication preserving the integrity of infected organ, further studies are required to better understand the underlying mechanism for NK cell impairment.

Since, DCs are potent activators of NK cells, in the present study we investigated whether the presence of HBV might affect dendritic cell functions, thus impairing NK/DCs cross-talk, resulting in a less efficient NK cell anti-viral response.

In our study we confirmed the alteration of DC maturation, observed by others. Additionally, we found that HBV have a direct role in DC impairment, influencing negatively DC functions in a time and dose-dependent manner. The presence of purified HBV determines a defective DC phenotype, as demonstrated by the low level expression of the most important maturation markers. Furthermore, DC ability to secrete IL-12 is drastically impaired. Accordingly with the reduced amount of IL-12, DCs fail to stimulate on optimal IFN-γ and TNF-α production by NK cell, following co-culture. Moreover, we found that DCs are substantially impaired, also in their ability to stimulate NK cell proliferation. Indoleamine 2,3 –dioxygenase, IDO, a tolerogenic enzyme known to suppresses adaptive T-cell immunity, was found significantly upregulated in DCs treated in vitro with HBV particles. Importantly, DCs, purified from CHB infected-patients show a significant increase in IDO gene expression. Our additional analyis revealed that IDO enzyme is involved, at least in part in the inhibition of DC-mediated NK cell expansion. Although, conflicting data exist about NK cell frequency in HBV infected-patients, studies have reported an increase in NK cell number (143) However, several study desribed a decrease in NK cell absolute number in course of CHB infection (144); hence, this data might provide a mechanism by which the observed reduction of NK cell number could be explained.

Importantly, antiviral therapy with nucleotide analogues improved DC functions in both maturation and IL-12 production as our analysis revealed. These data suggest a significant correlation between DC impairment and increased sera viral load. Moreover, this finding might suggest that the re-established IL-12 release by DCs may play a crucial role in the IFN-γ production recovery by NK cell, described following anti-viral therapy.

Additionally, we found that DCs conditioned by HBV, despite their immature phenotype and function, show comparable level expression of HLA-I, by which they might escape from NK cell lysis, thus avoid Editing physiological process. This data may imply that DCs, not susceptible to NK lysis, could circulate in a immature and potentially tolerogenic state in CHB infected patients.

DCs are highly specialized professional antigen presenting cells that have a central role in the initiation of adaptive immune responses. This DC functional activity is exerted by the activation of Immunoproteasome, compose of IFN-γ inducible catalytic subunits. In our study, we revealed defective expression of specific catylitic subunits, LPM-2, and at lesser extent LMP-7 and Tapasine, suggesting that the antigen-presenting potential of DC might be seriously impaired in the course of HBV infection.

To identify the cellular factors that inhibit HBV replication, Wieland et al. performed a gene expression analysis utilizing HBV transgenic mice and an immortalized hepatocyte cell line derived from these mice. Among the various classes of genes analysed, IFN-inducible proteasome catalytic subunits LMP2 and LMP7 are considered important. Following these data, Robek et al (145) have demonstrated that for an appropriate antiviral effect of IFN against HBV replication in vitro, proteasome activity is necessarily required. Considering these findings and our data, it is possible to speculate that the inhibition of iproteasome subunits expression by HBV might significantly alter DC potential to shape adaptive immune response. Additionally, the defective NK/DC cross talk, resulting in a less amount of IFN-γ production by NK cells, may lead to a less efficient DC iproteasome activation, thus establishing a self-perpetuating loop.

HBV, compromising innate immunity pathways, compromises in turn the quality of adaptive immune response, in favor of its survival.

Therefore, it is important to keep in account that the efficient activation of innate immunity induce interferon production that lead to viral clearance but not killing of infected hepatocytes, thus limiting tissue damage. Considering that the innate immunity responses are linked to a favorable clinical outcome and subsequent robust adaptive immune responses, a better understanding of the mechanisms by which HBV affects various components of the innate immune system, might help to consider new strategies to counteract HBV infection. To this regard, immune strategies aimed at making more efficient NK/DC axis may pave the way for new approach in the treatment of chronic HBV infection.

Bibliography

1. Liang TJ. Hepatitis B: the virus and disease. Hepatology. 2009;49:13–21.
2. Busca A, Kumar A. Innate immune responses in hepatitis B virus (HBV) infection. Virology journal. 2014;11:22 doi: 10.1186/1743-422X-11-22.
3. Untergasser A., Zedler U., Langenkamp A., Hosel M., Quasdorff M., Esser K., Dienes H.P., Tappertzhofen B., Kolanus W., Protzer U. Dendritic cells take up viral antigens but do not support the early steps of hepatitis B virus infection. Hepatology. 2006;43:539–547.
4. Takeuchi O., Akira S. Innate immunity to virus infection. Immunol. Rev. 2009;227:75–86. doi: 10.1111/j.1600-065X.2008.00737.x.
5. Akira, S., S. Uematsu, and O. Takeuchi. 2006. Pathogen recognition and innate immunity. Cell 124:783-801.
6. Medzhitov R. Recognition of microorganisms and activation of the immune response. Nature. 2007;449:819–26.
7. Sato S, et al. Toll/IL-1 receptor domain-containing adaptor inducing IFN-beta (TRIF) associates with TNF receptor-associated factor 6 and TANK-binding kinase 1, and activates two distinct transcription factors, NF-kappa B and IFN-regulatory factor-3, in the Toll-like receptor signaling. J Immunol. 2003;171:4304–10.
8. Schroder M, Bowie AG. TLR3 in antiviral immunity: key player or bystander? Trends Immunol (2005) 26(9):462–8.10.1016/j.it.2005.07.002
9. Hewson C. A., Jardine A., Edwards M. R., Laza-Stanca V., and Johnston S. L. (2005) Toll-like receptor 3 is induced by and mediates antiviral activity against rhinovirus infection of human bronchial epithelial cells. J. Virol. 79, 12273–12279.
10. Schulz, O., Diebold S.S., Chen M., et al. Toll-like receptor 3 promotes cross-priming to virus-infected cells, Nature, 433 (2005), pp. 887-892.
11. Wieland, S. F., L. G. Guidotti, and F. V. Chisari. 2000. Intrahepatic induction of alpha/beta interferon eliminates viral RNA-containing capsids in hepatitis B virus transgenic mice. J. Virol. 74:4165–4173.
12. Isogawa M, Robek MD, Furuichi Y, Chisari FV. Toll-like receptor signaling inhibits hepatitis B virus replication in vivo. J Virol 2005; 79: 7269–7272.
13. Pasquetto, V., S. F. Wieland, S. L. Uprichard, M. Tripodi, and F. V. Chisari. 2002. Cytokine-sensitive replication of hepatitis B virus in immortalized mouse hepatocyte cultures. J. Virol. 76:5646-5653.
14. Zhang E, Lu M: Toll-like receptor (TLR)-mediated innate immune responses in the control of hepatitis B virus (HBV) infection. Med Microbiol Immunol. 2015; 204(1): 11–20.
15. Huang YW, Lin SC, Wei SC, Hu JT, Chang HY, Huang SH, Chen DS, Chen PJ, Hsu PN, Yang SS, Kao JH. Reduced Toll-like receptor 3 expression in chronic hepatitis B patients and its restoration by interferon therapy. Antivir Ther. 2013;18:877–884. doi: 10.3851/IMP2630.
16. Lu Y, Xu Y, Yang D, Kemper T, Roggendorf M, Lu M. Molecular characterization of woodchuck type I interferons and their expression by woodchuck peripheral blood lymphocytes. Cytokine. 2008;41:127–135. doi: 10.1016/j.cyto.2007.11.002.
17. Ferlazzo G, Morandi B. Cross-talks between natural killer cells and distinct subsets of dendritic cells. Front Immunol (2014) 5:159.10.3389/fimmu.2014.00159.
18. A. Mildner, S. Jung. Development and function of dendritic cell subsets. Immunity, 40 (2014), pp. 642-656.
19. Merad M, Sathe P, Helft J, Miller J, Mortha A. The dendritic cell lineage: ontogeny and function of dendritic cells and their subsets in the steady state and the inflamed setting. Annu Rev Immunol (2013) 31:563–604.10.1146/annurev-immunol-020711-074950.
20. Colonna M, Trinchieri G, Liu YJ. Plasmacytoid dendritic cells in immunity. Nature immunology. 2004;5:1219–1226. doi: 10.1038/ni1141.
21. Banchereau, J. & Steinman, R. M. (1998). Dendritic cells and the control of immunity. Nature 392, 245–252.
22. Trombetta, E. S. & Mellman, I. (2005).Cell biology of antigen processing in vitro and in vivo. Annu. Rev. Immunol. 23, 975–1028.
23. Albert, M. L. & Bhardwaj, N. (1998) Resurrecting the dead: DCs cross-present antigen derived from apoptotic cells on MHC I. Immunologist 6, 194–198.
24. Heath, W. R. & Carbone, F. R. (2001).Cross-presentation, dendritic cells, tolerance and immunity. Annu. Rev. Immunol. 19, 47–64.
25. Itano, A. A. & Jenkins, M. K. (2003).Antigen presentation to naive CD4 T cells in the lymph node. Nature Immunol. 4, 733–739.

26. Bergtold, A., Desai, D. D., Gavhane, A. & Clynes, R. (2005).Cell surface recycling of internalized antigen permits dendritic cell priming of B cells. Immunity 23, 503–514.
27. M. Crowley, K. Inaba, M. Witmer-Pack, R.M. Steinman. The cell surface of mouse dendritic cells: FACS analyses of dendritic cells from different tissues including thymus. Cell. Immunol., 118 (1989), pp. 108-125.
28. D. Vremec, J. Pooley, H. Hochrein, L. Wu, K. Shortman. CD4 and CD8 expression by dendritic cell subtypes in mouse thymus and spleen. J. Immunol., 164 (2000), pp. 2978-2986.
29. F.M. Burnet. A modification of Jerne's theory of antibody production using the concept of clonal selection. Aust. J. Sci., 20 (1957), pp. 67-69.
30. M.-L. del Rio, J.-I. Rodriguez-Barbosa, E. Kremmer, R. Förster. CD103- and CD103+ bronchial lymph node dendritic cells are specialized in presenting and cross-presenting innocuous antigen to CD4+ and CD8+ T cells. J. Immunol., 178 (2007), pp. 6861-6866.
31. B.T. Edelson, T.R. Bradstreet, K. Hildner, J.A. Carrero, K.E. Frederick, W. KC, R. Belizaire, T. Aoshi, R.D. Schreiber, M.J. Miller, et al. CD8α(+) dendritic cells are an obligate cellular entry point for productive infection by Listeria monocytogenes. Immunity, 35 (2011), pp. 236-248.
32. A. Bachem, S. Güttler, E. Hartung, F. Ebstein, M. Schaefer, A. Tannert, A. Salama, K. Movassaghi, C. Opitz, H.W. Mages, et al. Superior antigen cross-presentation and XCR1 expression define human CD11c+CD141+ cells as homologues of mouse CD8+ dendritic cells. J. Exp. Med., 207 (2010), pp. 1273-1281.
33. K. Crozat, R. Guiton, V. Contreras, V. Feuillet, C.-A. Dutertre, E. Ventre, T.-P. Vu Manh, T. Baranek, A.K. Storset, J. Marvel, et al. The XC chemokine receptor 1 is a conserved selective marker of mammalian cells homologous to mouse CD8alpha+ dendritic cells. J. Exp. Med., 207 (2010), pp. 1283-1292.
34. J.M. den Haan, S.M. Lehar, M.J. Bevan. CD8(+) but not CD8(-) dendritic cells cross-prime cytotoxic T cells in vivo. J. Exp. Med., 192 (2000), pp. 1685-1696.
35. D. Dudziak, A.O. Kamphorst, G.F. Heidkamp, V.R. Buchholz, C. Trumpfheller, S. Yamazaki, C. Cheong, K. Liu, H.W. Lee, C.G. Park, et al. Differential antigen processing by dendritic cell subsets in vivo. Science, 315 (2007), pp. 107-111.
36. K.L. Lewis, M.L. Caton, M. Bogunovic, M. Greter, L.T. Grajkowska, D. Ng, A. Klinakis, I.F. Charo, S. Jung, J.L. Gommerman, et al. Notch2 receptor signaling controls functional differentiation of dendritic cells in the spleen and intestine. Immunity, 35 (2011), pp. 780-791.
37. B. Vander Lugt, A.A. Khan, J.A. Hackney, S. Agrawal, J. Lesch, M. Zhou, W.P. Lee, S. Park, M. Xu, J. DeVoss, et al. Transcriptional programming of dendritic cells for enhanced MHC class II antigen presentation. Nat. Immunol., 15 (2014), pp. 161-167.
38. E.K. Persson, H. Uronen-Hansson, M. Semmrich, A. Rivollier, K. Hägerbrand, J. Marsal, S. Gudjonsson, U. Håkansson, B. Reizis, K. Kotarsky, W.W. Agace. IRF4 transcription-factor-dependent CD103(+)CD11b(+) dendritic cells drive mucosal T helper 17 cell differentiation. Immunity, 38 (2013), pp. 958-969.
39. A. Schlitzer, N. McGovern, P. Teo, T. Zelante, K. Atarashi, D. Low, A.W.S. Ho, P. See, A. Shin, P.S. Wasan, et al. IRF4 transcription factor-dependent CD11b+ dendritic cells in human and mouse control mucosal IL-17 cytokine responses. Immunity, 38 (2013), pp. 970-983.
40. A.I. Proietto, M.H. Lahoud, L. Wu. Distinct functional capacities of mouse thymic and splenic dendritic cell populations. Immunol. Cell Biol., 86 (2008), pp. 700-708.
41. Liu K, Victora GD, Schwickert TA, Guermonprez P, Meredith MM, et al. In vivo analysis of dendritic cell development and homeostasis. Science. 2009;324:392–97.
42. Varol C, Vallon-Eberhard A, Elinav E, Aychek T, Shapira Y, et al. Intestinal lamina propria dendritic cell subsets have different origin and functions. Immunity. 2009;31:502–12.
43. Bogunovic M, Ginhoux F, Helft J, Shang L, Hashimoto D, et al. Origin of the lamina propria dendritic cell network. Immunity. 2009;31:513–25.
44. Diao J, Zhao J, Winter E, Cattral MS. Recruitment and differentiation of conventional dendritic cell precursors in tumors. J Immunol. 2010;184(3):1261–1267. doi: 10.4049/jimmunol.0903050.
45. Iyoda T., Shimoyama S., Liu K., Omatsu Y., Akiyama Y., Maeda Y., Takahara K., Steinman R.M., and Inaba K. 2002. The CD8+ dendritic cell subset selectively endocytoses dying cells in culture and in vivo. J. Exp. Med. 195:1289–1302. 10.1084/jem.20020161.
46. Henri S., Vremec D., Kamath A., Waithman J., Williams S., Benoist C., Burnham K., Saeland S., Handman E., Shortman K. The dendritic cell populations of mouse lymph nodes. J. Immunol. 2001;167:741–748.
47. Shortman K, Heath WR. The CD8+ dendritic cell subset. Immunol Rev. 2010;234:18–31.

48. M.C. Dieu, B. Vanbervliet, A. Vicari, J.M. Bridon, E. Oldham, S. Aït-Yahia, F. Brière, A. Zlotnik, S. Lebecque, C. Caux. Selective recruitment of immature and mature dendritic cells by distinct chemokines expressed in different anatomic sites. J. Exp. Med., 188 (1998), pp. 373-386
49. Romani N., Clausen B. E., Stoitzner P. Langerhans cells and more: langerin-expressing dendritic cell subsets in the skin. Immunological Reviews. 2010;234(1):120–141. doi: 10.1111/j.0105-2896.2009.00886.x.
50. Boltjes A, van Wijk F. Human dendritic cell functional specialization in steady-state and inflammation. Frontiers in Immunology. 2014;5:131 doi: 10.3389/fimmu.2014.00131
51. Rahman AH, Aloman C. Dendritic cells and liver fibrosis. Biochim Biophys Acta 2013;1832:998–1004. doi: 10.1016/j.bbadis.2013.01.005
52. 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 (Baltimore, Md.) 2012.
53. Sozzani S, Allavena P, D'Amico G, Luini W, Bianchi G, Kataura M, Imai T, Yoshie O, Bonecchi R, Mantovani A. Cutting edge: Differential regulation of chemokine receptors during dendritic cell maturation: A model for their trafficking properties. J. Immunol. 1998;161:1083–1086.
54. Merad M, Manz MG, Karsunky H, Wagers A, Peters W, Charo I, Weissman IL, Cyster JG, Engleman EG. Langerhans cells renew in the skin throughout life under steady-state conditions. Nat. Immunol. 2002;3:1135–1141.
55. Geissmann F, Jung S, Littman DR. Blood monocytes consist of two principal subsets with distinct migratory properties. Immunity. 2003;19:71–82.
56. Stumbles PA, Strickland DH, Pimm CL, Proksch SF, Marsh AM, McWilliam AS, Bosco A, Tobagus I, Thomas JA, Napoli S, et al. Regulation of dendritic cell recruitment into resting and inflamed airway epithelium: use of alternative chemokine receptors as a function of inducing stimulus. J. Immunol. 2001;167:228–234.
57. Merad M, Hoffmann P, Ranheim E, Slaymaker S, Manz MG, Lira SA, Charo I, Cook DN, Weissman IL, Strober S, Engleman EG. Depletion of host Langerhans cells before transplantation of donor alloreactive T cells prevents skin graft-versus-host disease. Nat. Med. 2004;10:510–517.
58. de Fougerolles AR, Stacker SA, Schwarting R, Springer TA. 1991. Characterization of ICAM-2 and evidence for a third counter-receptor for LFA-1. J Exp Med 174:253–267. doi:10.1084/jem.174.1.253.
59. Granucci F, Ferrero E, Foti M, Aggujaro D, Vettoretto K, Ricciardi-Castagnoli P. Early events in dendritic cell maturation induced by LPS. Microbes Infect. 1999;1:1079–1084.
60. Ten Broeke T, Wubbolts R, Stoorvogel W. MHC class II antigen presentation by dendritic cells regulated through endosomal sorting. Cold Spring Harb Perspect Biol. 2013;5:a016873.
61. de Witte L, Nabatov A, Geijtenbeek TB. Distinct roles for DC-SIGN+-dendritic cells and Langerhans cells in HIV-1 transmission. Trends Mol Med. 2008;14:12–19.
62. Banchereau J, Briere F, Caux C, et al. Immunobiology of dendritic cells. Annu Rev Immunol. 2000;18:767–811.
63. Verhasselt V, Buelens C, Willems F, De Groote D, Haeffner-Cavaillon N, Goldman M. 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. J Immunol. 1997;158:2919–25.
64. Edwards AD, Manickasingham SP, Sporri R, et al. Microbial recognition via Toll-like receptor-dependent and -independent pathways determines the cytokine response of murine dendritic cell subsets to CD40 triggering. J Immunol. 2002;169:3652–60.
65. Dreher D, Kok M, Cochand L, et al. Genetic background of attenuated *Salmonella typhimurium* has profound influence on infection and cytokine patterns in human dendritic cells. J Leukoc Biol. 2001;69:583–9.
66. Geijtenbeek TB, Van Vliet SJ, Koppel EA, et al. Mycobacteria target DC-SIGN to suppress dendritic cell function. J Exp Med. 2003;197:7–17.
67. Carreno B. M., Bennett F., Chau T. A., Ling V., Luxenberg D., Jussif J., et al. (2000). CTLA-4 (CD152) can inhibit T cell activation by two different mechanisms depending on its level of cell surface expression. J. Immunol. 165, 1352–1356.
68. Lenschow DJ, Walunas TL, Bluestone JA. CD28/B7 system of T cell costimulation. Annu Rev Immunol 1996; 14: 233–258.
69. Quezada SA, Jarvinen LZ, Lind EF, Noelle RJ. CD40/CD154 interactions at the interface of tolerance and immunity. Annu Rev Immunol (2004) 22:307–28.10.1146/annurev.immunol.22.012703.104533.

70. Steinbrink K, Jonuleit H, Muller G, Schuler G, Knop J, Enk AH. Interleukin-10-treated human dendritic cells induce a melanoma-antigen-specific anergy in CD8(+) T cells resulting in a failure to lyse tumor cells. Blood 1999; 93: 1634–1642.
71. Tosello V., Zamarchi R., Merlo A., Gorza M., Piovan E., Mandruzzato S., Bronte V., Wang X., Ferrone S., Amadori A., et al. Differential expression of constitutive and inducible proteasome subunits in human monocyte-derived dc differentiated in the presence of IFN-alpha or IL-4. Eur. J. Immunol. 2009;39:56–66. doi: 10.1002/eji.200738098.
72. Hensley SE, Zanker D, Dolan BP, David A, Hickman HD, Embry AC, et al. Unexpected role for the immunoproteasome subunit LMP2 in antiviral humoral and innate immune responses. J Immunol. 2010;184:4115–4122.
73. van Helden MJ, de Graaf N, Bekker CP, Boog CJ, Zaiss DM, Sijts AJ. Immunoproteasome-deficiency has no effects on NK cell education, but confers lymphocytes into targets for NK cells in infected wild-type mice. PLoS One. 2011;6:e23769.
74. Moretta L., Ferlazzo G., Mingari M. C., Melioli G., Moretta A. (2003). Human natural killer cell function and their interactions with dendritic cells. *Vaccine* 21(Suppl. 2) S38–S42.
75. Vivier E., Raulet D. H., Moretta A., Caligiuri M. A., Zitvogel L., Lanier L. L., et al. (2011). Innate or adaptive immunity? The example of natural killer cells. *Science* 331 44–49
76. Ferlazzo G. (2005). Natural killer and dendritic cell liaison: recent insights and open questions. *Immunol. Lett.* 101 12–17.
77. Poli A, Michel T, Theresine M, Andres E, Hentges F, Zimmer J. CD56bright natural killer (NK) cells: an important NK cell subset. Immunology (2009) 126(4):458–65.10.1111/j.1365-2567.2008.03027.x
78. Cooper MA, Fehniger TA, Caligiuri MA. The biology of human natural killer-cell subsets. Trends Immunol. 2001;22:633–40.
79. Caligiuri M. Human natural killer cells. Blood. 2008;112:461–9.
80. Schleypen J. S., Baur N., Kammerer R., Nelson P. J., Rohrmann K., Grone E. F., et al. (2006). Cytotoxic markers and frequency predict functional capacity of natural killer cells infiltrating renal cell carcinoma. *Clin. Cancer Res.* 12 718–725.
81. Dalbeth N., Gundle R., Davies R. J., Lee Y. C., McMichael A. J., Callan M. F. (2004). CD56bright NK cells are enriched at inflammatory sites and can engage with monocytes in a reciprocal program of activation. *J. Immunol.* 173 6418–6426
82. Cella M., Fuchs A., Vermi W., Facchetti F., Otero K., Lennerz J. K., et al. (2009). A human natural killer cell subset provides an innate source of IL-22 for mucosal immunity. *Nature* 457 722–725
83. Doherty D. G, O'Farrelly C. (2000). Innate and adaptive lymphoid cells in the human liver. *Immunol. Rev.* 174 5–20
84. Rueger P. D., Lassen M. G., Qiao H., Hahn Y. S. (2011). Regulation of NK cell repertoire and function in the liver. *Crit. Rev. Immunol.* 31 43–52
85. Carrega P., Morandi B., Costa R., Frumento G., Forte G., Altavilla G., et al. (2008). Natural killer cells infiltrating human nonsmall-cell lung cancer are enriched in CD56 bright CD16- cells and display an impaired capability to kill tumor cells. *Cancer* 112 863–875.
86. Montalto E., Mangraviti S., Costa G., Carrega P., Morandi B., Pezzino G., et al. (2010). Seroma fluid subsequent to axillary lymph node dissection for breast cancer derives from an accumulation of afferent lymph. *Immunol. Lett.* 131 67–72
87. Berahovich RD, Lai NL, Wei Z, Lanier LL, Schall TJ. Evidence for NK cell subsets based on chemokine receptor expression. J Immunol (2006) 177(11):7833–40.10.4049/jimmunol.177.11.7833
88. Ponzetta A, Benigni G, Antonangeli F, Sciume G, Sanseviero E, Zingoni A, et al. Multiple myeloma impairs bone marrow localization of effector natural killer cells by altering the chemokine microenvironment. Cancer Res (2015) 75(22):4766–77.10.1158/0008-5472.CAN-15-1320
89. Riva A, Laird M, Casrouge A, Ambrozaitis A, Williams R, Naoumov NV, et al. Truncated CXCL10 is associated with failure to achieve spontaneous clearance of acute hepatitis C infection. Hepatology (2014) 60(2):487–96.10.1002/hep.27139.
90. Miller JS, Alley KA, McGlave P. Differentiation of natural killer (NK) cells from human primitive marrow progenitors in a stroma-based long-term culture system: identification of a CD34+7+ NK progenitor. Blood. 1994;83:2594–2601.
91. Freud AG, Yu J, Caligiuri MA. Human natural killer cell development in secondary lymphoid tissues. Semin Immunol (2014) 26(2):132–7.10.1016/j.smim.2014.02.008.
92. Fehniger TA, Cooper MA, Nuovo GJ, et al. CD56bright natural killer cells are present in human lymph nodes and are activated by T cell-derived IL-2: a potential new link between adaptive and innate immunity. Blood. 2003;101:3052–3057.
93. Caligiuri MA. Human natural killer cells. Blood (2008) 112(3):461–9.10.1182/blood-2007-09-077438

94. Carson WE, et al. A potential role for interleukin-15 in the regulation of human natural killer cell survival. J Clin Invest. 1997;99:937–943.
95. Mrozek E, Anderson P, Caligiuri MA. Role of interleukin-15 in the development of human CD56+ natural killer cells from CD34+ hematopoietic progenitor cells. Blood. 1996;87:2632–2640.
96. Dubois S, Mariner J, Waldmann TA, Tagaya Y. IL-15Ralpha recycles and presents IL-15 In trans to neighboring cells. Immunity. 2002;17:537–547.
97. Stonier SW, Schluns KS. Trans-presentation: a novel mechanism regulating IL-15 delivery and responses. Immunol Lett. 2010;127:85–92
98. Sugamura K, et al. The common gamma-chain for multiple cytokine receptors. Adv Immunol. 1995;59:225–277
99. Freud AG, Yokohama A, Becknell B, Lee MT, Mao HC, Ferketich AK, et al. Evidence for discrete stages of human natural killer cell differentiation in vivo. J Exp Med (2006) 203(4):1033–43.10.1084/jem.20052507.
100. Yu H, Fehniger TA, Fuchshuber P, Thiel KS, Vivier E, Carson WE, et al. Flt3 ligand promotes the generation of a distinct CD34(+) human natural killer cell progenitor that responds to interleukin-15. Blood (1998) 92(10):3647–57
101. Ljunggren HG, Karre K. In search of the 'missing self': MHC molecules and NK cell recognition. Immunol Today. 1990;11(7):237–244.
102. Raulet DH, Held W, Correa I, Dorfman JR, Wu MF, Corral L. Specificity, tolerance and developmental regulation of natural killer cells defined by expression of class I-specific Ly49 receptors. Immunol Rev. 1997;155:41–52. doi: 10.1111/j.1600-065X.1997.tb00938.x
103. Pegram HJ, Andrews DM, Smyth MJ, Darcy PK, Kershaw MH. Activating and inhibitory receptors of natural killer cells. Immunol Cell Biol (2011) 89:216–24.10.1038/icb.2010.78
104. Vivier E, Nunès JA, Vély F. Natural killer cell signaling pathways. Science. 2004;306(5701):1517–1519. doi: 10.1126/science.1103478.
105. Lanier LL. NK cell recognition. Annu Rev Immunol. 2005;23:225–274. doi: 10.1146/annurev.immunol.23.021704.115526.
106. Horton NC, Mathew PA. NKp44 and natural cytotoxicity receptors as damage-associated molecular pattern recognition receptors. Front Immunol (2015) 6:31.10.3389/fimmu.2015.00031
107. Vacca P, Montaldo E, Croxatto D, Loiacono F, Canegallo F, Venturini PL, et al. Identification of diverse innate lymphoid cells in human decidua. Mucosal Immunol (2014).10.1038/mi.2014.63
108. Hanna J, Goldman-Wohl D, Hamani Y, Avraham I, Greenfield C, Natanson-Yaron S, et al. Decidual NK cells regulate key developmental processes at the human fetal-maternal interface. Nat Med (2006) 12:1065–74.10.1038/nm1452
109. Pietra G, Manzini C, Rivara S, Vitale M, Cantoni C, Petretto A, et al. Melanoma cells inhibit natural killer cell function by modulating the expression of activating receptors and cytolytic activity. Cancer Res (2012) 72:1407–15.10.1158/0008-5472.CAN-11-2544
110. Baychelier F, Sennepin A, Ermonval M, Dorgham K, Debré P, Vieillard V. Identification of a cellular ligand for the natural cytotoxicity receptor NKp44. Blood (2013) 122(17):2935–42.10.1182/blood-2013-03-489054
111. Ferlazzo G, Tsang M, Moretta L, Melioli G, Steinman R, Munz C. Human dendritic cells activate resting natural killer (NK) cells and are recognized via the NKp30 receptor by activated NK cells. J Exp Med. 2002;195:343–51
112. Vitale M, Della Chiesa M, Carlomagno S, Pende D, Arico M, Moretta L, et al. NK-dependent DC maturation is mediated by TNFalpha and IFNgamma released upon engagement of the NKp30 triggering receptor. Blood (2005) 106(2):566–71.10.1182/blood-2004-10-4035
113. Sivori S, Vitale M, Morelli L, Sanseverino L, Augugliaro R, Bottino C, Moretta L, Moretta A (1997) p46, a novel natural killer cell-specific surface molecule which mediates cell activation. J Exp Med 186: 1129–1136
114. Della Chiesa M. et al. . The tryptophan catabolite L-kynurenine inhibits the surface expression of NKp46- and NKG2D-activating receptors and regulates NK-cell function. Blood 108, 4118–4125, doi: 10.1182/blood-2006-03-006700 (2006).
115. Bryceson YT, March ME, Ljunggren HG, Long EO. Synergy among receptors on resting NK cells for the activation of natural cytotoxicity and cytokine secretion. Blood. 2006;107:159–166.
116. Vitale M, Falco M, Castriconi R, Parolini S, Zambello R, Semenzato G, et al. Identification of NKp80, a novel triggering molecule expressed by human NK cells. Eur J Immunol (2001) 31(1):233–42.10.1002/1521-4141(200101)31:1<233::AID-IMMU233>3.0.CO;2-4

117. Moretta A, Pende D, Locatelli F, Moretta L. Activating and inhibitory killer immunoglobulin-like receptors (KIR) in haploidentical haematopoietic stem cell transplantation to cure high-risk leukemias. Clin Exp Immunol (2009) 157:325–31.10.3389/fmed.2015.00093.1
118. Pende D, Castriconi R, Romagnani P, Spaggiari GM, Marcenaro S, et al. Expression of the DNAM-1 ligands, Nectin-2 (CD112) and poliovirus receptor (CD155), on dendritic cells: relevance for natural killer-dendritic cell interaction. Blood. 2006;107:2030–-6.
119. Raulet DH. Roles of the NKG2D immunoreceptor and its ligands. Nat. Rev. Immunol. 2003;3:781–-90.
120. Walzer T., Dalod M., Robbins S. H., Zitvogel L., Vivier E. Natural-killer cells and dendritic cells: 'l'union fait la force' Blood. 2005;106(7):2252–2258. doi: 10.1182/blood-2005-03-1154.
121. Zanoni I, Foti M, Ricciardi-Castagnoli P, Granucci F (2005) TLR-dependent activation stimuli associated with Th1 responses confer NK cell stimulatory capacity to mouse dendritic cells. J Immunol 175: 286–292
122. Ferlazzo G, Pack M, Thomas D, et al. : Distinct roles of IL-12 and IL-15 in human natural killer cell activation by dendritic cells from secondary lymphoid organs. Proc Natl Acad Sci U S A. 2004;101(47):16606–11. 10.1073/pnas.0407522101
123. Borg C, Jalil A, Laderach D, Maruyama K, Wakasugi H, Charrier S, et al. NK cell activation by dendritic cells (DCs) requires the formation of a synapse leading to IL-12 polarization in DCs. Blood (2004) 104(10):3267–75.10.1182/blood-2004-01-0380
124. Tosi D, Valenti R, Cova A, Sovena G, Huber V, Pilla L, et al. Role of cross-talk between IFN-alpha-induced monocyte-derived dendritic cells and NK cells in priming CD8+ T cell responses against human tumor antigens. J Immunol (2004) 172(9):5363–70
125. Andrews DM, Scalzo AA, Yokoyama WM, Smyth MJ, Degli-Esposti MA. Functional interactions between dendritic cells and NK cells during viral infection. Nat Immunol. 2003;4:175–81. doi: 10.1038/ni880.
126. Morandi B, Mortara L, Chiossone L, Accolla RS, Mingari MC, Moretta L, et al. Dendritic cell editing by activated natural killer cells results in a more protective cancer-specific immune response. PLoS One. 2012;7:e39170. doi: 10.1371/journal.pone.0039170.
127. Hagen TM, Huang S, Curnutte J, Fowler P, Martinez V, Wehr CM, Ames BN, Chisari FV. Extensive oxidative DNA damage in hepatocytes of transgenic mice with chronic active hepatitis destined to develop hepatocellular carcinoma. Proc Natl Acad Sci U S A. 1994;91(26):12808–12812.
128. Pollicino T, Belloni L, Raffa G, Pediconi N, Squadrito G, Raimondo G, Levrero M. Hepatitis B virus replication is regulated by the acetylation status of hepatitis B virus cccDNA-bound H3 andhistones. Gastroenterology. 2006;130:823–837.
129. Watashi K, Urban S, Li W, Wakita T. NTCP and beyond: opening the door to unveil hepatitis B virus entry. Int J Mol Sci. 2014;15:2892–2905. doi: 10.3390/ijms15022892.
130. Fisicaro P, Barili V, Montanini B, et al. Targeting mitochondrial dysfunction can restore antiviral activity of exhausted HBV-specific CD8 T cells in chronic hepatitis B. Nat Med 2017;23:327–36.doi:10.1038/nm.4275
131. Dunn C., Peppa D., Khanna P., Nebbia G., Jones M., Brendish N., Lascar R.M., Brown D., Gilson R.J., Tedder R.J., et al. Temporal analysis of early immune responses in patients with acute hepatitis B virus infection. Gastroenterology. 2009;137:1289–1300. doi: 10.1053/j.gastro.2009.06.054.
132. Tjwa E. T., van Oord G. W., Hegmans J. P., Janssen H. L., Woltman A. M. (2011). Viral load reduction improves activation and function of natural killer cells in patients with chronic hepatitis B. J. Hepatol. 54, 209–21810.1016/S0168-8278(11)60511-4
133. Oliviero B., Varchetta S., Paudice E., Michelone G., Zaramella M., Mavilio D., et al. (2009). Natural killer cell functional dichotomy in chronic hepatitis B and chronic hepatitis C virus infections. Gastroenterology 137, 1151–116010.1053/j.gastro.2009.05.047
134. Peppa D., Micco L., Javaid A., Kennedy P. T., Schurich A., Dunn C., et al. (2010). Blockade of immunosuppressive cytokines restores NK cell antiviral function in chronic hepatitis B virus infection. PLoS Pathog. 6:e1001227.10.1371/journal.ppat.1001227
135. Dunn C., Brunetto M., Reynolds G., Christophides T., Kennedy P. T., Lampertico P., et al. (2007). Cytokines induced during chronic hepatitis B virus infection promote a pathway for NK cell-mediated liver damage. J. Exp. Med. 204, 667–68010.1084/jem.20061839
136. Zhang Z., Zhang S., Zou Z., Shi J., Zhao J., Fan R., et al. (2011). Hypercytolytic activity of hepatic natural killer cells correlates with liver injury in chronic hepatitis B patients. Hepatology 53, 73–8510.1002/hep.24200

137. Micco L, Peppa D, Loggi E, Schurich A, Jefferson L, Cursaro C, et al. Differential boosting of innate and adaptive antiviral responses during pegylated-interferon-alpha therapy of chronic hepatitis B. J Hepatol. 2013;58(2):225–33. pmid:23046671
138. Shi CC, Tjwa ET, Biesta PJ, Boonstra A, Xie Q, Janssen HL, Woltman AM. Hepatitis B virus suppresses the functional interaction between natural killer cells and plasmacytoid dendritic cells. J Viral Hepat. 2012;19:e26–e33
139. Beckebaum S, Cicinnati VR, Zhang X, Ferencik S, Frilling A, Grosse-Wilde H, Broelsch CE, Gerken G. Hepatitis B virus-induced defect of monocyte-derived dendritic cells leads to impaired T helper type 1 response in vitro: mechanisms for viral immune escape. Immunology. 2003;109:487–495.
140. Tavakoli S, Mederacke I, Herzog-Hauff S, Glebe D, Grün S, Strand D et al. Peripheral blood dendritic cells are phenotypically and functionally intact in chronic hepatitis B virus (HBV) infection. Clin Exp Immunol 2007; 151: 61–70
141. Ferlazzo G, Moretta L. Dendritic cell editing by natural killer cells. Crit Rev Oncog (2014) 19(1–2):67–75.10.1615/CritRevOncog.2014010827
142. Guidotti LG, Ishikawa T, Hobbs MV, Matzke B, Schreiber R, Chisari FV. Intracellular inactivation of the hepatitis B virus by cytotoxic T lymphocytes. Immunity (1996) 4(1):25–36.10.1016/S1074-7613(00)80295-2
143. Oliviero B, Varchetta S, Paudice E, Michelone G, Zaramella M, Mavilio D, De Filippi F, Bruno S, Mondelli MU. Natural killer cell functional dichotomy in chronic hepatitis B and chronic hepatitis C virusinfections. Gastroenterology. 2009;137:1151–1160.
144. Liu, F. et al. Lower number and decreased function of natural killer cells in hepatitis B virus related acute-on-chronic liver failure. Clin Res Hepatol Gastroenterol (2016).
145. Robek MD, Wieland SF, Chisari FV. Inhibition of hepatitis B virus replication by interferon requires proteasome activity. J Virol 2002; 76: 3570–3574.